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Enzymes in Water-in-oil Microemulsions ('Reversed Micelles'): Principles and Applications

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Introduction

Microemulsions are thermodynamically stable, single-phase mixtures of oil, water and surfactant. They form spontaneously, usually within seconds, simply by shaking the components together in appropriate proportions. In these respects they differ fundamentally from conventional emulsions. The exact structure of the microemulsion depends on the kind of surfactant used and on the exact composition (relative amounts of water, oil and surfactant), but a common defining feature is that the surfactant is located almost exclusively at the oil–water interface, forming a close-packed monolayer such that the polar or charged headgroups are hydrated on the aqueous side of the interface and the apolar tails are likewise favourably solvated on the oil side. When the (water+surfactant) volume fraction is not too high (<0.2), the microemulsion is oil-continuous and for many, but not all, systems the water is dispersed in the form of tiny (nanometer diameter), approximately spherical, droplets which diffuse randomly and independently with the continuous oil medium. These systems are referred to as water-in-oil (w/o) microemulsions, or as reversed micellar systems (since the orientation of the surfactant molecules, with the headgroups pointing inwards, is opposite to that encountered in classical aqueous micelles). A characteristic feature of w/o

Abbreviations: AOT, Aerosol-OT; BTP, bis-tris-propane; CD, circular dichroic spectroscopy; CSTR, continually stirred tank reactor; α-CT, α-chymotrypsin; CTAB, cetyltrimethylammonium bromide; DDAB, didodecyldimethylammonium bromide; EPR, electron paramagnetic resonance; FFA, free fatty acid; GPNA, glutaryphenylalanyl-4-nitroanilide; GuHCl, guanidine hydrochloride; HLADH, horse-liver alcohol dehydrogenase; HLB, hydrophile-lyophile balance; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; LipOH, liposamide dehydrogenase; MBGs, microemulsion-based gels; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PCS, photon correlation spectroscopy; pit, phase-inversion temperature; SAAP, *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide; SANS, small-angle neutron scattering; SAXS, small-angle X-ray scattering; SDS, sodium dodecyl sulphate; t-CNI, *N-trans* cinnamoyl imidazole; TG, triglyceride; TOMAC, trioctyl ammonium chloride; TP, temperature-phase; TTN, total turnover number.

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microemulsions is that they are almost always optically transparent since, due to their small size, the droplets do not scatter visible light.

Water-in-oil microemulsions have attracted much interest from biotechnologists in recent years, since the discovery that enzymes can be dissolved in them with retention of activity and, in large measure, stability. The concept of the enzyme-containing water droplet as a microreactor stably dispersed within a continuous oil medium has proven to be an extremely attractive concept for those interested in biotransformations involving apolar (water-insoluble) substances. Consequently the development of these systems for such purposes is an area of great interest and active research (reviewed by Luisi and Magid, 1986; Martinek *et al.*, 1986, 1989; Luisi *et al.*, 1988).

Biotransformation is an interdisciplinary science which relies on a combination of gene-manipulation techniques to improve the activity/stability characteristics of existing biocatalysts, of fermentation techniques to meet the demand for the biocatalyst, and of medium-engineering to provide solvent systems appropriate for the biotransformation. The application of microemulsions quite clearly falls within the medium-engineering side of this equation. A well-engineered medium is expected (1) to support and stabilize biocatalyst activity, (2) to be a good solvent for reactants, products and any additional cofactors, and (3) to be accessible to post-reaction separations techniques permitting product recovery. A fundamental objective of this chapter is to give an indication of the inherent flexibility in microemulsion engineering which permits each of these requirements to be addressed in a systematic and holistic fashion. Their properties in this context will be reviewed in terms of the progress that has been made over the past few years in understanding the activity and stability characteristics of solubilized enzymes, and in the peculiarities of microemulsion structure and phase-behaviour which complement this application by facilitating cofactor regeneration, reaction monitoring and product recovery.

As a consequence of the great activity in this area, a number of new possibilities for the application of microemulsions which go beyond the basic medium-engineering concept, have become apparent. In one active area of research, microemulsions are being applied for the selective extraction of added-value products (amino acids, antibiotics and proteins) from conjugate aqueous phases such as culture-broths; in another, the biotechnological applications of microemulsion gels (that is, microemulsions with the rheological properties of highly viscous gels) are being explored. New results and trends in these fields are reported here, also.

This chapter is therefore divided into four sections, as follows: The first discusses phase-behaviour, structure and dynamics of microemulsions. This information is considered essential for a full appreciation of their applications and potential in biotransformations. The second is a discussion of the properties and behaviour (structure, activity, stability) of microemulsified enzymes. The third discusses recent advances in process engineering of microemulsions with a view to industrial-scale biotransformations and covers practical considerations such as provision of cofactors and product separation. The fourth deals with new research and topics, the final section summarizes and gives an indication of trends and future prospects.

Although warranted, there is unfortunately no room here for a detailed comparison of microemulsions with other methods of stabilizing enzyme activities in non-

aqueous media (including two-phase (oil+water) systems (Lilly, 1983), enzymes rendered oil-soluble by covalent modification (Inada *et al.*, 1986) and dry (or immobilized) enzyme suspensions in organic solvents (Klibanov, 1989). Where possible, reference will be made to a few studies where direct comparisons have been attempted. See recent reviews (Dordick, 1991; Gupta, 1992) for an appreciation of current status of these other technologies.

Physical chemistry

SOME FUNDAMENTAL PROPERTIES OF MICROEMULSIONS

Definition of a microemulsion

Danielsson and Lindman (1981) give the following comprehensive definition: 'A microemulsion is defined as a system of water, oil and amphiphile which is a single phase, optically isotropic and thermodynamically stable liquid solution'. This definition reasonably excludes from consideration:

1. real emulsions (which, by definition, are unstable with respect to the corresponding two-phase systems);
2. solutions of surfactant in a single solvent (i.e. binary molecular and micellar or reversed-micellar systems);
3. liquid-crystalline systems; and
4. amphiphile-free systems of any description.

The three basic microemulsion types are shown in *Figure 1*. The oil-continuous (water-in-oil; w/o) type microemulsions, already described, consist of surfactant-coated droplets of water in an oil-continuous phase. Water-continuous (oil-in-water; o/w) microemulsions are dispersions of surfactant-coated oil droplets in a water-continuous phase. The droplets-type structure is typical for compositions which are predominantly (>80% v/v) oil or water. To a good first-approximation, the droplets in a given preparation are the same size, i.e. microemulsions are characteristically monodisperse. Bicontinuous microemulsions contain approximately equal volumes of water and oil (and a correspondingly large amount of surfactant); they are distinct from w/o and o/w systems in that the oil, water and surfactant subphases are physically continuous throughout the sample, the interfacial surfactant forming a dividing surface between the oil and water domains. It is rather more difficult to assign a structural icon for these systems, since a number of different structures may be envisaged, each consistent with the thermodynamic constraints imposed by the bicontinuous condition (Scriven, 1976). Probably, examples of both highly randomized disordered structures (*Figure 1c*) and various kinds of semi-ordered structures with elements of tetragonal (*Figure 1d*) or cubic (*Figure 1e*) topology exist, depending on the particular microemulsion system and its exact composition (Zemb *et al.*, 1987; Lindman *et al.*, 1989).

Droplets-type microemulsions are usually optically transparent, although preparations with larger droplets (radii of 10 nm or greater) and bicontinuous microemulsions often show Tyndall scattering. All microemulsions, however, are optically isotropic.

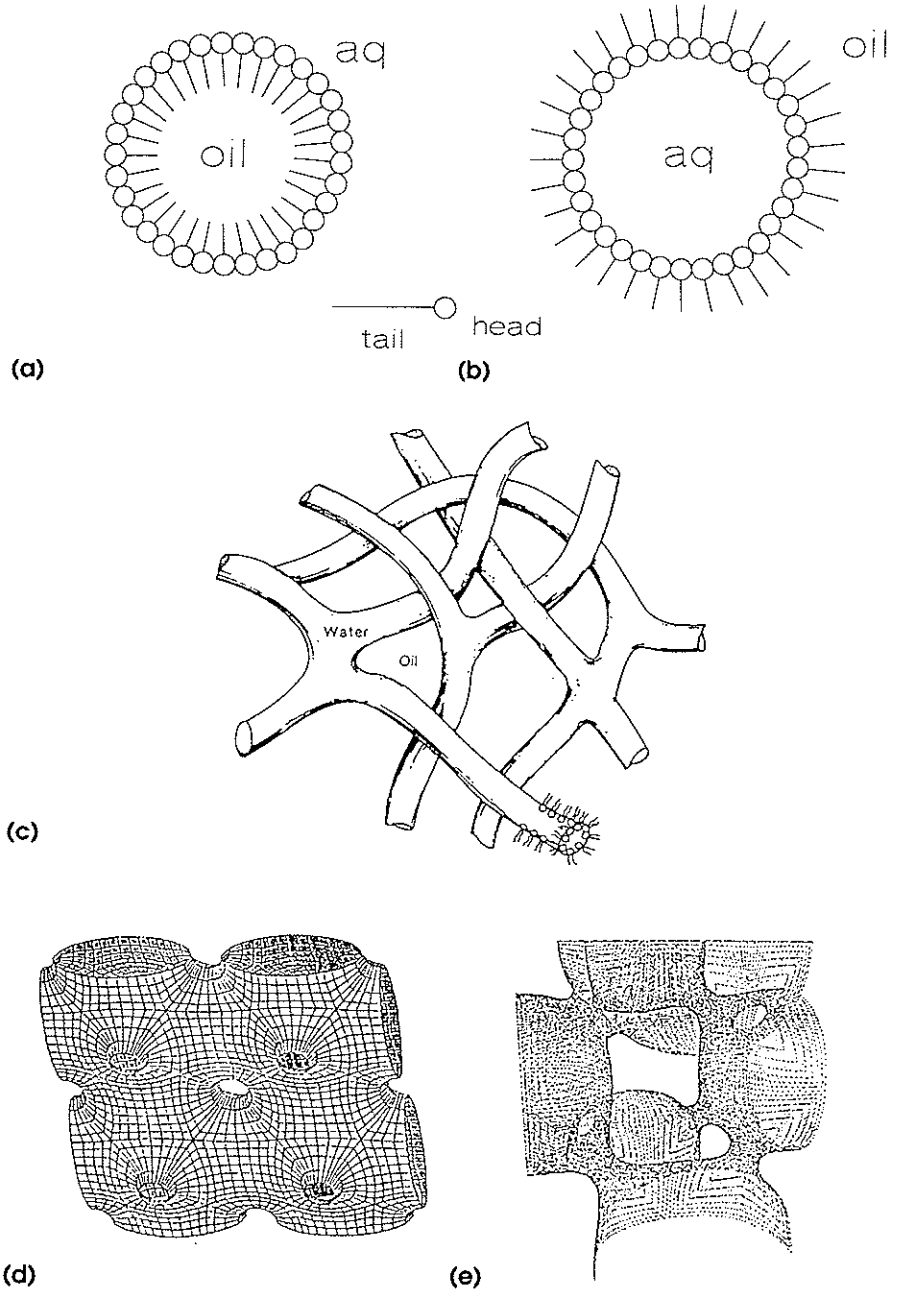


Figure 1. Examples of microemulsion structural motifs (not to scale). (a) Water droplets in an oil-continuous system; (b) oil-droplets in a water-continuous system; (c) an open, disordered bicontinuous system (Chen, S.J. Evans, D.F., Ninham, B.W., Mitchell, D.J., Blum, F.D. and Pickup, S. (1986) *Journal of Physical Chemistry* **90**, 842–847 © (1986) Americal Chemical Society); (d) one example of a system possessing low mean curvature with tetragonal symmetry; and (e) one example of a system possessing low mean curvature with body-centred cubic symmetry (Lindman, B., Shinoda, K., Olsson, U., Anderson, D., Karlström, G. and Wennerström, H. (1989) *Colloids and Surfaces* **38**, 205–224). The bicontinuous structures should be envisaged as highly dynamic, or 'melted'. Figures reproduced with permission.

Winsor systems

In addition to single-phase microemulsions, there exist two- and three-phase systems in which one of the phases is a microemulsion (*Figure 2*). These multiphase systems are referred to using the classification of Winsor (1954). The single-phase systems already described (o/w, w/o and bicontinuous) are Winsor type IV microemulsions. Type I systems consist of an o/w microemulsion in equilibrium with an excess oil phase; type II systems consist of a w/o microemulsion in equilibrium with an excess water phase; and type III systems consist of a microemulsion phase in equilibrium with both an excess water and an excess oil phase. In the last case the microemulsion is usually the middle phase and is usually bicontinuous in nature. As discussed in more detail on pp. 265–270, these systems may be interconverted by manipulating the temperature or the ionic strength and this has important implications in areas such as product recovery and selective extraction processes.

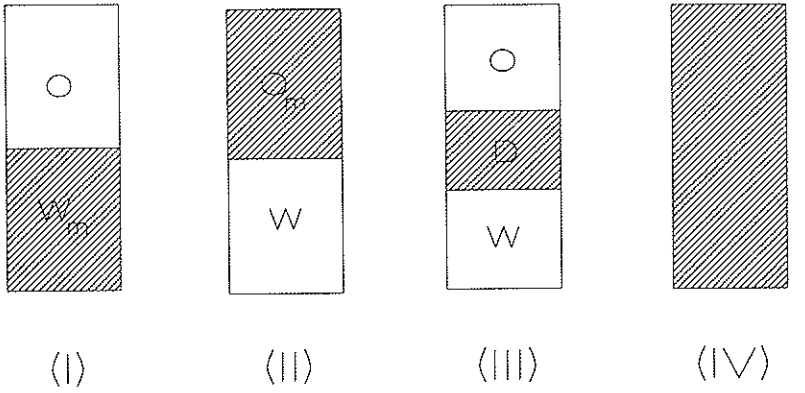


Figure 2. Winsor systems. The Winsor (I) system consists of a water-continuous microemulsion (W_m) in equilibrium with an excess oil (O) phase; The Winsor (II) system consists of an oil-continuous microemulsion (O_m) in equilibrium with an excess water phase (W). The Winsor (III) consists of a surfactant-rich phase (bicontinuous microemulsion, D), in equilibrium with excess oil and water phases. The Winsor (IV) system is a single-phase microemulsion which may be oil-, water- or bicontinuous.

Dispersion properties of microemulsions

All microemulsion phases, whether droplets-type or bicontinuous, are characterized by the fact that the surfactant is located almost entirely at the oil–water interface (the surfactant layer, with its associated water and oil of solvation, is conveniently referred to as the 'interphase'). Consequently, microemulsions contain an enormous amount of interfacial surface area, much greater than that generated in a simple emulsion. The interfacial area is dependent only on the amount of surfactant present and on the surface area occupied per surfactant molecule. For example, a typical w/o microemulsion consists of 9.5 ml oil (e.g. *n*-heptane), 0.5 ml water and 0.1 mol dm^{-3} of a surfactant. If each surfactant molecule occupies 0.5 nm^2 at the interface (a typical value), simple calculation shows that the total surface area is about 30 m^2 per cm^3 of microemulsion. The composition of a w/o microemulsion is usually related by stating the surfactant concentration and the ratio R, where,

$$R = \frac{[H_2O]}{[AOT]} \qquad \text{Eq. (1)}$$

(the concentration units are mol per litre of microemulsion, M^m). The droplet size is directly proportional to R, for many w/o microemulsion systems. In the preparation just described, $R = 28$ and a typical value for the radius of the water droplets is 5 nm (pp. 000–000). The surface area:volume ratio is therefore of the order of 10^8 m^{-1}. There are about 10^{17} droplets per cm^3. A droplet of radius 5 nm is roughly 11 times larger (in terms of volume) than the roughly spherical α-chymotrypsin (α-CT) molecule ($M_r = 25$ kDa), or about the same size as a horse-liver alcohol dehydrogenase (HLADH) molecule ($M_r = 80$ kDa). Since the dimensions of these droplets are very small compared with those of visible light (400–700 nm), light-scattering by the droplets is minimal and the microemulsion is optically transparent. Calculations such as these serve to show the incredible degree of dispersion achieved and the important consequence that interfacial surface area cannot be a limiting factor in determining phase-transfer rates between the water and bulk-oil phases.

Role of the surfactant in determining structure

An idealized microemulsion-forming surfactant is one which is insoluble in, and also cannot micellize in, either of the bulk phases (Overbeek, 1978). On addition to an oil-water two-phase system, therefore, the surfactant spontaneously migrates to the interface, which expands until all of the surfactant is accommodated. This process is driven by the favourable free-energy of solvation of the surfactant at the interface and by the exclusion of direct oil–water contact, and is accompanied by a dramatic lowering of interfacial surface tension (to very small positive values, of the order of 10^{-2} mN m^{-1}, compared with 50 mN m^{-1} for the tension at an oil–water interface (Aveyard *et al.*, 1986; Aveyard, Binks and Mead, 1986; Langevin, Guest and Meunier, 1986)).

As already indicated, even relatively small amounts of surfactant require an enormous amount of surface area and the exact way in which this area is accommodated within the volume of the sample (i.e. the microstructure of the microemulsion phase) is determined by the preference of the surfactant film to spontaneously adopt a certain 'natural' radius of curvature, r_N, which has both direction and magnitude, corresponding to a minimum surface free-energy (Safran and Turkevitch, 1983; Mukherjee, Miller and Fort, 1983; Lam, Falk and Schechter, 1987). Hence surfactant films which spontaneously curve such that the headgroups face 'inwards' (negative curvature) will form oil-continuous microemulsions, films which spontaneously curve with the headgroups facing 'outwards' (positive curvature) will form water-continuous microemulsions, and those which spontaneously form films with low, or zero (mean) radii of curvature, favour the open, extended surfactant films found in bicontinuous microemulsions (Auvray *et al.*, 1984; Lindman *et al.*, 1989).

The reduction in surface free-energy due to surfactant saturation, and the preference to adopt a certain curvature, are sufficient for a qualitative understanding of the stability of microemulsion phases. For example, w/o microemulsions form because the equilibrium interfacial free-energy is extremely low and because the surfactant film has negative curvature. The combined surface free-energy of the droplets,

though positive (which in itself predicates phase separation), is small enough that it is counterbalanced by the entropy of dispersion of the droplets within the continuous phase (Ruckenstein and Chi, 1975). Hence there is a delicate balance between droplet surface energy and dispersion entropy, and a microemulsion will form only if the surfactant used is capable of lowering the surface energy to values which can be compensated for by the dispersion entropy. (Certain surfactants which, acting alone, cannot stabilize microemulsions may do so in the presence of a second amphipathic compound – a co-surfactant; p. 268. The action of the co-surfactant complements that of the surfactant by further lowering the surface tension, to below the threshold value for microemulsion formation.) Similarly, oil and water alone do not form microemulsions because the interfacial tension is so high that it cannot be so compensated, even for very high concentrations of droplets (high dispersion entropies). The same kind of argument applies to water-continuous microemulsions, except that the surfactant spontaneously curves positive, and for bicontinuous systems, except that zero mean curvature is preferred and the entropy factor arises from the extreme torsional flexibility of the interface.

Curvature is a powerful tool for understanding and predicting microstructure. For example, the existence of Winsor II systems is a consequence of the fact that the microemulsion phase will take up only a finite amount of water, until the radius of curvature, $r \approx r_N$ ($R = R_{max}$). Then, further water can only be incorporated at the expense of a further decrease in the radius of curvature ($r<r_N$), and an unfavourable increase in the curvature energy. The excess water therefore remains as a separate phase in (dynamic) equilibrium with the internalized phase. A similar argument applies to Winsor I systems. Winsor III systems are formed as the surfactant phase takes up (approximately) equal amounts of both oil and water to establish the preferred zero-mean curvature, leaving two excess phases.

The formation of single-phase w/o microemulsions is consistent with the amount of the water in the system being such that $R<R_{max}$. It follows that $r>r_N$, given that all the surfactant is located interfacially, and the difference, (r_N-r) must increase as R decreases. This observation is consistent with the inverse proportionality between size and concentration in droplets-type microemulsions (p. 272, equation 7b) observed when R is varied at constant surfacant concentration (constant total interfacial area): As R decreases the droplet concentration increases, so providing increased entropic compensation for the increased curvature energy of the smaller droplets. The balance between radius of curvature and droplet concentration is also consistent with the relatively low polydispersity index of droplets-type microemulsions (Kotlarchyk *et al.*, 1982; Robinson *et al.*, 1984).

The natural radius of curvature may be considered to be set, in magnitude and direction, by the balance of opposing forces within the surfactant film (Bowcott and Schulman, 1955). A very simple model considers the surfactant film as a bilayer composed of a layer of headgroups fused to layer of tails. On each side, the various kinds of intermolecular interactions (dispersion, dipole–dipole, electrostatic) determine an optimal separation between adjacent groups within a plane, and curvature arises when this optimal distance is different for the layer of heads, compared with that for the layer of tails. The curving interface permits interactions on both sides to be optimized under the bilayer constraint. Factors such as the type of oil, the electrolyte concentration and the temperature strongly influence the value of r_N for

any given surfactant, and herein lies the influence of these parameters on Winsor phase equilibria as already mentioned.

Progress towards a quantitative description of structure and phase-behaviour in surfactant systems on purely thermodynamic grounds is impeded by a lack of quantitative understanding of the nature of the forces within the surfactant layer and how they interplay to determine curvature. A simpler, and very successful approach to this problem involves considering the geometrical properties – the shape – of the surfactant molecule (Oakenfull, 1980; Mitchell and Ninham, 1981). The curvature preference for a particular surfactant molecule can be determined by calculating the surfactant packing parameter, *Sp* (Mitchell and Ninham, 1981):

$$Sp = \frac{v}{a_0 \times l_c} \qquad \text{Eq. (2)}$$

where v is the volume occupied by the tail, l_c is its length (measured radially from the interface) and a_0 is the interfacial surface area occupied per molecule. *Sp* is really a measure of the ratio of hydrophobic to hydrophilic surface area, and in this sense is similar to the older hydrophile–lyophile balance (HLB) system (Becher, 1984). Microemulsion-forming surfactants are characterized by *Sp* values around unity, corresponding to the HLB concept of a balanced surfactant (i.e. one which is insoluble in bulk phases and hence preferentially migrates to the interface, the essential prerequisite, *vide supra*, for a microemulsion-forming surfactant). Functionally $Sp \approx 1$ (HLB = 8–10) surfactants are most likely to stabilize bicontinuous microemulsions, since their effective hydrophobic and hydrophilic surface areas are equal. Surfactants which are functionally $Sp > 1$ (HLB 4–6) form negatively curving structures (in this way the excess volume occupied by the tails is accommodated) and hence stabilize w/o microemulsions, and those which are functionally $Sp < 1$ (HLB 12–15) form positively curving structures in order to accommodate the excess volume of the headgroups. The condition $Sp >> 1$ (very low HLB) and $Sp << 1$ (very high HLB) describes surfactants which are very oil- or water-soluble, and which therefore do not saturate interfaces (Becher, 1984). The term 'functional' is important because parameters such as temperature, electrolyte concentration and oil type determine the actual value of *Sp*, and this may be very different from a first-approximation value calculated, for example, using algorithms based on atomic volumes. Usually this difference arises as a consequence oil- or co-surfactant penetration into (solvation of) the tailgroups (increasing v and/or l_c); in the case of ionic surfactants a_0 is a function of the electrolyte concentration (a_0 decreases as the degree of electrostatic shielding increases). The corollary of this argument is that microemulsion structure can be modulated by the use of additives (co-surfactants, electrolytes) which alter *Sp* (pp. 267–270).

The 'thermodynamic' (natural curvature) and 'geometric' (packing) approaches to predicting phase behaviour and structure in surfactant systems should be considered to be complementary. The thermodynamic approach seeks to explain microstructure by elucidating the nature and balance of the forces in the surfactant layer, whereas the packing approach acknowledges the fact that molecular shape largely determines the overall shape of structures formed by non-covalent assembly (Evans, Mitchell and Ninham, 1986).

STRUCTURE AND PHASE-BEHAVIOUR OF W/O MICROEMULSIONS

Microemulsion-forming surfactants

Surfactants which approximate the $Sp \sim 1$ condition for a microemulsion-former include sodium *bis*-2-ethylhexyl sulphosuccinate (Aerosol-OT, AOT), didodecyldimethylammonium bromide (DDAB) and trioctyl ammonium chloride (TOMAC). Many other common surfactants are too hydrophilic to stabilize w/o microemulsions (*Sp* too low), although some of these will do so in the presence of a co-surfactant (p. 000). Examples include members of the Brij and Lubrol W series of polyoxyethylene ethers of aliphatic alcohols, the Triton-X and Nonidet-P series of polyoxyethylene ethers of 4-*t*-octylphenol and the Tween series of polyoxyethylene sorbitol esters (all non-ionic surfactants), the alkyl sulphates (e.g. sodium dodecyl sulphate, SDS; anionic) the alkyltrimethylammonium halides (e.g. cetyl-trimethylammonium bromide, CTAB; cationic) and phosphatidylcholines (lecithins), all of which are familiar to biologists (Helenius and Simons, 1975).

AOT as a model surfactant

The most commonly used surfactant for enzymological studies is AOT. This surfactant solubilizes large amounts of water (at least 60 mol mol^{-1}, at ambient temperatures) in a range of oil solvents which include alphatic alcohols (octanol, decanol), aromatics (benzene, xylene) and alkanes (*n*-heptane, iso-octane, cyclohexane), without the requirement for a co-surfactant.

A space-filling model of AOT (*Figure 3*) gives a visual indication of why this molecule is a good w/o microemulsion-forming surfactant. The sulphosuccinate headgroup is small in comparison with the bulk of the molecule, the alkyl tails are of medium length and are branched, so that v (equation 2) is large for a modest value of l_c. The consequence is that this surfactant preferentially forms films with negative curvature, permitting the accommodation of the bulky hydrophobic groups at the interface.

The AOT molecule possesses a further interesting structural feature, the potential for considerable rotational flexibility about the C–C bonds of the ethane skeleton of the succinate moiety. It has been shown using ^1H NMR spectroscopy (Maitra and Eicke, 1979; Maitre, 1984) and ^{13}C NMR and infra-red spectroscopy (Martin and Magid, 1981) that the equilibrium population of rotamers varies with R. Specifically, the population of the more hydrophilic (lower *Sp*) *trans* isomer increases at the expense of the more hydrophobic *gauche* isomer with increasing R. This isomerism permits the *Sp* to increase by a factor of two, from about 1.5 to about 3, as R is increased from 0 to 50. The headgroup surface area (a_0) is essentially independent of R and the increase in *Sp* is achieved mainly by this alteration in the relative orientations of the two hydrocarbon tails (i.e. *Sp* is explicitly a function of v). Thus it appears that AOT is capable of altering its wedge-angle (within limits) to suit the radius of curvature of the droplet formed (Kubik *et al.*, 1982; Maitra, 1984).

The presence of several medium-length alkyl tails (high v/l ratio) and a small charged headgroup is a common motif among surfactants which stabilize

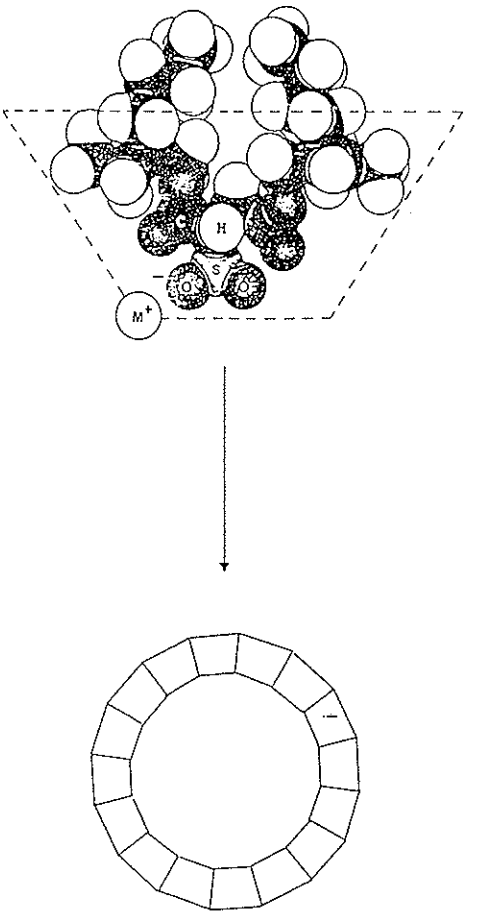


Figure 3. A space-filling model of an AOT molecule. The shape favours the formation of negatively curving structures.

microemulsions in the absence of a cosurfactant. For example, DDAB has two tails and TOMAC has three.

Phase diagram for AOT systems

Phase diagrams are used to relate structure–composition relationships in colloidal systems. Phase information for a three-component (oil, water, surfactant) system at a given temperature is presented in the form of a Gibbs phase triangle. Any point within the triangle represents a unique combination (volume, weight, or mol-fraction) of the three components. The phase-behaviour of several ternary AOT–water–oil systems has been characterized in detail (Ekwall, Mandell and Fontell, 1970; Kunieda and Shinoda, 1979, 1980; Shinoda and Kunieda, 1987).

The ternary phase diagram for the AOT–water–iso-octane system at 15°C is shown in *Figure 4* (Kunieda and Shinoda, 1979). A large area, O_m, of oil-continuous Winsor IV (single-phase) microemulsion extends from the oil-corner deep into the phase

diagram. The O_m is bordered on the water-rich side by a Winsor II domain, O_m+W (oil-continuous microemulsion in equilibrium with an excess water phase) and, on the AOT-rich side, by a reversed hexagonal liquid-crystalline phase. This is distinguishable from the adjacent O_m by its optical birefringence, indicating a non-isotropic composition. As the water volume-fraction increases in this lower region of the phase triangle, the reversed hexagonal phase evolves into a lamellar liquid-crystalline phase. Extending from the water-corner of the phase triangle, in the direction of the oil-corner is a W_m phase of water-continuous microemulsion and of Winsor I W_m+O (water-continuous microemulsion in equilibrium with excess water). AOT is not a good o/w microemulsion-former, and this simply reflects the difficulty of fitting this wedge-shaped molecule on to the surface of a sphere in the 'normal' micellar configuration, as can be seen from *Figure 3*.

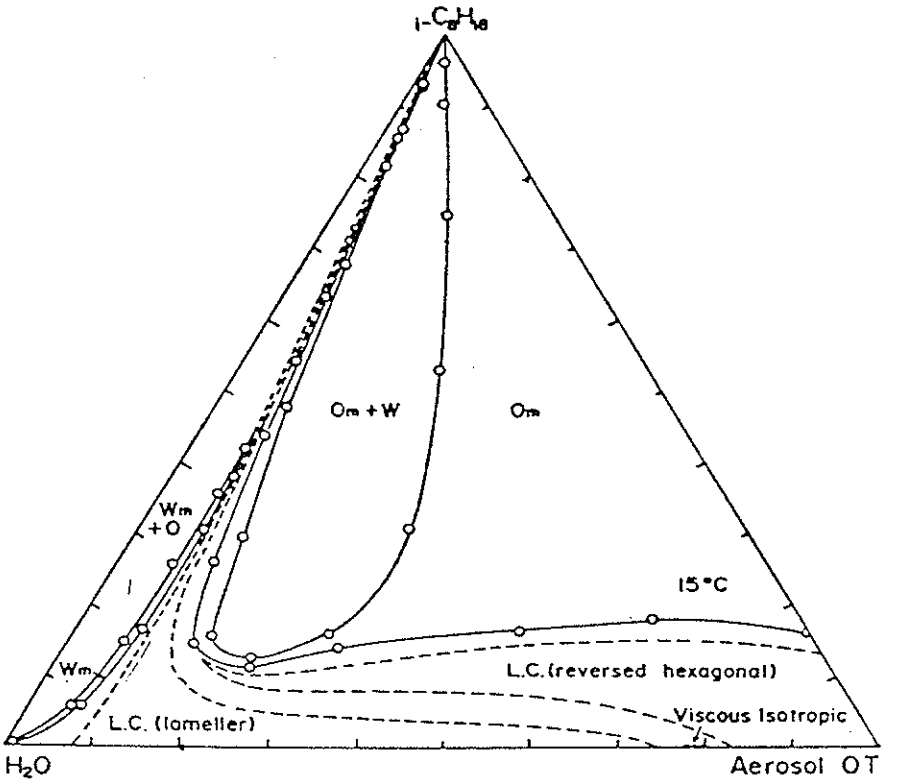


Figure 4. Ternary phase diagram for the water–AOT–iso-octane system at 15°C. Om, oil-continuous (w/o) microemulsion; Om+W, oil-continuous microemulsion plus conjugate aqueous phase; Wm, water-continuous microemulsion; Wm+O, water-continuous microemulsion plus excess oil-phase; l.c., liquid-crystalline phase. The phase diagram is pseudo-ternary since a salt (Na_2SO_4) is present at a constant molar AOT:Na_2SO_4 ratio of approximately 50:1. (Reproduced from Kunieda, H. and Shinoda, K. (1979) *Colloid and Interface Science* **70**, 577–583, with permission.)

Effects of temperature, salt on phase-behaviour

The Gibbs phase triangle gives information about the phase-behaviour at a single temperature. The temperature dependence information can be built-in by extending a

fourth (temperature) axis, perpendicular to those of the Gibbs triangle, to make a phase prism. A temperature-phase (TP) plot for the AOT–iso-octane system is shown in *Figure 5* (Shinoda and Kunieda, 1987). This plot corresponds to a section through the phase prism parallel to the oil–water axis. The AOT concentration is constant at 3.5 wt% (80 mmol dm^{-3}) which falls within the range (50–200 mmol dm^{-3}) used in most published enzymological studies. In this very oil+water-rich part of the phase triangle the single-phase region occupies a relatively small area and, except at very low (<0.05) or very high (<0.9) water:oil ratios, is confined within a narrow channel located parallel to the abscissa, at about 42°C (the HLB, or phase-inversion temperature (pit)). This channel, extending as it does right across the phase plot, implies an evolution of w/o → o/w, moving from right to left. In the major part of the diagram, therefore, the effect of increasing the temperature up to, and then beyond, 42°C is to drive a Winsor I → IV → II phase transition. The effect of temperature on the ternary phase diagram is shown in *Figure 6*. As the temperature increases, the single-phase region shifts from the oil-rich to the water-rich region, as the surfactant adopts a more hydrophilic (lower *Sp*) character. The tie-lines show that the excess phase is almost pure water (below the HLB temperature) and almost pure oil (above).

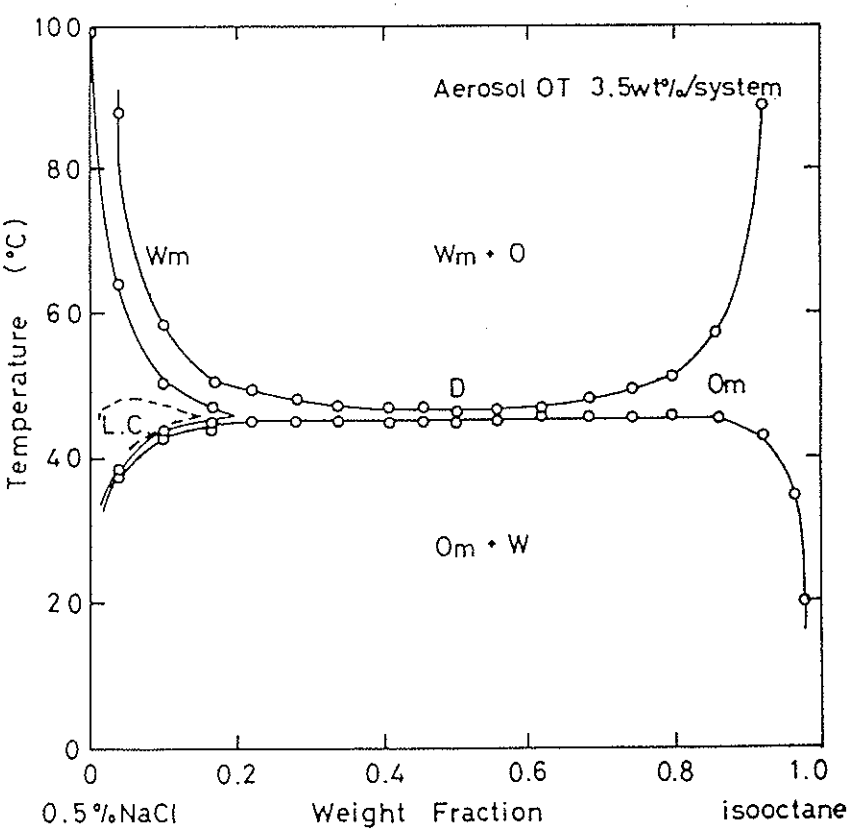


Figure 5. Temperature-phase plot for the AOT–water–isooctane system. Abbreviations are as for *Figure 4*. (Reproduced from Shinoda, K. and Kunieda, H. *Journal of Colloid and Interface Science* **118**, 586–589 (1987), with permission.)

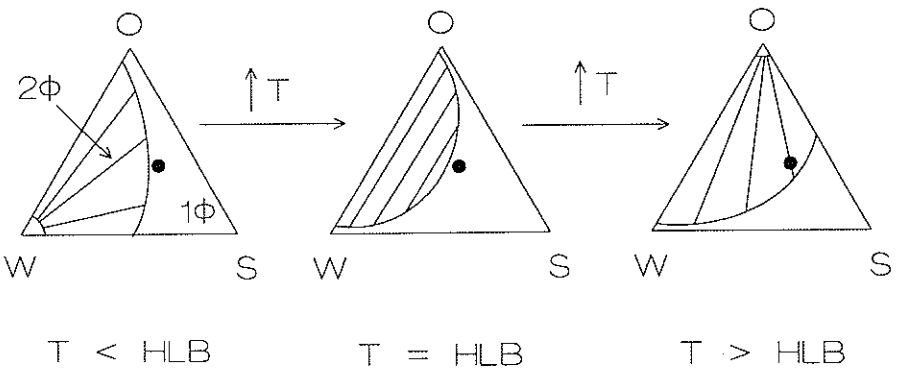


Figure 6. The effect of increasing the temperature on the ternary phase diagram for an oil–water–ionic surfactant system. The phase diagram is simplified to include only one- and two-phase regions. Tie-lines in the two-phase region indicate the compositions of the two phases. T < HLB temperature, the two-phase region consists of a w/o microemulsion phase in equilibrium with an almost pure aqueous phase. T> HLB, the two-phase region consists of an o/w microemulsion in equilibrium with an almost pure oil phase. The point (●) is in the single-phase region when T < HLB, and in the two-phase region when T > HLB.

The effect of temperature on the phase-behaviour is understandable in terms of its effect on the natural curvature of the surfactant film. Increasing the temperature is thought to increase the degree of dissociation of the counterions and surfactant headgroups, leading to decreased headgroup shielding and therefore to a decrease in *Sp* (increased a_0). As the temperature is increased from a point well below the HLB temperature, therefore (*Figure 5*), the natural radius of curvature decreases continuously, passing through zero mean-curvature at the HLB temperature in the single-phase region and becoming positive above it. In order to maintain the natural radius of curvature, r_N, at any temperature, the microemulsion phase takes up, or expels, some of the internalized phase. Thus as the temperature is increased to the HLB temperature, the oil-continuous microemulsion phase takes up water. The volume of water solubilized is maximal in the single-phase region where, in order to maintain zero mean-curvature, it is roughly equal to the volume of the oil-phase. Above the HLB temperature the microemulsion phase becomes water-continuous and expels oil.

Compared with AOT and other ionic surfactant systems, the temperature-phase diagram for non-ionic surfactant systems is inverted so that increasing the temperature drives a Winsor II → IV → I transition, as described by Lichterfield, Schmeling and Strey (1986) for $C_{12}E_5$ systems. This is probably because the effect of increasing temperature is to dehydrate the polar headgroups, so causing a decrease in a_0 (increase in *Sp*). In other words, the effect of increasing temperature is to make the surfactant more hydrophobic, instead of more hydrophilic.

The presence of electrolyte also has a profound effect on the phase-behaviour of ionic surfactant systems (Ghosh and Miller, 1987; Shinoda and Kunieda, 1987). A salt-phase plot (2 wt% AOT, 42°C) is shown in *Figure 7*. The plot is characterized by a three-phase channel running parallel to the abscissa and centred at approximately 0.7 wt% (120 mM) NaCl. The effect of increasing the salt concentration is to drive a Winsor I → III → II phase transition. Once again, the natural radius of curvature is achieved by uptake/expulsion of the internalized phase. The salt effect can also be explained in terms of its effect on the natural curvature of the surfactant film. Increasing the salt concentration causes an increase in *Sp* (decrease in a_0 due to more

effective shielding). Therefore curvature increases from a negative value (favouring water-continuous microemulsions) to a positive one (favouring oil-continuous), passing through zero mean-curvature in the Winsor III channel, as the salt concentration is increased.

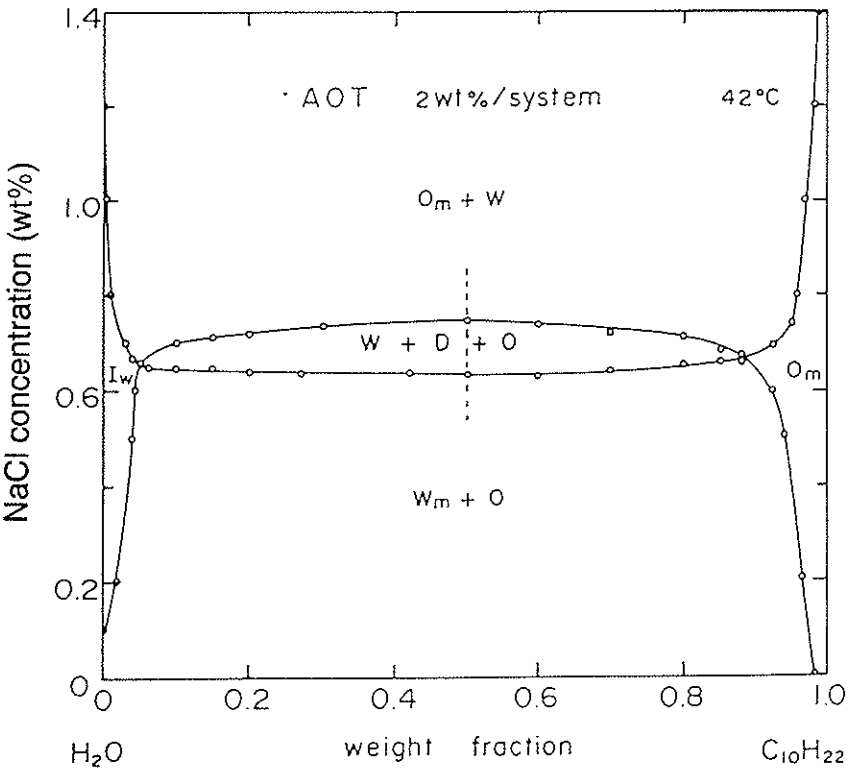


Figure 7. Salt-phase plot for the water–AOT–decane system at 42°C. Nomenclature as for *Figure 4*. (From Shinoda, K. and Kunieda, H. *Journal of Colloid and Interface Science* **118**, 586–589 (1987), with permission.)

Quaternary systems: the effect of co-surfactants

Another way of modulating surfactant curvature is by the addition of a co-surfactant. Typical co-surfactants for w/o microemulsions include medium-chain alcohols and polarizable small molecules such as chloroform and benzene. Like surfactants, co-surfactants are amphipathic but are much more oil soluble and partition extensively between the interphase and the bulk oil-phase. The interfacial surfactant:co-surfactant ratio can be measured, at least for compositions close to the O_m phase-boundary, using a titration method (Bowcott and Schulman, 1955). The interphase surfactant:co-surfactant ratio is usually relatively low. Values of around 2, are reported for both the oleate–benzene–water–hexanol system (Bowcott and Schulman, 1955) and the CTAB–octane–water–hexanol system (Hilhorst *et al.*, 1984).

Co-surfactants act at two levels. In the first place they complement the action of a surfactant in lowering the surface tension sufficiently for microemulsion formation to

occur. Secondly, they dictate structure in the surfactant phase by modulating interfacial curvature. This second property is easily understood in terms of the *Sp* of the surfactant/co-surfactant combination. In the case of alcohols, for example, the hydroxyl group contributes to a_0 and there is a contribution to v and l_c from the alkyl chain. Short-chain alcohols make only a small contribution to v and l_c from the alkyl chain. Short-chain alcohols make only a small contribution to v and the net result is that *Sp* decreases. As the chain-length increases, the contribution to v increases (that to a_0 remains constant) and *Sp* increases. When the co-surfactant chain-length exceeds that of the surfactant itself there is a contribution l_c, as well as to a_0 and v, and *Sp* decreases again. Hence *Sp* passes through a maximum (most likely to stabilize oil-continuous microemulsions) as the alkanol chain-length is increased.

The effects of temperature and additives on surfactant curvature are summarized in *Table 1*.

Table 1. Factors influencing the curvature and *Sp* (equation 2) of surfactant monolayers (after Fletcher and Parrot, 1989)

Variable	Effect on curvature	Reason
Electrolyte concentration	More negative (higher *Sp*) as electrolyte concentration increases	More effective shielding reduces a_0
Oil (alkane) type	More positive (lower *Sp*) as alkane chain-length increases	Alkane penetration (solvation) of surfactant tails decreases with increasing chain-length
Co-surfactant (alkanol) chain-length	More negative (higher *Sp*) as chain-length increases until $l_{ALKANOL} = l_{SURFACTANT}$; then more positive	Co-surfactant headgroup contribution to a_0 constant; tail contribution to v increases with $l_{ALKANOL}$; *Sp* increases. l_c also increases when $l_{ALKANOL} > l_{SURFACTANT}$, then *Sp* decreases again
Temperature (ionic surfactants)	More positive with increasing temperature	Surfactant/counterion dissociation increases
Temperature (non-ionic surfactants)	More negative with increase temperature	Headgroup size reduced due to dehydration

Consideration of phase-behaviour is extremely important from an applications point-of-view. For example, in the design of a microemulsion reactor system with provision for product recovery by phase separation, a composition within the single-phase channel (*Figure 5*) is ideal, since post-reaction phase separation can be initiated by changing the temperature by just a few degrees. In this way the energy input required to effect the phase separation is minimized (obviously, much larger temperature changes are necessary to induce phase separation at compositions far from phase boundaries). In the case of AOT, and other systems prepared with ionic surfactants, an increase in temperature (triggering IV $\rightarrow$ II) is appropriate for isolating a nearly pure (water, surfactant-free) oil-phase containing apolar reaction products (*Figure 6*). In addition, the single-phase channel, in extending right across the diagram, offers the opportunity for close optimization of composition to suit the reaction under consideration (through, for example, optimization of biocatalyst activity and stability; substrate and cofactor solubility).

Further opportunities for fine-tuning phase-behaviour include changing the type of oil (in the case of AOT microemulsions, for example, the HLB temperature shifts downwards with increasing alkane chain-length) (Fletcher, Howe and Robinson, 1987). Changing an ionic for a non-ionic surfactant provides the opportunity to induce phase separation (IV → II) by decreasing, rather than increasing, the temperature. Non-ionic surfactants may therefore be used if product extraction with recovery and re-use of thermolabile enzymes is desirable. In the case of the Brij/Lubrol W type surfactants (*CiEj*), there is the opportunity for optimization of HLB temperature by systematic variation of both alkyl tail length, *i*, and headgroup length, *j*.

The effect of salt on phase-behaviour is important in the design of Winsor II systems, e.g. for protein purification by liquid–liquid extraction; one method of modulating phase-transfer characteristics is to alter the ionic strength, (p. 310). Here an understanding of phase behaviour is necessary to avoid phase inversion with loss of the w/o microemulsion phase.

Structure in the O_m domain

The presence of a transparent, isotropic, O_m phase is consistent at the macroscopic level with structural types ranging from 'pure' droplets (as originally conceptualized by Hoar and Schulman, 1943) and evolving through various types of bicontinuous structure (Lindman *et al.*, 1989), as the (surfactant+water) volume fraction increases (i.e. as one moves away from the oil-corner of the phase diagram). Detailed physical studies are required to establish the relationship between structure and composition. Scattering techniques (neutrons, light or X-rays) are usually employed to give a first-approximation analysis. However, since these techniques usually require the fitting of data to a structural model, the results are not necessarily unambiguous (Langevin, 1989; Lindman *et al.*, 1989). For this reason, the best-understood microemulsions are those for which more than one of the above techniques has been applied, and for which basic structural analyses have been complemented with additional information, the most useful being macroscopic self-diffusion coefficients measured using Fourier transform ^{13}C NMR relaxation spectroscopy (Lindman, Stilbs and Moseley, 1981; Lindman *et al.*, 1983). This technique permits the simultaneous measurement of diffusion coefficients of all the components of the system over distances (several hundred micrometres) which are large compared with the dimensions of microemulsion structural domains, hence providing information about the dynamic behaviour of the microemulsion components which is missing from the static picture obtained from scattering measurements. The principle is that molecules in the continuous phase(s) will have macroscopic self-diffusion coefficients approximately equal to that in a structureless fluid (of the order of 10^{-9} m^2 s^{-1}), whereas those contained within a domain will diffuse perhaps 10^2 times more slowly. Furthermore, in the bicontinuous case, it is possible to distinguish between alternative structures since each of these has a characteristic set of diffusion coefficients (Lindman *et al.*, 1989).

In the particular case of the AOT system, low-angle X-ray scattering (Ekwall, Mandell and Fontell, 1970), NMR self-diffusion (Lindman, Stilbs and Moseley, 1981) and SANS (Kotlarchyk *et al.*, 1984; Huang and Kotlarchyk, 1986) support a droplets-type structure deep into the ternary phase diagram, at ambient temperatures and moderate-to-high ionic strengths. This behaviour is quite remarkable when it is

considered that it seems to be the case even when the (surfactant+water) volume fraction exceeds 0.74, the packing maximum for a body-centred cubic arrangement of spheres (Ekwall, Mandell and Fontell, 1970). It seems that under these conditions the droplets become distorted (non-spherical) in shape, rather than simply fusing together to form a bicontinuous structure. For the majority of systems, however, NMR self-diffusion measurements clearly indicate an evolution from droplets to bicontinuous structure as the (water+surfactant) volume-fraction increases (Lindman, Stilbs and Moseley, 1981; Lindman *et al.*, 1983).

Several other ionic and non-ionic systems consist of droplets at low (surfactant+water) volume-fractions, including the CTAB–water–(chloroform+iso-octane) system (Lang *et al.*, 1990), water–hexane microemulsions stabilized by the alkylpolyoxyethylenes $C_{10}E_4$ and $C_{12}E_5$ (Fletcher and Horsup, 1992) and the poly(oxyethylene) sorbitan trioleate (Tween 85)–water–hexane–isopropanol system (Komives, Lilley and Russell, 1994). Detailed studies on ternary DDAB microemulsions indicate droplets at low (water+DDAB) volume fractions; at higher volume fractions (precise values depend on the oil), structure is best described in terms of a bicontinuous structure consisting of interconnected water channels of the type shown in *Figure 1*$_c$. The dimensions (radius of curvature) of the channels, is are strongly dependent on the oil type (Chen *et al.*, 1986). The water–Triton-X-100–(hexane+benzene 1:1v/v)) system seems to behave similarly (Zhu, Wu and Schelly, 1992).

Droplets-type microemulsions: the aggregation model

AOT microemulsions obey a very well-known structural model which may be referred to as the 'aggregation' model (Nicholson and Clark, 1984), or as the 'equipartition' model (Zulauf and Eicke, 1979; Eicke and Kubik, 1980). It has the following features:

1. the discontinuous subphase is a monodispersion of spherical water droplets, each bounded by a monolayer of surfactant;
2. all surfactant is located at the surface of the water droplet;
3. each surfactant molecule occupies the same surface area at the droplet surface;
4. each water molecule occupies the same average volume.

Microemulsions obeying this model have structural features which can be deduced from purely geometrical considerations (Hoar and Schulman, 1943; Luisi *et al.*, 1988). The radius, r_{wp}, of the water-pool is given by:

$$r_{wp} = \frac{3v_{H_2O}}{a_0} . R \qquad \text{Eq. (3)}$$

where v_{H_2O} is the volume of a single water molecule (usually taken to be 0.03 nm^3). If v_{H_2O} and a_0 are independent of R, equation (3) predicts a linear dependence of *rwp* on the compositional parameter R. The hydrodynamic radius, *rh* of the droplet is

$$r_h = r_{wp} + l_c. \qquad \text{Eq. (4)}$$

The number of water molecules per droplet is

$$N_{H_2O} = \frac{4\pi r^3_{wp}}{3v_{H_2O}} \qquad \text{Eq. (5)}$$

The number of surfactant molecules per droplet is

$$N_{AOT} = \frac{N_{H_2O}}{R} \qquad \text{Eq. (6)}$$

and the molar droplet concentration is

$$[\bigcirc]_T = \frac{[AOT]}{N_{AOT}} = \frac{[H_2O]}{N_{H_2O}} \qquad \text{Eq. (7a)}$$

It is also worth noting that (combining equations 3 and 5 and substituting for N_{H_2O} in equation 7a),

$$[\bigcirc]_T = \frac{K[H_2O]}{R^3} \qquad \text{Eq. (7b)}$$

where constant $K = a_0^3/36\pi v_{H_2O}^2$. It follows from equations (7a) and (7b) that the droplet concentration and R may be varied independently of each other.

It is useful to relate these various manipulations to the phase diagram (*Figure 4*). If one traverses the O_m along a linear path which begins at a point on the water–surfactant axis and passes through the oil-corner, R, and therefore the droplet size, remains constant and the droplet concentration changes (decreasing in the direction of the oil-corner). The value of R is determined by the point of origin of the line on the water–surfactant axis. On the other hand, if one follows a linear path originating at the oil–surfactant axis and passing through the water-corner, R increases in direct proportion to the water volume-fraction at constant surfactant concentration. From mass-balance considerations, the droplet concentration also decreases with increasing R along this path. These are the two most common systematic variations of microemulsion composition adopted for experimental purposes. Indeed, the validity or otherwise of the aggregation model may be checked, for any given system, by measuring the droplet size along these paths. Along the oil-path, the droplets do not change in size; along the water-path, the water-pool size is directly proportional to R (equation 3).

Droplet sizes, measured using small-angle neutron scattering (SANS) (Robinson *et al.*, 1984; Huang and Kotlarchyk, 1986); Huang, Sung and Wu, 1989), photon correlation spectroscopy (PCS) (Zulauf and Eicke, 1979; Nicholson and Clarke, 1984; Aveyard, Binks and Mead, 1986; Aveyard *et al.*, 1986); small-angle X-ray scattering (SAXS) (North *et al.*, 1986; Pileni, 1989), and nuclear magnetic resonance (NMR) (Maitra, 1984) are consistent with the aggregation model. Thus, at ambient temperatures, for $R = 5$–50, the water–AOT–*n*-heptane system obeys very well the equation $r_{wp} = 0.15\,R$ nm and the water–AOT–decane system obeys the equation $r_{wp} = 0.12\,R$ nm. From equation (3), values for a_0 of approximately 0.58 nm^2 and 0.75 nm^2 are obtained for the heptane and decane systems, respectively, assuming $v_{H_2O} = 0.03$ nm^3.

Time-resolved quenching of flurorescence, or of hydrated electrons (generated by

pulsed radiolysis), provides a direct measure of the droplet concentration, rather than of the droplet size (which can then be estimated from mass-balance considerations). Droplet sizes calculated for AOT microemulsions using these techniques are in very good agreement with those measured using scattering techniques (Bridge and Fletcher, 1983; Pileni *et al.*, 1984).

Use of equations (3)–(7) permits the calculation of N_{AOT}, N_{H_2O} and the droplet concentration given size data. Calculations of this kind are most illuminating. Although the droplet radius is directly proportional to R, the number of water molecules per droplet is directly proportional to $R^{1/3}$, hence the droplets 'grow' very quickly as R is increased (Nicholson and Clarke, 1984). For example, $R = 10$ droplets have a radius of 1.5 nm and therefore contain about 470 molecules (equation 5); $R = 20$ droplets have twice the radius and contain around 3800 molecules. $R = 50$ droplets each contain about 60 000 water molecules. At the same time, mass conservation requires that, assuming the surfactant concentration is held constant as R is varied, the droplet concentration decreases. Thus if [AOT] = 0.1 M (a typical working concentration), the droplet concentrations are 2100, 520 and 85 μM at R = 10, 20 and 50. Aggregation of water has important consequences for the dependence of the water activity, and hence for the rates of chemical and enzyme-catalysed reactions, on R.

The effects of temperature on phase-behaviour are reflected at the microscopic level on the structure. In AOT microemulsions, for example, phase separation at high temperatures arises because droplets grow very large and ultimately coalesce, probably because the surfactant becomes sufficiently hydrophilic (very high HLB; $Sp >> 1$) that it becomes soluble in the aqueous phase and desorbs from the interface (Zulauf and Eicke, 1979). Similarly the phase separation at low temperatures occurs because the surfactant becomes oil-soluble.

Phase-behaviour in phospholipid microemulsions

A number of amphipathic compounds produced by living organisms have been shown to be capable of stabilizing w/o microemulsions, including lecithin (Misiorowski and Wells, 1974; Poon and Wells, 1974) and triacylglucolipids (Lindblom *et al.*, 1993, reviewed by Walde *et al.*, 1990). This class of microemulsions is becoming increasingly important due to the status of phospholipids as biological surfactants since they are more likely to be approved in connection with food- and drug-related applications than are synthetic surfactants. In addition, lecithin itself is an important foodstuff and there is much interest in biotransformations involving this material (for example, in the upgrading of low-quality lecithins, (e.g. palm oil), to food-grade cocoa butter by lipase-catalysed trans- or interesterification reactions (Macrae, 1985)). Here microemulsions have a role to play in facilitating such biotransformations since the surface-active substrate molecules forming the interfacial monolayer are equally accessible to the biocatalyst in the aqueous subphase.

A number of studies of the phase-behaviour of lecithin–water–organic solvent systems have been published. Most studies utilize purified (triglyceride-free) lecithin from egg or soybean. The purest commercially available soybean lecithin is about 97% phosphatidylcholines, the remainder being other phosphatidic acid derivatives. Naturally occurring phosphatidylcholines are somewhat heterogeneous with respect to the

fatty acyl content (chain-length and degree of unsaturation). Synthetic phospholipids of defined fatty acyl composition are available have been used in a few studies.

Lecithin yields an O_m phase in water–oil ternary systems with a wide range of oils of varying polarities, including diethylether, benzene and cyclohexane, and aliphatics such as hexane, octane and decane (Shervani *et al.*, 1991). With relatively polar solvents (benzene, diethylether) the O_m is a non-viscous isotropic oil-continuous droplets type microemulsion (Kumar and Raghunathan, 1986). In alkanes, the phase-behaviour is more complex, and the O_m is a liquid microemulsion only at low R. As R is increased above a critical (alkane-specific) value, the system becomes first viscous, due to the formation of non-spherical structures, and then an optically transparent, isotropic gel. The liquid–gel transition occurs over a very narrow range ($<2R$). For cyclohexane the transition occurs around $R = 4$; for octane around $R = 2$. The transition is depressed (shifted to higher R) on addition of co-surfactants; and is reversed by increasing the temperature.

Synthetic phosphatidyl cholines prepared with short- or medium-chain fatty acyl groups ($C < 8$) do not form gels and much higher degrees of water-solubilization are obtained with these 'balanced' surfactants. Lecithin carrying two pentanoate (C_5) chains can solubilize up to 80 mol water per mol lipid (in the presence of a medium-chain alcohol co-surfactant). Longer-chain (C_6, C_7) lecithins are much less effective, maximally solubilizing about 20 mol water mol^{-1} (Peng and Luisi, 1990).

The gels, like liquid microemulsions, are transparent and isotropic (non-birefringent) and therefore are not liquid-crystalline in nature, a result confirmed by SANS and SAXS (Luisi *et al.*, 1990). However, they are characterized by extremely high shear viscosities (up to 10^6 cp), even at relatively low (lecithin+water) volume fractions (Scartazzini and Luisi, 1988). A combined light- and neutron-scattering study is consistent with the existence of extended cylindrical and semiflexible rods (Capitani *et al.*, 1991; Schurtenberger *et al.*, 1992).

Waterless microemulsions, surfactantless microemulsions

In addition to water, several other polar, oil-immiscible solvents are known to form microemulsions. Microemulsion phases have been mapped for the CTAB–formamide–(cyclohexane+butan-1-ol) system (Rico and Lattes, 1984) and for the AOT–glycerol–*n*-heptane and CTAB–glycerol–(*n*-heptane+chloroform) systems (Fletcher, Galal and Robinson, 1984, 1985). The two glycerol systems apparently obey the aggregation model, with $r_h = 1.72 + 0.88\,R$ and $1.8 + 1.2\,R$ nm, respectively. Microemulsions with polar subphases consisting of glycerol–water mixtures in various ratios may also be prepared.

Surfactantless microemulsions are prepared with relatively large amounts of a co-surfactant. Typically, the amphipath is a medium chain-length aliphatic alcohol such as propan-2-ol. The ternary phase diagram for the hexane/propan-2-ol/water system is shown in *Figure 8* (Khmelnitsky *et al.*, 1986, 1989b). The phase-diagram is divided into four major domains, a three-phase region (A), a single-phase oil+surfactant-continuous microemulsion region (B), a single-phase region which consists of loosely associated H-bonded aggregates of hydrated alcohol (C) and true ternary solutions (D). The shaded region indicates an apparently droplets-like region within the microemulsion domain. These systems are not much studied. The absence of

surfactant may be important in certain applications where even traces of surfactants are undesirable. Possibilities for post-reaction product recovery lie in the possibility for inducing a phase transition (B) → (A), for example, by addition of more amphipath or water, as required.

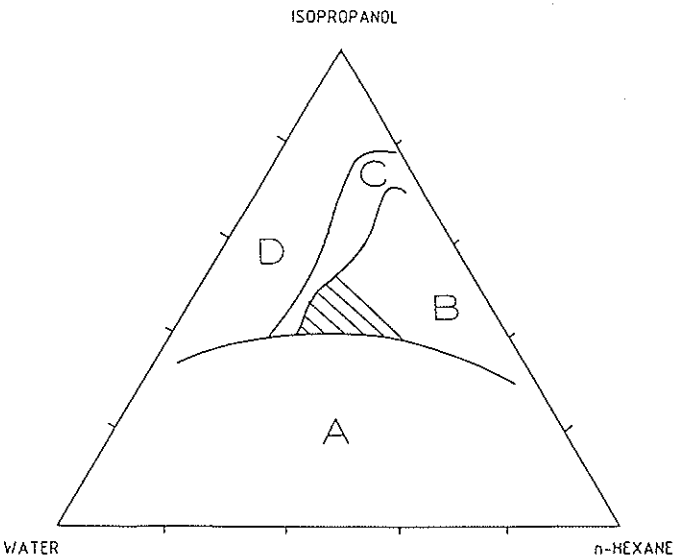


Figure 8. Ternary phase diagram for the hexane/isopropanol/water system. The apparently droplets-type microemulsion region is shaded. (Redrawn from Khmelnitsky Yu. L., van Hoek, A. Veeger, C. and Visser, A.J.W.G. (1989b) *Journal of Physical Chemistry* **93**, 872–878 © (1989) American Chemical Society, with permission.)

DYNAMIC PROPERTIES

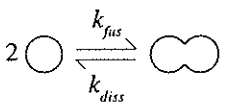
Droplet dynamics

Individual droplets are very mobile structures. Quasi-elastic neutron scattering studies indicate that water molecules diffuse rapidly within the confines of the droplet, and the AOT molecules diffuse laterally over the droplet surface, on the sub-nanosecond time scale (Fletcher, Robinson and Tabony, 1986). ^{13}C NMR studies suggest that the AOT molecules additionally undergo restricted rotational motions about the long-axis. The droplets are in continuous random (Brownian) motion and are frequently involved in diffusion-controlled inelastic collisions with each other. The interdroplet collisions can be described essentially as hard-sphere interactions, with a small attractive potential, believed to be due to van der Waals interactions between the surfactant tails of the colliding droplets (Brouwer, Nieuwenhuis and Kops-Werkhoven, 1980; Cazabat and Langevin, 1981; Huang *et al.*, 1984).

Exchange

Perhaps the most important dynamic element of microemulsion behaviour is the exchange process which permits water-soluble solutes to transfer between droplets. Exchange can be demonstrated by choosing a chemical reaction of the type A + B →

P, where A and B are water soluble. If two microemulsions, one containing A and the other B, are mixed, formation of product, P, will be observed (Eicke, Shepherd and Steinemann, 1976). Fast-reaction techniques have been used to study the exchange mechanism for AOT microemulsions in some detail (Atik and Thomas, 1981; Fletcher and Robinson, 1981; Fletcher, Howe and Robinson, 1987). Data for several different kinds of fast chemical reaction are consistent with the formation of a fused dimer in sufficiently energetic colisions between pairs of droplets. The dimer breaks down rapidly to regenerate a pair of 'daughter' droplets compositionally identical to the original pair. The second-order rate constant for dimer formation, k_{fus} (k-fusion) is of the order of 10^7 M^{-1} s^{-1} at 25°C, which may be compared with a value of *c.* 10^{10} M^{-1} s^{-1} for the diffusion-controlled encounter rate-constant, indicating approximately 0.1% efficiency for dimer formation. This corresponds to a between-collisions lifetime on the millisecond scale, given typical microemulsion droplet concentrations. The lifetime of the dimer has never been measured directly, but probably lies in the microsecond range, long enough for thorough intermixing of the dimer contents, but too short to undergo further collision events which might lead to the formation of trimers, etc. In effect, droplets and dimers and their contents are in equilibrium,



where $k_{eq} = k_{diss}/k_{fus} << 1$. Measurements of activation parameters imply a 'dumbell'-shaped transition structure, with a relatively small contact area, rather than a spherical unit (Eicke, Shepherd and Steinemenn, 1976; Fletcher, Howe and Robinson, 1987).

Some measurements of k_{fus} have been made for other microemulsions. Using fluorescence quenching of Zn-porphyrin-cytochrome *c* by ferricyanide, Vos, Lavalette and Visser (1987) reported values of 1–5 × 10^7 M^{-1} s^{-1}, depending on *R*, for CTAB microemulsions with hexanol as a co-surfactant. Using time-resolved fluorescence quenching of Tris-(2,2'bipyridyl)ruthenium(II) by methylviologen, Fletcher and Horsup (1992) reported values of the order of 10^8–10^9 M^{-1} s^{-1} for several different alkylpolyoxyether (i.e. Brij/Lubrol W) surfactants. Note that in the last case the exchange rates are close to the diffusion-limited maximum.

Assuming that exchange of solutes between droplets occurs by the mechanism just described, equations can be derived for the dependence of the rates of chemical and enzyme-catalysed reactions involving water-soluble reactants (i.e. confined within the aqueous subphase), on the droplet size and concentration and on the exchange rate. The key to the solution of the rate equation is to relate the observed rate to the rate within the droplet. Intuitively, the observed rate must be a function of the rate of the reaction within the droplet and of the number of droplets (per unit volume of microemulsion) in which the reaction is occurring (per unit time). This can be done using the steady-state approximation, i.e. by assuming that the number of droplets within which the reaction is occurring is constant during the period over which the rate is measured (Oldfield, 1991). *Figure 9* shows the scheme for the reaction, A + B → P. The reaction between A and B, initially confined within separate droplets (which is inevitably the case under steady-state conditions) requires that A and B first exchange into the same droplet. The reaction may occur either in the dimer (type (i)

kinetics (Oldfield *et al.*, 1992), or later, in a daughter droplet (type (ii) kinetics), depending on the rate of dimer dissociation relative to that of the chemical reaction. The solution yields an expression for the apparent second-order rate constant, k_{ex}, for the chemical reaction, expressed in 'overall' concentration units ($M^{-1m}\,s^{-1}$, that is, litre microemulsion per mol per second), in terms of k_{+1}^{obs}, and k_{+2}^{obs}, (units, s^{-1}) the first-order rate constants for the conversion of droplet (A,B) to (P) and of (A,B) to (P), respectively. The equation is completed by introducing an expression for the rate within a single droplet. In the simplest case, when there is only one each of A and B in the droplet, k_{+1}^{obs} is related to k_{+1}^{d}, the second-order rate constants for the reaction within the droplet (units, $M^{-1d}\,s^{-1}$; that is, litre water (within a single droplet) per mol per second), by the expression,

$$k_{+1}^{obs} = k_{+1}^{d}\ \frac{1}{N_A V_d} \qquad \text{Eq. (8)}$$

where V_d is the volume of the droplet and N_A is Avogadro's number. The same relationship holds for k_{+2}^{d} and k_{+1}^{obs}. The complete equation is,

$$k_{ex} = \left(\frac{\dfrac{k_{+1}^{d}}{N_A 2V_d} + \dfrac{k_{+2}^{d} k_{diss}}{k_{+2}^{d} + k_{fus}\phi^{aq}}}{\dfrac{2k_{diss}}{k_{fus}} + \dfrac{k_{+1}^{d}}{k_{fus} N_A 2V_d} - \dfrac{k_{diss}\phi^{aq}}{k_{+2}^{d} + k_{fus}\phi^{aq}}} \right) \qquad \text{Eq. (9)}$$

and ϕ^{aq} replaces the product [◯] T. $N_A V_d$ (the volume fraction of the aqueous subphase is the product of the molar volume of each droplet and the total droplet concentration). The equation in this form only holds for droplets containing at most one each of A and B, and requires some modification in order to accommodate higher-order occupancies.

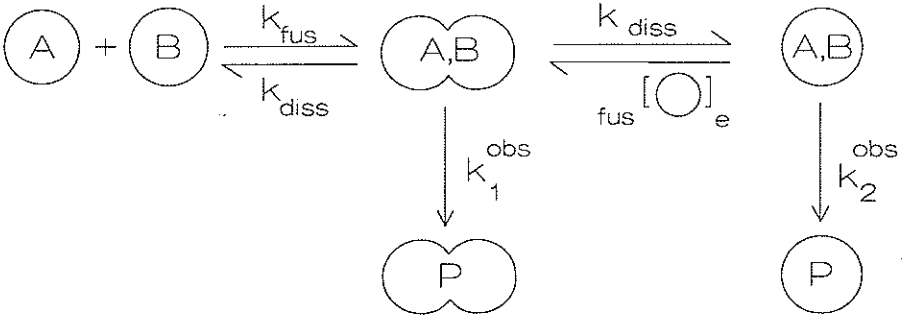


Figure 9. Scheme for the chemical reaction, A + B → P in an oil-continuous microemulsion. (Reproduced from Oldfield, C., Otero, C., Rua, M.L. and Ballesteros, A. (1992). In *Biocatalysis in Non-Conventional Media* (J. Tramper, Ed.), pp. 189–198. Elsevier, Amsterdam, with permission.

There are two interesting reductions of equation (9). When $k_{diss} << k_{+1}^{d}/(N_A V_d)$, such that the reaction can occur only within the dimer,

$$k_{ex} = k_{fus}. \qquad \text{Eq. (10)}$$

Hence this condition must be satisfied by a chemical reaction in order to make it useful for the measurement of k_{fus}. On the other hand, if $k_{diss} >> k_{+1}^{d}/(N_A V_d)$, so that the reaction can only occur after the dimer has dissociated (i.e. within one of the daughter-droplets derived from it) then it is expected that,

$$k_{ex} = \frac{k_{+2}^{d}}{\frac{2k_{+2}^{a}}{k_{fus}} + \phi^{aq}}. \qquad \text{Eq. (11)}$$

The factor '2' appears as a statistical factor since there are two alternative fates for (A,B) dissociation, to (A,B) + ◯ or to (A) + (B), only the former being potentially product-forming. Equation (11) predicts a linear dependence of $1/k_{ex}$ on ϕ^{aq}. Thus k_{ex} is expected to decrease as ϕ^{aq} increases, i.e. as the droplet concentration, and hence the rate of the non-productive exchange-out reaction, (A,B) → (A) + (B) ($= k_{fus}$ [(A,B)][◯]$_T$), increases. The rate of product formation therefore decreases, reaching a minimum when $\phi^{aq} = 1$, the (hypothetical) microemulsion with the highest possible droplet concentration. Substituting a smaller number of larger droplets at constant ϕ^{aq} does not help because the rate of P-formation within the larger droplet (equation 8) is correspondingly slower. Equation (10) therefore predicts maximal reaction rates in microemulsion preparations containing a low concentration of small droplets (minimal exchange-out rate; maximal rate of reaction between A and B within a droplet). since there is no compartmentalization in aqueous solution, and hence no equivalent intermediate (A,B), second-order rate constants in microemulsions and in aqueous solution cannot be compared directly. These observations have important implications for the rates of enzyme-catalysed reactions involving water-soluble substrates (pp. 297–301).

NATURE OF THE SOLUBILIZED WATER

Water structure

A large number of studies have been made which indicate that, in comparison with bulk water, the structure and other physical properties of microemulsified water is perturbed to an extent which depends on R. Direct measurement of water activity, a_w, in AOT microemulsions (Higuchi and Mistra, 1962; Kubik, Eicke and Jönsson, 1982) clearly show that the activity increases steeply and asymptotically, from about 0.4 at $R = 1$, approximating that of bulk water when $R > 12$ (*Figure 10*). This behaviour is consistent with the involvement of several water molecules in the hydration of the surfactant headgroup and its sodium counterion (this 'bound' water has a lower vapour pressure than non-bound water). Direct measurement of the hydration requirement of the AOT and its sodium counterion in w/o microemulsions, using NMR, electron paramagnetic resonance (EPR) and differential scanning calorimetry (Wong, Thomas and Nowak, 1977; Hauser *et al.*, 1989) indicates that between two and six water molecules are relatively tightly bound to Na-AOT, with several others being more weakly bound.

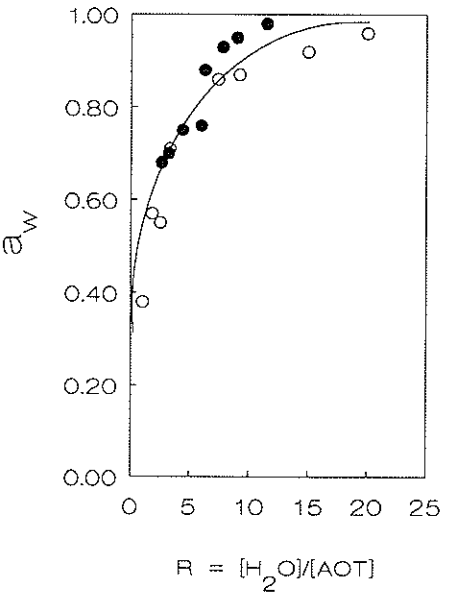


Figure 10. Dependence of water activity (a_w) on R (=[H_2O]/[AOT]). (O) Measured by vapour pressure osmometry (Kubik, Eicke and Jönsson (1982)); (●) Derived from proton transfer rates. Redrawn from Bardez, E., Goguillon, B.-T., Keh, E. and Valeur, B. (1984). *Journal of Physical Chemistry* **88**, 1909–1913 © (1984) American Chemical Society, with permission.

Water molecules involved in hydration of ions experience steric constraints which prevent the formation of the preferred tetrahedral clusters characteristic of bulk water, and this is clearly seen in ^{1}H NMR studies, which show chemical shifts characteristic of a non-hydrogen-bonded water structure (Wong, Thomas and Nowak, 1977; Gierasch *et al.*, 1984), in the spectral behaviour of hydrogen bond-sensitive probes, which indicate an excess of hydrogen bonding capacity (Wong, Thomas nd Nowak, 1977; Oldfield, Robinson and Freedman, 1990). Steady-state fluorescence and EPR studies of dissolved probes indicate a reduction in solvent dielectric (Wong, Thomas and Grätzel, 1976; Haering, Luisi and Hauser, 1988). As with the activity, all of these parameters relax asymptotically to near bulk-water values as R increases.

In general, it is considered that bulk-like water is absent at $R < 10$ in AOT microemulsions, but accumulates rapidly, as R increases beyond this threshold, predominating at $R > 20$. In these higher-R droplets water molecules can be considered to exchange rapidly (on the sub-nanosecond time scale) between the 'bound' (peripheral, solvating) and non-bound (bulk-water) states (Fletcher, Robinson and Tabony, 1986). The peripheral layer is considered to consist of the surfactant headgroups, those tightly bound ions forming the Helmholtz plane, and their water of hydration. The thickness of the peripheral layer is about 0.6–0.8 nm, for AOT systems (Zinsli, 1979).

The aggregation model helps to explain why the physical properties of the microdispersed water asymptote steeply to bulk-water values with increasing R. At $R = 10$, there can be no 'free' water, if the hydration requirement is 10 mol water per mol AOT; hence all of the 470 water molecules per droplet (equation 5) are involved in

solvation. On the other hand, in the $R = 15$ droplet ($N_{AOT} = 166$; $N_{H_2O} = 2500$) the droplet may reasonably be expected to contain about 800 free water molecules. At $R = 20$ ($N_{AOT} = 190$; $N_{H_2O} = 3800$), the droplet should contain about 1800 free water molecules; and in the $R = 50$ droplet ($N_{AOT} = 1200$; $N_{H_2O} = 60\,000$) there should be a pool of about 48 000 free water molecules.

The same general properties, strong association of water with surfactant headgroups, changes in hydrogen bonding structure and micropolarity, characterize microemulsions prepared with other surfactants, including phosphatidylcholine (Wells, 1974; Boicelli, Giomini and Giuliani, 1984; Kumar and Raghunathan, 1986), oleate (Senatra, Galorielli and Guarini, 1986) and Triton X-100 (Zhu, Wu and Schelly, 1992a).

Ion distribution within the water-pool

^{23}Na NMR studies (Wong, Thomas and Grätzel, 1977) indicate that most (at least 70–80%) of the total sodium counterions are rather tightly bound to the sulphonate headgroups of the AOT molecules, forming a Helmholtz double-layer (a large degree of charge neutralization at the interface is, *a priori*, an essential prerequisite for the stability of the interface). By analogy with classical theories of charged interfaces, it is expected that the remaining, more mobile, ions are distributed radially through the droplet, along the exponential decay of the electrical field generated by the charged interface, so forming a semi-diffuse Gouy–Chapman layer. The form of this distribution is also expected to determine the radial dependence of the solvent polarity and viscosity.

A theoretical study (Karpe and Ruckenstein, 1989) indicates that the field decay, and hence the radial dependence of the sodium ion concentration, is relatively steep, such that most of the ions are confined within the peripheral water layer. The calculated pool concentrations are much lower (a few tens to a few hundred millimolar) than those calculated if the sodium ions were assumed to be evenly distributed through the water-pool (amounting to several molar). Even so, this kind of calculation ignores the rather vexed question of the activity (electrochemical potential) of the ions, and hence the ionic strength within the droplet, a knowledge of which is essential for a real understanding of the influence of the microemulsion environment on reaction rates.

pH determination and control

Another potential consequence of the disruption of water structure is a change in acid–base behaviour of the solubilized water, and hence of dissolved weak acids and bases (buffers, indicators, amino acids). Measurements of the rates of excited state pyranine deprotonation (Bardez *et al.*, 1984) are consistent with the microdispersed water behaving as a progressively weaker base (poorer proton acceptor), as R is decreased below 12. Above $R = 12$ the deprotonation rate is identical to that in bulk water. There is an excellent correlation between a_w values derived from these measurements with the vapour-pressure measurements already referred to (*Figure 10*).

Comparing the pH-dependence of the EPR order-parameter for doxylstearate (a surface-active spin-probe) in buffered and unbuffered AOT microemulsions ($R = 50$), Haering, Luisi and Hauser (1988) concluded that the pH of unbuffered water increases by approximately 1.2 units on microemulsification. This result is consistent

with increases in base:acid ratio of the highly water-soluble indicators, pyranine and 4-nitrophenol-2-sulphonate (author's unpublished work), and is most likely due to ion-exchange of protons for sodium ions at the interface, with an increase in pH (decrease in a_{H^+}), which can be buffered out (El Seoud, Chinelatto and Shimizu, 1982; El Seoud and Chinelatto, 1983). It should be emphasized, however, that changes in acid:base ratio may reflect changes in the indicator pK_a, in the pH, or both, and that these alternatives cannot be distinguished without additional information.

A number of authors have discussed acid–base equilibria in terms of a pH gradient across the droplet following the decay of the interfacial field gradient (El Seoud, Chinelatto and Shimizu, 1982; El Seoud and Chinelatto, 1983; Karpe and Ruckenstein, 1989). This seems unreasonable, since although there clearly should be a concentration gradient (for H^+, Na^+ and all other ions), the thermodynamic activity (electrochemical potential) of H^+ (a_{H^+}), and Na^+ (a_{Na^+}) must be the same at all points within the microemulsion (implying an inverse relation between the field gradient and the activity coefficient), since the microemulsion is an equilibrium system. It seems more reasonable to think in terms of a single microemulsion pH ($pH = -\log 10 a_{H^+}$), with apparent pK_as for solubilized dyes determined by their (weighted) distribution between the subphases of the microemulsion (Oldfield, Robinson and Freedman, 1990). In this context the problem of pH measurement reduces from the apparently complex one of measuring the pH in a tiny water-droplet, to one of designing a probe which faithfully reports a_{H^+} of the bulk microemulsion. It has been reported that the pH, measured using the glass electrode, correlates well with the pH of the aqueous buffer solution used to prepare it (Menger and Saito, 1978). However, it is not obvious that the electrode should reliably report pH in this predominantly non-aqueous system. No further studies along this line have been reported.

van Dijk *et al.* (1992) have described a method of measuring microemulsion pH using the enzyme hydrogenase to catalyse the equilibrium between microemulsion H^+ and the standard hydrogen electrode. The technique cannot be described as routine but the thorough study carried out by these authors (covering four different microemulsion systems and five different buffers in the range pH 5–9), indicates that for the most part only small changes in pH occur when buffered aqueous solutions are solubilized. These results are consistent with ^{31}P NMR studies in AOT and in lecithin microemulsions, which show only minor changes in the base:acid ratio of solubilized phosphate buffers (Smith and Luisi, 1980; Peng and Luisi, 1990). On this basis it was recommended by Smith and Luisi (1980), and has since become convention, that the microemulsion pH should be reported as the pH of the original buffered aqueous solution used to prepare it. This convention should not be extended to unbuffered microemulsions, *vide supra*.

As a practical matter, highly water-soluble buffers are preferable over more apolar buffers since partitioning into the surfactant phase may change its pK_a. The zwitterionic Good's-type buffers are best suited for this purpose (Good *et al.*, 1966). These buffers were originally developed for the purpose of buffering biological systems containing membrane preparations since they are not soluble in phospholipid membranes (and therefore experience no pK_a shift due to partitioning). By direct analogy, therefore, they are expected also to be effective buffers for microemulsions prepared with anionic surfactants. They seem to be equally acceptable for buffering microemulsions prepared with cationic surfactants (Oldfield and Freedman, 1989).

Some early work in AOT microemulsions indicated large upward shifts in pH-profiles for several different enzymes. This phenomenon was traced to the presence of relatively large amounts of acidic contaminants (sodium hydrogen sulphate) arising from the manufacturing process, sufficient to titrate-out added buffer (reviewed by Luisi and Magid, 1986). This acidity of AOT preparations can be checked using a titration technique (Magid, Kon-no and Martin, 1981). The quality of commercially available AOT has improved considerably in recent years and no longer contains significant amounts of titratable impurities.

Enzymes in microemulsions

HISTORICAL OVERVIEW

The first example of an enzyme operating in a microemulsion environment is phospholipase A_2. An assay for this enzyme based on the hydrolysis of phosphati-

Table 2. Main examples of enzymes studied in w/o microemulsions stabilized by AOT

Enzyme	Oil	Nature of study	Reference
α-Chymotrypsin	*n*-Octane	First paper describing enzyme activity in an AOT microemulsion	Martinek *et al.* (1977)
Ribonuclease, HLADH α-chymotrypsin	Iso-octane, *n*-octane	Analytical ultracentrifugation	Bonner, Wolf and Luisi (1980) Levashov *et al.* (1982)
α-Chymotrypsin	*n*-Octane	Kinetics of N-*trans* cinnamoyl imidazole deacylation	Levashov, Klyachko and Martinek (1981)
α-Chymotrypsin	*n*-Octane	Active site spin-labelling	Belonogova *et al.* (1983)
Horseradish peroxidase	*n*-Octane	Kinetics, 'superactivity'	Klyachko, Levashov and Martinek (1984)
Chromobacterium viscosum lipase	*n*-Heptane, *n*-dodecane	Kinetics and stability	Fletcher, Galal and Robinson (1985)
HLADH	Cyclohexane	Kinetics and stability	Larsson, Adlercreutz and Mattiasson (1987)
HLADH	Isooctane	Product recovery, enzyme recycling	Larsson, Adlercreutz and Mattiasson (1990)
α-Chymotrypsin	Toluene+octane	Entrapment in nanogel particles	Khmelnitsky *et al.* (1989a)
Tyrosinase	Iso-octane	Kinetics	Bru *et al.* (1989b)
Rhizopus delemar lipase	Iso-octane	Synthesis of polyol-fatty acid esters	Hayes and Gulari (1992)
Δ^5-3-Ketosteroid isomerase	Iso-octane	Kinetics, stability	Levashov *et al.* (1992)
Penicillium simplissimum lipase	Iso-octane	Stereospecific synthesis of fatty acyl menthol esters	Stamatis, Xenakis and Kolisis (1993)
Horseradish peroxidase	Iso-octane	Synthesis of polyethyl-phenols	Rao *et al.* (1993)
Rhizopus arrhizus lipase	Iso-octane	Kinetics, spectroscopy	Brown, Yada and Marangoni (1993)

dylcholine (PC) in diethylether–PC–water microemulsion system described by Wells and co-workers (Misiorowski and Wells, 1974; Poon and Wells, 1974; Wells, 1974). Interestingly, the surfactant is also the substrate for the enzyme.

The first report of an enzyme functional in a microemulsion system prepared with a synthetic surfactant was α-chymotrypsin in an AOT-stabilized system (Martinek *et al.*, 1977), and confirmed by Menger and Yamada (1979). There followed a rapid succession of papers detailing the solubilization and properties of ribonuclease (Wolf and Luisi, 1979), cytochrome *c* (Douzou, Keh and Balny, 1979), HLADH (Meier and Luisi, 1980) and horseradish peroxidase (Levashov *et al.*, 1980), also in AOT microemulsions. Levashov *et al.* (1982) further demonstrated the activity of enzymes in microemulsions stabilized by CTAB and Brij 56, and the operation of multistep enzyme reactions, such as the conversion of phosphoenolpyruvate to lactate, via pyruvate, catalysed sequentially by pyruvate kinase and lactate dehydrogenase (see also Martinek *et al.*, 1981). These papers together served to give an indication of the potential of microemulsions as an important new class of organic solvent media for enzymes, and in doing so, opened up a new field of research.

By far, the majority of studies have been carried out using AOT microemulsions. In contrast to the several hundred studies of enzymes in these systems (see *Table 2* for some interesting examples), only a handful of studies exist for those stabilized by other kinds of surfactants (*Table 3*).

THE NATURE OF SOLUBILIZED ENZYMES

Incorporation of enzymes into microemulsions

In principle, enzymes may be introduced in three different ways (Luisi and Magid, 1986):

1. by dissolving dry enzyme powder in a pre-formed microemulsion (dry extraction);
2. by transfer from a conjugate aqueous phase (liquid extraction); and
3. by introducing the enzyme as a concentrated aqueous stock solution (injection).

The dry-extraction method is, at least in principle, the most reliable method of measuring the solubilization capacity of a single-phase microemulsion for a given protein; the liquid–liquid extraction method has found an important application in the differential extraction of proteins from aqueous phases as a method of protein purification (p. 308). The injection method is used routinely for the rapid preparation of microemulsions of defined composition.

The dry-extraction procedure simply involves stirring the pre-formed microemulsion with the enzyme powder. The progress of transfer can be followed by monitoring the A_{280} of the microemulsion phase. Excess protein is removed by low-speed centrifugation. The liquid-extraction procedure involves dissolving the protein in a Winsor II system. The system can be stirred to facilitate transfer (the microemulsion+water emulsion separates rapidly when mixing stops). For kinetic studies, the injection method is the fastest and most convenient method. Usually an aliquot of aqueous enzyme stock is injected into a pre-formed microemulsion. This additional material is rapidly incorporated into the microemulsion phase (within a

Table 3. Main examples of enzymes studied in w/o microemulsions stabilized by surfactants other than AOT

Surfactant/co-surfactant	Enzyme	Nature of study	Reference
Phosphatidylcholine	Phospholipase A_2	Routine assay of enzyme activity	Misiorowski and Wells (1974)
CTAB	20β-Hydroxysteroid dehydrogenase	Cofactor regeneration study	Hilhorst *et al.* (1984)
Brij 96	Luciferase	Kinetics	Belyaeva *et al.* (1983)
Triton X-100	Cholesterol oxidase	Kinetics and stability	Lee and Biellmann (1986)
CTAB	Bilirubin oxidase	Kinetics	Oldfield and Freedman (1989)
Brij 96	Polyphenol-oxidase	Kinetics	Sanchez-Ferrar, Bru and Garcia Carmona (1988)
Brij 56	Tryptophanase	Tryptophan synthesis: process engineering	Eggers and Blanch (1988)
TOMAC	Several	Selective protein extraction	Wolbert *et al.* (1989)
$C_{12}E_5$[a]	HLADH	Enzyme/cofactor recycling study	Larsson, Adlercreutz and Matiasson (1990)
Phosphatidylcholine	Trypsin, α-chymotrypsin	Kinetics and stability	Peng and Luisi (1990)
Phosphatidylcholine	Lipase	Entrampment in lecithin microemulsion gels	Scartazzini and Luisi (1990)
Triton X35 + Nonidet P40	Lipoxygenase	Kinetics	Piazza (1992)
Tween 85	Organophosphorus hydrolase	Kinetics	Komives, Lilley and Russell (1994); Komives, Osborne and Russell (1994)

[a] The surfactant used in this example was a single homologue of the Brij/Lubrol W class. The molecular formula is $CH_3(CH_2)_{11}(OCH_2CH_2)_5OH$, abbreviated to $C_{12}E_5$ by convention.

few seconds). This approach minimizes enzyme inactivation arising from exposure to the pre-microemulsion mixture. In some cases (e.g. in fast kinetic studies) it is desirable to prepare enzyme and substrate in separate microemulsions, which may then be mixed together (p. 276).

Size and composition of protein-containing droplets

The types of studies which have been undertaken to study the nature of the solubilized water–protein–surfactant aggregates include (1) measurements of the solubilization capacity of the microemulsion for a protein and (2) application of physical techniques including analytical centrifugation, dynamic light scattering (PCS) and small-angle neutron scattering (SANS) to determine the composition of the host structure.

Detailed studies of solubilization capacity of AOT microemulsions for α-chymotrypsin and LADH were made by Matzke *et al.* (1992). In these experiments the proteins were incorporated, using either solid-phase extraction or injection, into microemulsions buffered at pH 8. The results for both proteins show that, as R is increased, there is a

sudden rise in solubilization beyond a point where the 'original' droplet size (i.e. that in a microemulsion containing no protein, but of otherwise the same composition) very roughly corresponds to the size of the enzyme (*Figure 11*). Different results were obtained for microemulsions prepared by the injection method, especially at low R, where considerably more protein was retained by the microemulsion than anticipated on the basis of the solid-phase extraction data. Most likely microemulsions prepared in this way are metastable (overloaded systems which are kinetically slow to lose excess protein). The differences in the microemulsion solubilization capacities for the two proteins simply reflect the differences in aqueous solubilities.

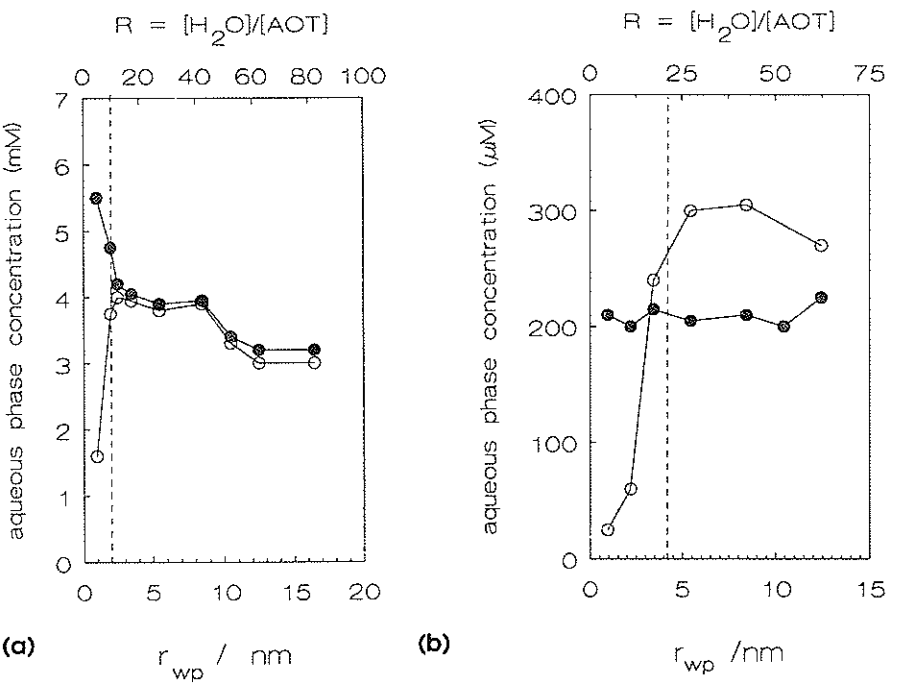


Figure 11. Solubilization of proteins as a function of original droplet size by AOT-microemulsions. (a) α-Chymotrypsin; (b) horse-liver alcohol dehydrogenase. (O) Prepared by dry-extraction; (●) prepared by injection. [AOT] = 50 mM, pH = 8.0 (200 mM[aq] Tris-HCl). The dotted line shows the droplet radius equivalent to that of the enzyme considered as a sphere. (Redrawn from Matzke, S.F., Creagh, A.L., Haynes, C.A., Prausnitz, J.M. and Blanch, H.W. (1992). *Biotechnology and Bioengineering* **40**, 91, by permission of John Wiley & Sons, Inc.)

It is useful to compare the molar solubilization capacities (determined using the solid-extraction method) with the 'original' molar droplet concentration i.e. the concentration in microemulsions prepared with no protein.. The results obtained by Matzke *et al.* (1992) show that at low R the nominal α-chymotrypsin droplets ratio is $<<1$; rising to unity only when R reaches 30. Above $R = 30$, there is (nominally) more than one per original droplet. The solubilization capacity for LADH is always much lower than the droplet concentration (*Figure 12*). The assumption that the droplet concentration remains unchanged on addition of protein is unreasonable, especially in situations where the original droplet size is comparable with, or less than, that of the

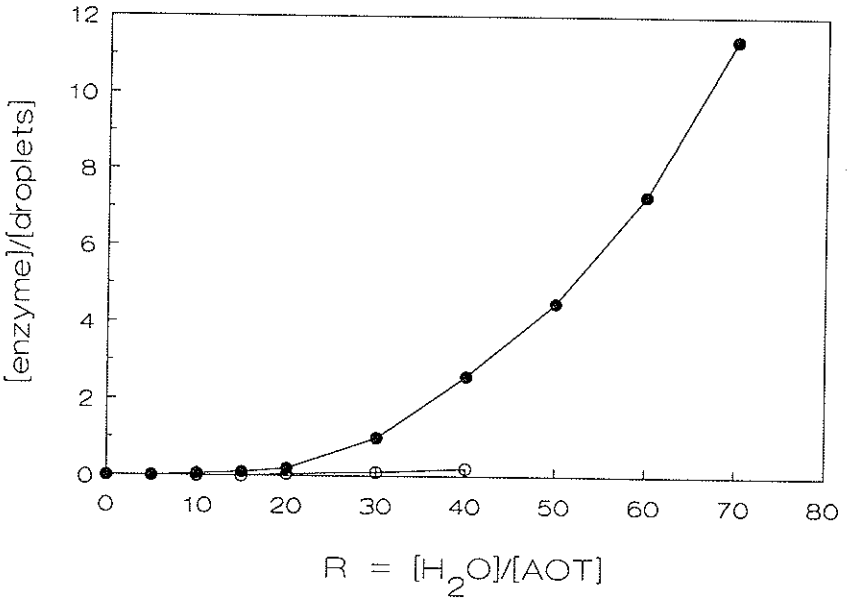


Figure 12. Apparent enzyme droplet ratio for AOT microemulsions saturated with α-chymotrypsin (●) or horse-liver alcohol dehydrogenase (O). (Redrawn from Matzke, S.F., Creagh, A.L., Haynes, C.A., Prausnitz, J.M. and Blanch, H.W. (1992). *Biotechnology and Bioengineering* **40**, 91, by permission of John Wiley & Sons, Inc.)

protein molecule, when solubilization might be expected to involve the aggregation of several droplets. Nevertheless the calculations show that, as a general rule, solubilization is not simply a function of the droplet concentration.

The precise size and composition of enzyme-containing droplets has been studied using analytical ultracentrifugation, which is a powerful tool for studying the sedimentation behaviour of a specific species in a mixed-droplet population. The protein-containing fraction, for example, may be followed by u.v. absorbance at 280 nm. These studies show that, for small proteins such as ribonuclease, lysozyme and myelin basic protein (Bonner, Wolf and Luisi, 1980; Zampieri, Jäckle and Luisi, 1986) and α-chymotrypsin (Levashov *et al.*, 1982), and at high R, the enzyme effectively sits within an 'original' droplet, and there is no massive re-distribution of water and surfactant. At lower R (the original droplet is similar in size, or smaller than, the protein), the data are consistent with the recruitment of water and surfactant from a small number (perhaps two or three) original droplets, in order to host the enzyme molecule. It seems that the enzyme-containing droplet is more water-rich than the original (i.e. the droplet value of R is larger than the nominal value, implying that R is lower for the 'unfilled' droplets (Zampieri, Jäckle and Luisi, 1986). This is reasonable given the considerable hydration requirements of the protein molecule; a quasi-elastic neutron-scattering study indicates that in AOT microemulsions at $R = 20$, a single α-chymotrypsin molecule is solvated by 250–500 molecules of water, which is roughly the same as that in bulk aqueous solution (Fletcher, Robinson and Tabony, 1986).

The size and composition of α-chymotrypsin-containing droplets has also been studied by SANS (Rahaman and Hatton, 1991). These workers find that, in AOT

microemulsions prepared either by injection or by liquid extraction, the size of the enzyme-containing droplet is independent of R. The origin of the fundamental discrepancy between this result and that obtained in the ultracentrifuge study of Levashov *et al.* (1982) is not clear, and further investigation is required. It might be pointed out that analysis of scattering data for mixed populations is not necessarily a straightforward task, even for a two-component system, especially if the ratios of the two components are far from unity. In addition, the particular droplet population containing the enzyme cannot be identified from the scattering data alone.

The bulk of data are consistent with the 'water-shell' model (Bonner, Wolf and Luisi, 1980; Zampieri, Jäckle and Luisi, 1986) which may be described as follows:

1. Enzymes are insoluble in oils and in binary reversed micellar solutions of surfactants in oils (see, for example, Nicot *et al.*, 1985; Leser *et al.*, 1986) and therefore solubilization models in which the microdispersed water does not play a primary role as a solvent cannot be important.
2. The protein molecule is considered to be dissolved in the water-pool, which is itself contained within a close-packed surfactant monolayer.
3. No constraint is placed on the hydrodynamic radius of the droplet, and surfactant and available water are permitted to redistribute between protein-containing, and 'empty droplets'.

This model is probably adequate to describe the protein-droplet structures for globular proteins. Data for a few non-globular proteins suggest more complex solubilization behaviour, involving the formation of highly asymmetric droplet structures in order to accommodate these proteins (reviewed by Luisi and Magid, 1986). Solubilization of gelatin in AOT microemulsions leads to the formation of gels (p. 312).

The water-shell model according to Zampieri, Jäckle and Luisi (1986) might be referred to as non-conservative, since redistribution of water and surfactant, and changes in droplet dimensions, are allowed. In contrast, Martinek and co-workers (Levashov *et al.*, 1982; Martinek *et al.*, 1989) argue for a model (the 'induced fit' model) that is absolutely conservative, with no redistribution of water or surfactant between droplets, and no change in hydrodynamic radius until the original droplet size falls below that of the enzyme. Only when the corresponding pool size is less than that of the enzyme is redistribution of water and surfactant between droplets and, presumably, a change in droplet dimensions, permitted. The problem with this model, which has been criticized before (Luisi and Magid, 1986), is the fate of the water displaced by the enzyme. The water cannot leave the droplet, yet the hydrodynamic volume of the droplet cannot change, and this implies zero volume (infinite density) for this water (Levashov *et al.*, 1982). In defending the model, Martinek and his colleagues have suggested that the displaced water is expelled somewhat into the surfactant layer, and provide ^{13}C NMR data which imply greater hydration of the carbonyl carbons and of the first methylene carbons of the ethylhexyl chains of AOT in preparations containing α-chymotrypsin (Shapiro *et al.*, 1989). However, simple calculation will shows that as R decreases, so that the radii of the original water-pools and of the protein molecules converge, the volume of water displaced becomes so great that it would extend beyond the thickness of the surfactant layer, if reasonable values for the density of the surfactant and of the displaced water are used, thus contradicting the fixed radius element of the model.

Protein conformation; localization within the droplet

A number of spectroscopic studies have been carried out to determine how the enzyme conformation changes on solubilization, using cytochromes P_{450} and b_5 (Erjomin and Metelitza, 1983), cytochrome *c* (Vos *et al.*, 1987a), lysozyme (Steinmann, Jäcle and Luisi, 1986), Horse-liver alcohol dehydrogenase (Vos *et al.*, 1987b) and α-chymotrypsin (Dorovska-Taran, Veeger and Visser, 1993a). Most of the data pertain to studies in AOT and CTAB microemulsions.

In general, solubilization in microemulsions has a negligible effect on the UV-spectrum of proteins. Steady-state fluorescence emission spectra, on the other hand, usually show a fairly significant blue shift and increase in quantum yield, both of which are more pronounced at lower *R* (Luisi and Magid, 1986; Marzola and Gratton, 1991; Brown, Yada and Marangoni, 1993). These results suggest conformational rearrangements and interactions of the the chromophores with the surfactant layer, either or both of which result in the shifting of the chromophore to a non-polar environment. The shifts are usually relatively small (1–2 nm). Larger shifts can be expected for denatured proteins.

Secondary structure, examined using circular dichroic spectroscopy (CD), is usually only slightly perturbed, and again, the largest changes are observed at a low *R*. Major changes in CD spectra have been observed in a few cases. Lysozyme, for example, rapidly unfolds with loss of activity, in AOT microemulsions (Steinmann, Jäcle and Luisi, 1986). In contrast, solubilization of *Rhizopus arrhizus* lipase is accompanied by a large increase in secondary structure (β-sheets and turns). The enzyme remains active (Brown, Yada and Marangoni, 1993). Cytochrome *c* is solubilized in AOT microemulsions with considerable loss of conformation at high *R* ($R = 40$), whereas conformation is largely retained at lower *R* (Vos *et al.*, 1987b).

A number of studies have been carried out to determine the extent of the interactions of solubilized protein with the interphase. Petit, Brochette and Pileni (1986) studied the location of cytochrome *c*, α-chymotrypsin and ribonuclease using hydrated electron quenching and Vos *et al.* (1987a,b) studied cytochrome *c* and HLADH using fluoresence anisotropy. The interpretation of these experiments has proven not to be straightforward, but the general conclusion which may be drawn is that proteins interact with more strongly with the interphase when the pH is such that the net charge on the protein is opposite to that of the surfactant layer. Thus, at least for microemulsions prepared with ionic surfactants, electrostatic interactions are important in determining the degree of interaction.

KINETIC BEHAVIOUR OF ENZYMES IN MICROEMULSIONS

Michaelis–Menten kinetics

In microemulsions held at otherwise constant composition, enzymes usually obey Michaelis–Menten kinetics, i.e. the rate at constant substrate concentration is directly proportional to the enzyme concentration, and the rate, at constant enzyme concentration, is a rectangular-hyperbolic function of the substrate concentration. Values of k_{cat} and K_m may therefore be derived using the standard procedures. Good examples of Michaelis–Menten behaviour in AOT microemulsions include the hydrolysis of palm

oil by *Rhizopus arrhizus* lipase (Kim and Cheung, 1989), of Crambe oil by several different lipases (Derksen and Cuperus, 1992) and the reduction of acetaldehyde by HLADH/NADH (Meier and Luisi, 1980).

This apparently straightforward situation is complicated by the fact that k_{cat} and K_m are found to depend rather strongly on the composition of the microemulsion and that the precise nature of this dependence is a function of the particular microemulsion system and of the enzyme and substrate combination in question (in particular, whether the substrate is oil- or water-soluble). It is reasonable to assume, *a priori*, that the actual values of k_{cat} and K_m will be complex functions of the various (qualitatively definable) elements of microemulsion structure already introduced: surfactant interactions and changes in water structure, which modulate enzyme conformation; and partitioning and exchange-related factors, which serve to influence the local substrate concentration in the microenvironment of the enzyme.

The various patterns of behaviour which have been observed are best related by considering how the kinetics vary with R and with the droplet concentration, and on whether the substrates are oil- or water-soluble, as discussed in the following sections.

Kinetics as a function of R

Enzyme activity in microemulsions is usually a strong function of R. In AOT systems the most common manifestation is the so-called bell-shaped plot, in which the activity passes through a distinct maximum. Good examples are α-chymotrypsin (Belonogova *et al.*, 1983) (*Figure 13*), *Chromobacterium viscosum* lipase (Fletcher *et al.*, 1985a) (*Figure 14*), horseradish peroxidase (Klyachko, Levashov and Martinek, 1984; Das and Maitra, 1989), *Candida rugosa* lipase (Han and Rhee, 1986), alkaline phosphatase (Gonnelli and Strambini, 1988), *Rhizopus arrhizus* lipase (Kim and Cheung, 1989) and Δ^5-3-ketosteroid isomerase (Levashov *et al.*, 1992). Bell-shaped plots have been described for enzymes in a few other microemulsion systems, including pyrophosphatase and lactate dehydrogenase in a Brij 56 system (Levashov *et al.*, 1980; Levashov, Klyachko and Martinek, 1981) and *Candida rugosa* lipase in a lecithin system (Chen and Chang, 1993) and *Rhizopus delamer* lipase, again in a lecithin system (Schmidli and Luisi, 1990).

In the case of the deacylation of *N*-transcinnamoyl α-chymotrypsin (*Figure 13*) k_{cat} rises to a maximum at $R = 10$, and then declines to a limiting high-R value which is approximately the same as that in aqueous solution. At $R = 10$, k_{cat} is about 40% higher than in aqueous solution at the same pH (the enzyme is said to be 'superactive'; p. 273). Further experiments have shown that the rotational correlation time ($1/\tau_c$) of an active-site spin label passes through a minimum at the optimal R (R_{OPT}), as shown in *Figure 13*, implying conformational changes which reduce the free volume within the active-site cleft. The kinetic and spin-label data together have been interpreted in terms of a conformational change which renders the enzyme more active than in aqueous solution. Noting that this optimal R corresponds fairly well with an 'original' droplet size which approximates that of the chymotrypsin molecule, Martinek and coworkers have concluded that the 'tight-fit' of the enzyme within this droplet is the source of the conformational restraint (the 'induced-fit' model, described above) (Belonogova *et al.*, 1983; Martinek *et al.*, 1989; Levashov *et al.*, 1990). These workers also report a linear correlation between R_{OPT} and the size of the enzyme for

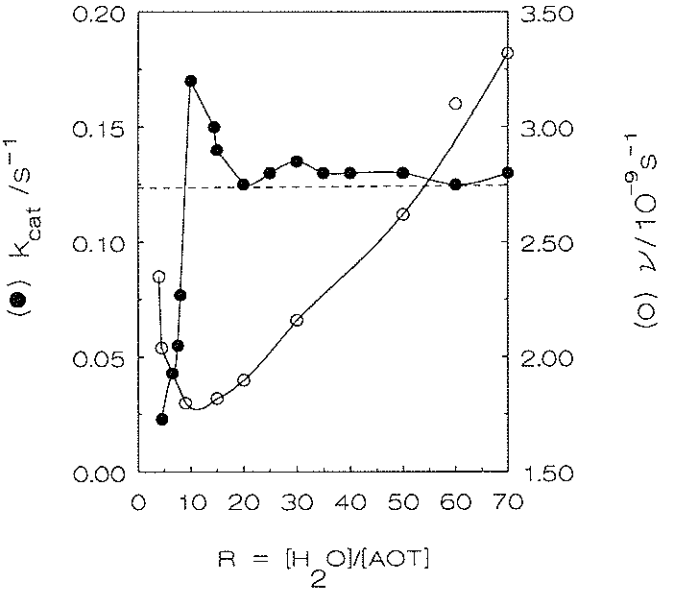


Figure 13. Dependence of k_{CAT} on R for the deacylation of *N-trans*-cinnamoylimidazole by α-chymotrypsin (●) and of the rotation frequency (O) of an active-site spin-label in an AOT microemulsion system. The broken line shows the value of k_{cat} in aqueous solution. (Reproduced from Belonogova *et al.* (1983), with permission.)

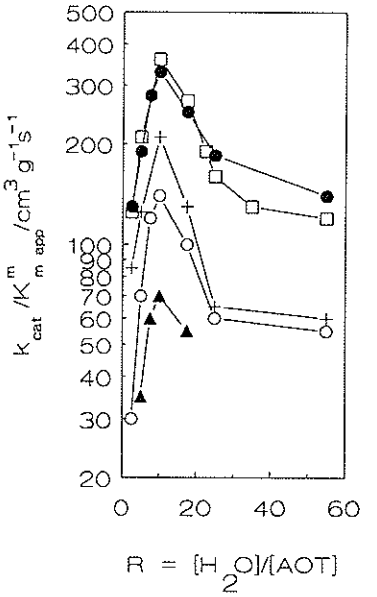


Figure 14. Dependence of k_{cat}/K for the hydrolysis of 4-nitrophenyl alkanoates by *Ch. viscosum* lipase in AOT-*n*-heptane microemulsions at pH 8. The substrates are (●) C_4, (+) C_6, (□) C_{16}, (▲) C_8 in an AOT–water–dodecane microemulsion. (Redrawn from Fletcher, P.D.I., Robinson, B.H., Freedman, R.B. and Oldfield, C. (1985a) *Journal of the Chemical Society – Faraday Transactions* **81**, 2667–2679, with permission.)

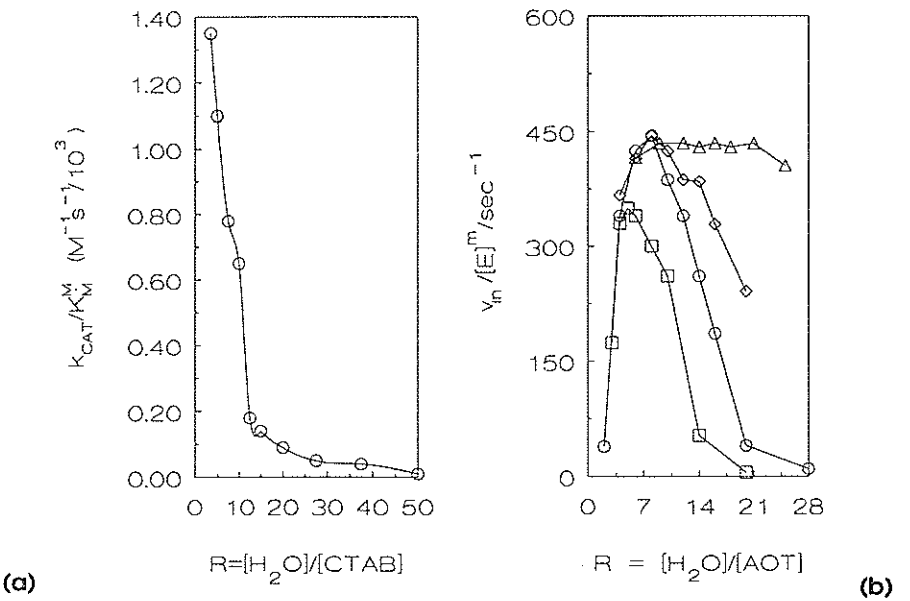
a large number of enzymes in the AOT system, implying that the induced-fit model holds for these also (Martinek *et al.*, 1989).

Elegant as this interpretation is, it is worth recalling that this induced-fit model has been criticized for being unphysical, as already discussed. In any case, it is not really necessary to invoke induced fit in order to formulate a reasonable hypothesis which explains the data. Enzymes are known to have a critical requirement for water of hydration in order to express catalytic activity, and the role of this water is generally accepted as being necessary to impart conformational mobility. Therefore in this system, as in others, it is reasonable to expect enzyme activity to increase with water content (water-activity), asymptoting to the bulk aqueous solution value as $a_w \rightarrow 1$. This underlying behaviour is recognizable in the kinetics (compare *Figures 10* and *13*). As for the superactive peak, this seems, at least in the case of α-chymotrypsin, to be a substrate-specific phenomenon. With resorufin butyrate as a substrate, k_{cat} rises sharply, to the aqueous solution value, at $R = 10$, and is thereafter constant (Dorovska-Taran *et al.*, 1993a) and with *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (SAAP) as a substrate that k_{cat} simply asymptotes to the aqueous solution value at high R (Bru and Walde, 1991). These curves are more nearly like those expected for a straightforward 'water-of-hydration' model.

It might be suggested that the availability of water as a substrate must play a role in determining the R-dependence of activity for hydrolytic enzymes such as α-chymotrypsin. However, this does not seem to be the case since non-hydrolytic enzymes (a good example is Δ^5-3-ketosteroid isomerase) (Levashov *et al.*, 1992) also behave in this fashion. Furthermore, several studies involving lipases reveal the same general trend (decreasing activity with decreasing R) for these enzymes operating in both synthetic (condensative) and hydrolytic modes (Fletcher *et al.*, 1985a; Prazeres, Garcia and Cabral, 1992; Stamatis, Xenakis and Kolisis, 1993; Stamatis *et al.*, 1993a,b).

Several enzymes, including lactate dehydrogenase (LDH), penicillin acylase, and γ-glutamyl synthetase, have been reported by one group to display a complex R-dependence, characterized by multiple activity peaks (Kabanov *et al.*, 1989; Kabakov *et al.*, 1992). The authors suggest that the peaks correspond to aggregates of different sizes and subunit compositions. The results for LDH are apparently not reproducible (Khmelnitsky *et al.*, 1993). The latter authors suggest that multiple peaks in the R-profile reported in the earlier study are artefacts caused by the instability of the enzyme in microemulsions.

Many enzymes display R-dependent kinetics other than bell-shaped. For example, Fletcher *et al.* (1985a) report that the activity of *Ch. viscosum* lipase (k_{cat}/K_m for the hydrolysis of 4-nitrophenyl alkanoates) is independent of R in a CTAB microemulsion, in contrast to the most definite bell-shaped kinetics obtained in an AOT microemulsion, shown in *Figure 14*. A similar result was obtained by Vos *et al.*, (1987b) for LADH. Again, in a CTAB system, $k_{cat}K_m$ for the oxidation of bilirubin by bilirubin oxidase decreases smoothly by a factor of 50, as R is increased from 3.5 to 25 (*Figure 15a*). This is a k_{cat} effect; K_m essentially remains constant (Oldfield and Freedman, 1989). Han, Walde and Luisi (1990) report that the bell-shaped dependence of the rate of olive-oil hydrolysis by *C. rugosa* lipase in an AOT microemulsion progressively disappears as the enzyme concentration is increased tenfold until, at the highest concentrations, the rate becomes an hyperbolic function of R (*Figure 15b*). Perhaps most intriguing of all is the observation that the bell-shaped R-dependence of the rate



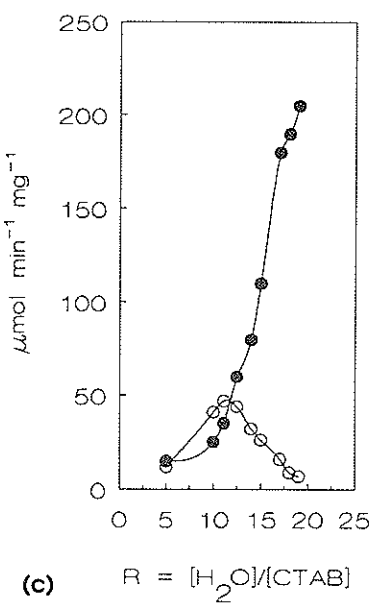


Figure 15. Dependence of rate parameters on R for different enzyme and microemulsion combinations. (a) Bilirubin oxidase (Oldfield, C. and Freedman, R.B. (1989). *European Journal of Biochemistry* **183**, 347–355); (b) *C. rugosa* lipase at overall concentrations of (□) 30, (O) 43, (♦), 91, and (Δ) 290 nM (Han, D., Walde, P. and Luisi, P.L. (1990). *Biocatalysis* **4**, 153–161); (c) pyruvate kinase in the presence (●) and absence (O) of the specific activator K^+ (Ramírez-Silva, L., Tuena de Gómez-Puyou, M. and Gómez-Puyou, A. (1993). *Biochemistry* **32**, 5332–5338 © (1993) American Chemical Society). Figures redrawn with permission.

of the pyruvate kinase-catalysed synthesis of pyruvate from phosphoenolpyruvate and ADP, in a CTAB microemulsion, is abolished on the addition of the specific activator, K^+ (*Figure 15c*); the kinetics are instead sigmoidal (Ramírez-Silva, Tuena de Gómez-Puyou and Gómez-Puyou, 1993).

It is often the case that enzymes have higher activity in microemulsions than in bulk aqueous solution. This 'superactivity' is a fairly common phenomenon, and the degree of activation is usually modest. As already shown, k_{cat} for the hydrolysis of N-*trans* cinnamoyl imidazole (t-CNI) is about 40% higher in the optimal $R = 10$ microemulsion than in aqueous solution at the same pH. As another example, Madamwar, Bhatt and Ray (1988) report an approximate fourfold increase in the activity of invertase (catalysing the hydrolysis of sucrose) in microemulsions stabilized by SDS or by sodium tauroglycholate.

A few enzymes appear to exhibit a much greater degree of superactivity in AOT microemulsions. The classic examples are mushroom tyrosinase (laccase), acid phosphatase and horseradish peroxidase (HRP), for which increases in k_{cat} of up to 50-, 100- and 200-fold, respectively, have been reported (Martinek *et al.*, 1986, 1989).

The origins of this kind of superactivity are not understood, but several attempts have been made to account for it and for R-dependent kinetics in general, through the construction of algorithms which are parameterized such that input of numerical values for each parameter will generate a model curve to fit the experimental data. The approach of Kabanov *et al.* (1988), extending the concept of the 'induced-fit' model, considers that droplet polydispersity accounts for the R-dependence, and the algorithm contains parameters representing droplet polydispersity and enzyme activity as a function of droplet size. On the other hand, Bru, Sánchez-Ferrar and Garcia-Carmona (1989a, 1990) considered that the enzyme activity is determined by partitioning of the enzyme between water domains characterized by three different water structures, and by partitioning of substrate between the subphases. The resulting algorithm contains a total of six adjustable parameters.

The problem with this kind of approach is that there is no independent experimental verification that the 'active' parameters actually influence enzyme activity in the way required by the model and there are no criteria for determining whether or not the values assigned to a given parameter are realistic. The algorithms have no predictive power, since they do not suggest new experiments to test the models; they are best regarded as rather elaborate curve-fitting procedures.

In another approach, Ruckenstein and Karpe (1990, 1991) considered that the (R-dependent) radial decay of the electrical field gradient generated by the fixed surfactant headgroups determines the radial distribution of solutes (hydrogen ions, counterions and charged substrate molecules), leading to pH, solvent dielectric and substrate concentration gradients within the droplet which combine to determine the intrinsic activity of the enzyme and the substrate concentration in the vicinity of the active site. Once again, there is no direct evidence to indicate the extent to which these factors are responsible for the observed R-dependencies and the question remains as to whether the concentrations, or the thermodynamic activities, of the solutes should be taken into consideration. In any case, for reactions where the enzyme operates under k_{cat} conditions (k_{cat} is a substrate concentration-independent parameter), or of enzyme-uncharged substrate combinations, the theory requires that the R-dependence of the kinetics must arise solely from pH or/and solvent dielectric effects. These

kinds of considerations once again highlight the need for a better understanding of the concept and measurement of pH in order to obtain a better understanding of enzyme behaviour.

Dependence of the kinetics on the droplet concentration

As long as the droplets remain in large excess over the enzyme, so that each molecule goes into a separate, identical host-droplet (*Figure 12*), a direct proportionality between reaction rate and enzyme concentration is expected and indeed observed, since the number of identical biocatalytic units simply increases on adding more enzyme. It is also reasonable to expect kinetic parameters (k_{cat} and K_m expressed in overall concentration units) to be independent of the total droplet concentration at fixed R, since the presence of unfilled droplets should have no effect on the activity of the enzyme (the notable exception being the case of a water-soluble, as opposed to an oil-soluble or surface active substrate, in which case theory predicts, and experiment supports, a proportionality between K_m and the droplet concentration (pp. 297–301). Early studies with α-chymotrypsin (measuring k_{cat} for the hydrolysis of glutaryphenylalanyl-4-nitroanilide (GPNA) and *Ch. viscosum* lipase (k_{cat}/K_m for the hydrolysis of 4-nitrophenyl alkanoates) (Fletcher *et al.*, 1984, 1985a) indicated that this is indeed the case, although more recent data indicate numerous exceptions, and that reaction rates can often be expected to decrease with increasing droplet concentration at fixed R (i.e. increasing $\phi^{aq}(R)$. Examples are HRP (Klyachko, Levashou and Martinek, 1984; Das and Maitra, 1989), Δ^5-3-ketosteroid isomerase (Levashov *et al.*, 1992), soybean lipoxygenase-1 (Shkarina, Khün and Schewe, 1992) and *Ch. viscosum* lipase with triolein as substrate (Prazeres, Garcia and Cabral, 1992). It has been suggested that these kinetics reflect a very strong dependence of the composition of the host droplet (aggregation numbers of enzyme, water and surfactant) on $\phi^{aq}(R)$ (Klyachko, Levashov and Martinek, 1984). If this is the case, an ultracentrifugation study should reveal such changes. In the particular case of *Ch. viscosum* lipase, substrate-specific effects must once again be considered since the enzyme activity, as assayed with 4-nitrophenyl alkanoates, is independent of $\phi^{aq}(R)$ (Fletcher *et al.*, 1985a), whereas the rate decreases markedly when assayed with triolein (Prazeres, Garcia and Cabral, 1992).

Reactions involving oil-soluble substrates

Solutes will distribute between the aqueous, oil and surfactant subphases, depending whether they are primarily oil or water soluble or surface active. The kinetics of chemical and enzyme-catalysed reactions should reflect this. For apolar and surface-active substrates (e.g. steroids, lipids), which partition between the oil and surfactant subphases, the pseudophase model (Biais *et al.*, 1981) is sufficient to interpret most kinetic data (Fletcher *et al.*, 1985a; Khmelnitsky *et al.*, 1990). This model simply ignores the microdispersed nature of the aqueous subphase, treating the system instead as a three-phase (oil–surfactant–water) system of (effectively) infinite interfacial surface area. Using the steady-state approximation, the rate equation for the enzyme-catalysed transformation of an apolar substrate, in which the enzyme 'sees' only that fraction of the substrate in solution in the aqueous subphase (*Figure 16*) gives (Oldfield, 1990):

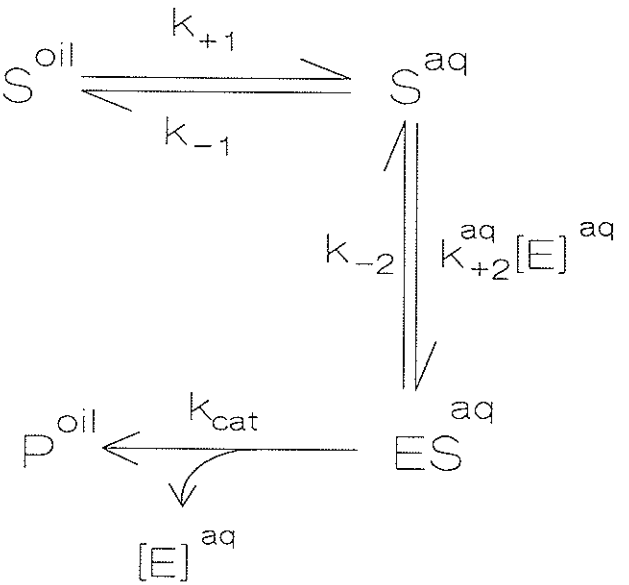


Figure 16. Kinetic scheme for the conversion of an oil-soluble substrate under pseudophase conditions. S^{aq}, substrate in the aqueous subphase; S^{oil}, substrate in the oil subphase; E^{aq}, enzyme in the aqueous subphase; ES^{aq}, enzyme–substrate complex in the aqueous subphase. The kinetics are simplified by assuming that the product, *P*, is also oil-soluble.

$$v^{aq} = \frac{k_{CAT}[E]_T^{aq}[S]^{aq}}{K_m^{aq}(K_{p\,app}\phi^{oil} + \phi^{aq}) + [E]_T^{aq}k_{cat}/k_{+1}\phi^{oil} + [S]^{aq}} \qquad \text{Eq. (12)}$$

(the volume fraction of the surfactant was ignored in this derivation). The superscript 'aq' denotes that the concentration units are mol per litre of the aqueous subphase. $K_{p\,app}$ is the apparent oil:water partition coefficient. It is extremely unlikely that phase transfer will ever be rate limiting, but the non-linear dependence of rate on the enzyme concentration predicted by this equation can be used to check for such behaviour. When phase transfer is not rate limiting ($k_{+1} >> k_{cat}$) the equation simplifies,

$$v^{aq} = \frac{k_{cat}[E]_T^{aq}[S]^{aq}}{K_{m\,app}^{aq} + [S]^{aq}} \qquad \text{Eq. (13)}$$

and Michaelis–Menten kinetics are predicted, where

$$K_{m\,app}^{aq} = K_m^{aq}(K_{p\,app}\phi^{oil} + \phi^{aq}); \qquad \text{Eq. (14)}$$

the apparent Michaelis constant, $K_{m\,app}^{aq}$ is larger than its actual value by a factor of $(K_{p\,app}\phi^{oil} + \phi^{aq})$. If the partitioning substrate is not surface active, then it is reasonable to equate $K_{p\,app}$ with the oil:water partition coefficient, $K_{OIL:AQ}$. If the substrate is surface active, then $K_{p\,app} = K_{OIL:INT} \times K_{INT:AQ}$, where $K_{OIL:INT}$ and $K_{INT:AQ}$ are respectively the oil:interphase and interphase:aqueous partition coefficients.

In a study of the catalytic behaviour of horse-liver alcohol dehydrogenase (HLADH) in AOT microemulsions, Khmelnitsky *et al.* (1990) measured $K_{OIL:INT}$ and $K_{INT:AQ}$ for the series of linear alkanols (*n*-butanol–*n*-decanol) using a calorimetric technique,

and were able to show that the differences between k_{cat}/K_m^{aq} and k_{cat}/K_m^{w} (the aqueous solution values) for oxidation of these alcohols is due almost entirely to partitioning, indicating that the enzyme itself remains fully functional on solubilization.

The same kinetics will also hold for enzymes which access the interphasic substrate pool. In this case $K_{p\,app} = K_{OIL:INT}$. A good example is *Ch. viscosum* lipase in AOT microemulsions (Fletcher *et al.*, 1985b). The activity ($k_{cat}/K_{m\,app}^{aq}$) towards members of the homologous series of 4-nitrophenyl alkanoates, varies by no more than a factor of five as the chain length increases from C_4 (butyrate) to C_{16} (palmitate) (*Figure 14*), while the *n*-heptane:water partition coefficient decreases by a factor of 10^7. This is interpreted in terms of an interfacial location for the substrate, with the enzyme extracting substrate directly from the surfactant layer.

A different approach to the problem of partitioning, which circumvents the technically difficult task of measuring partition coefficients, has been described by Laane and co-workers. The assumption is that the distribution behaviour of a solute between the continuous phase and the interphase can be determined by comparing the log *P* value of the solute (log P_s) with those for the continuous phase, log P_{cph} and the interphase, log P_{int} (log *P* is the $\log_{10}$ of the octanol:water partition coefficient). A similarity in log *P* values for a pair of compounds implies high mutual solubility; in a multiphase system a solute will partition most favourably into the phase with the most similar log *P*. The log *P* values of each component may be calculated from tables. In the case of the microemulsion continuous and interphases, an average log *P* is calculated, weighted according to the mol-fraction of each component present (Laane *et al.*, 1987a,b).

In one study, the activity of 20β-hydroxysteroid dehydrogenase (catalysing the reduction of progesterone or prednisone to the corresponding alcohol) in a CTAB–water–iso-octane–hexanol microemulsion was optimized using this approach (Hilhorst *et al.*, 1984). The composition of the interphase (i.e. the surfactant:co-surfactant ratio) and of the continuous phase (i.e. the oil:co-surfactant ratio) were estimated using the titration procedure of Bowcott and Schulman (1955). The reaction rate was optimized by adjusting log P_{int} (by changing the co-surfactant (hexanol) concentration), and by adjusting log P_{cph} (by changing the type of oil) so that the ($\log P_{OIL} - \log P_{SUBSTRATE}$) was maximized and ($\log P_{INTERPHASE} - \log P_{SUBSTRATE}$) was minimized (*Figure 17*). In this way the interphase concentration of substrate, hence the substrate concentration in the vicinity of the enzyme, was maximized. By the same reasoning, it should be possible to minimize product inhibition by manipulating ($\log P_{INTERPHASE} - \log P_{PRODUCT}$) and ($\log P_{OIL} - \log P_{PRODUCT}$) to minimize the interphase product concentration.

There are several other examples where choice of microemulsion components strongly influences reaction rates in a fashion which can be rationalized using log *P*. For example, in the *C. viscosum* lipase study already mentioned (Fletcher *et al.* (1985a), a large effect on the kinetics was obtained by substituting *n*-heptane (log *P* = 4.0) for dodecane (log *P* = 6.5); the decreased $k_{cat}/K_{m\,app}^{aq}$ for the C_8 substrate is consistent with a higher oil:interphase partition coefficient for the substrate (*Figure 14*). Stamatis, Xenakis and Kolisis (1993) and Stamatis *et al.* (1993b) have shown that the initial rate of (–) menthol oleate synthesis by *Penicillium simplicissimum* lipase in ternary AOT microemulsions is quite strongly dependent on log *P*, with a maximum at log *P* = 4.5 (iso-octane).

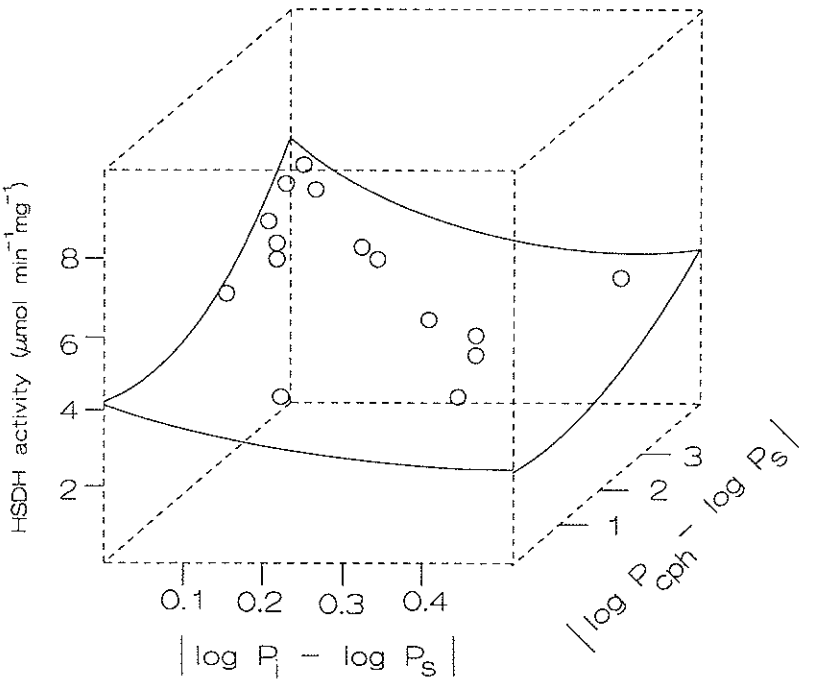


Figure 17. Dependence of the activity of 20β-hydroxysteroid dehydrogenase on log *P* of the substrate relative to that of the continuous subphase and interphase. The activity is maximal when the log *P*'s are balanced such that partitioning into the interphase is favoured. (Reproduced from Hilhorst, R., Spruijt, R., Laane, C. and Veeger, C. (1984). *European Journal of Biochemistry* **144**, 459–466, with permission.)

As far as reaction rates are concerned, partitioning arguments become irrelevant for enzymes which operate under conditions of substrate saturation (k_{cat}-kinetics). However, this may not always be possible to achieve and, in any case, process design optimization must take into account factors such as substrate and/or product inhibition. In this context these studies have been instrumental in demonstrating the possibilities for optimization of microemulsion composition using a rational design approach.

Water-soluble substrates

The case of water-soluble substrates is rather different from those of oil-soluble or surface-active ones (Oldfield, 1990). In this case, under steady-state conditions, the enzyme may only receive substrate molecules by exchange, and therefore it is reasonable to expect the rate of formation of the Michaelis complex to be dependent on the droplet size and concentration and on the exchange rate, just as for other bimolecular reactions. In principle, a kinetic scheme equivalent to that shown in *Figure 9*, for a bimolecular reaction, needs to be set up. However, the solution is unwieldy, and it is useful to consider type (i) and type (ii) kinetics separately (p. 277). The scheme for type (ii) kinetics is shown in *Figure 18*. The solution for a system with droplets containing a maximum of one substrate molecule per droplet is

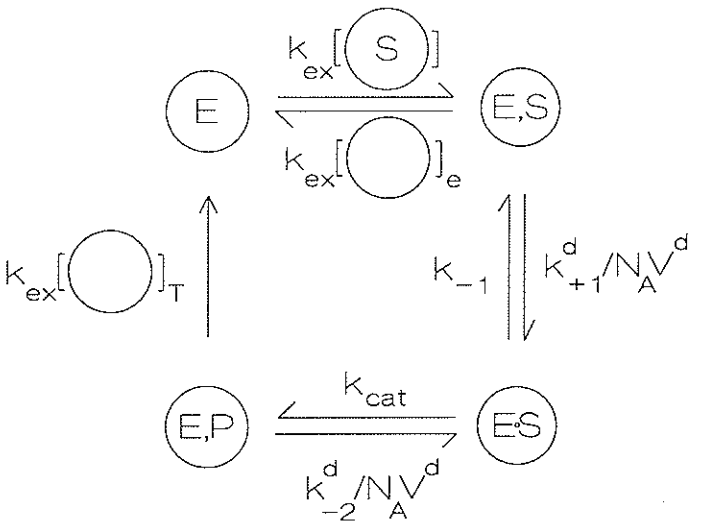


Figure 18. An enzyme-catalysed reaction obeying type (ii) kinetics in water-in-oil microemulsions. The criterion for type (ii) kinetics is that the enzyme–substrate (Michaelis complex) forms only after the transient dimer has dissociated, leaving enzyme and substrate together in a single daughter droplet (see *Figure 9*). The enzyme obeys Michaelis–Menten kinetics in aqueous solution. (E), droplet containing one molecule of enzyme, E; (S), droplet containing one molecule of substrate, S; (E,S), droplet containing one molecule each of E and S; (E•S), droplet containing a single Michaelis complex; (E•P), droplet containing one molecule each of E and product, P; ◯, empty droplet. (Reproduced from Oldfield, C., Otero, C., Rua, M.L. and Ballesteros, A. (1992). In *Biocatalysis in Non-Conventional Media* (J. Tramper, Ed.), pp. 189–198. Elsevier, Amsterdam, with permission.)

$$v3 = \frac{k_{\text{cat app}}[\text{E}]_T^{\text{m}}[\text{S}]^{\text{m}}}{K_{\text{m}}^{\text{m}} + [\text{S}]^{\text{m}}} \qquad \text{Eq. (15)}$$

(the superscript 'm' shows that the concentration units are mol per litre of microemulsion), where

$$k_{\text{cat app}} = k_{\text{cat}}^{\text{o}} - \frac{k_{-2}^{\text{d}} k_{\text{cat}}^{\text{o}}}{k_{-2} + k_{\text{ex}}\phi^{\text{aq}}} \qquad \text{Eq. (16)}$$

(the superscript 'd' indicates that the concentration units are mol per litre of microemulsion droplets and $k_{\text{cat}}^{\text{o}}$ is the maximum value of $k_{\text{cat app}}$, and

$$K_{\text{m}}^{\text{m}} = K_{\text{m}}^{\text{d}}\phi^{\text{aq}} + \frac{k_{\text{cat}}^{\text{o}}}{k_{\text{ex}}} \; ; \qquad \text{Eq. (17)}$$

ϕ^{aq} replaces the product ([◯]$_T$.N_AV_d), as in equation (9), and

$$K_{\text{m}}^{\text{d}} = \frac{k_{-1} + k_{\text{cat}}^{\text{o}}}{k_{+1}^{\text{d}}} \qquad \text{Eq. (18)}$$

K_{m}^{d} is equivalent to the Michaelis constant of aqueous solution kinetics, in the sense that it contains the same group of rate constants (equation 18). Equation (17)

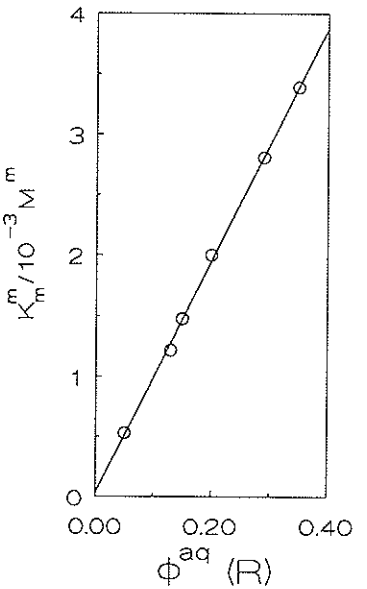


Figure 19. Dependence of K_m^m on ϕ^{aq} ($R = 25$) for the trypsin-catalysed hydrolysis of *N*-benzoyl DL-arginine *p*-nitroanilide in a CTAB microemulsion system. The system obeys the kinetics predicted by equation (17). For this system $k_{cat}.k_{ex} = 10^{-10}$ $M^{-1m}s^{-1}$ (Oldfield, 1990), and therefore there is direct proportionality between k_m and $\phi^{aq}(R)$ (Replotted from Martinek, K., Levashov, A.V., Klyachko, N.L., Pantin, V.I. and Berezin, I.V. (1981). *Biochimica et Biophysica Acta* **657**, 277–284, with permission.)

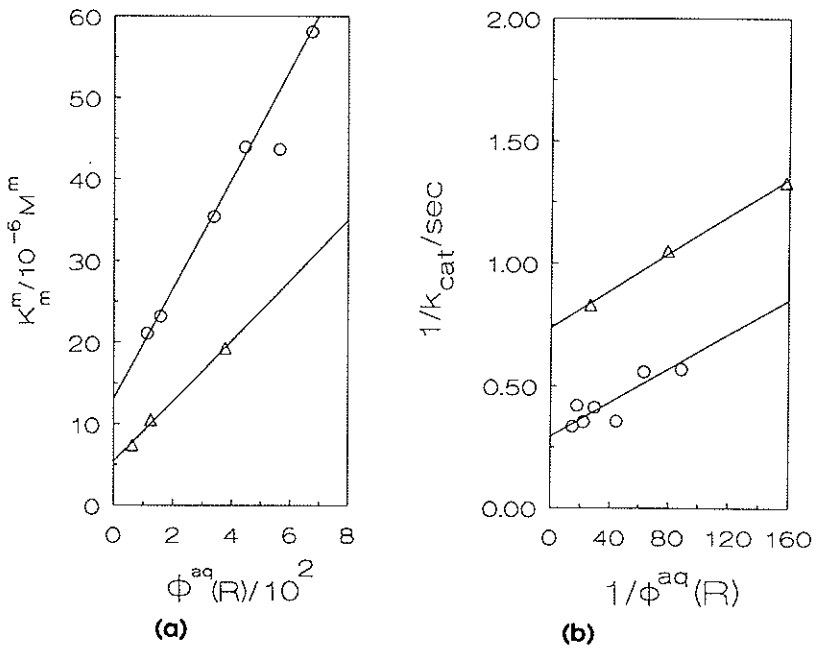


Figure 20. (a) Dependence of K_m^m on f^{aq} for the reduction of NAD^+ by horse-liver alcohol dehydrogenase (saturating concentrations of ethanol) in a CTAB microemulsion system; (b) dependence of $1/k_{cat}$ on $1/f^{aq}$ in the same system. (D) $R = 12.5$, (O) $R = 7.0$. (Replotted from Oldfield, C., Otero, C., Rua, M.L. and Ballesteros, A. (1992). In *Biocatalysis in Non-Conventional Media* (J. Tramper, Ed.), pp. 189–198. Elsevier, Amsterdam, with permission.)

shows that a plot of K_m^m v. ϕ^{aq} should be linear with positive slope and ordinate intercept k_{cat}/k_{ex}. Experimentally, it is better to alter ϕ^{aq} by changing the droplet concentration at constant R (i.e. changing $\phi^{aq}(R)$), rather than vice versa, because the linearity of the plot may be obscured by R-dependent effects on activity, as already discussed.

A linear dependence of K_m^m on $\phi^{aq}(R)$ was first described by Martinek *et al.* (1981), studying the kinetics of the hydrolysis of water-soluble substrates of trypsin in AOT microemulsions (*Figure 19*), and later by Fletcher *et al.* (1985b), for α-chymotrypsin in several different microemulsions, and by Oldfield *et al.* (1992), again for α-chymotrypsin in AOT microemulsions and also for the LADH-catalysed interconversion of NADH/NAD$^+$ in a CTAB system (*Figure 20a*).

The dependence of K_m^m on $\phi^{aq}(R)$ (i.e. on the droplets concentration) arises because of the effect of compartmentalization of enzyme and substrate on the rate of formation of the Michaelis complex, E•S, via the intermediate species (E,S), in the same way that the reaction A + B → C is determined by the rate of formation of the intermediate species (A,B). If the droplet concentration is increased at constant substrate concentration (i.e. if the [◯]$_e$:[S] ratio is increased), the rate at which E,S decomposes back to separate E and S droplets (= k_{ex}[◯]$_e$[(E,S)]) increases, whilst the rate of formation (= k_{ex}[E]$_T$[S]) remains unchanged. Therefore the effect of increasing the droplet concentration is to shift the E + S ⟶ E•S pre-equilibrium to the left, which is unfavourable for product formation. Increasing the droplet volume (i.e. increasing R) at constant droplet concentration has a similar effect, since the rate of E•S formation within (E,S) is inversely proportional to the droplet volume (equation 8). For the same total droplet concentration, therefore, these larger, longer-lived (E,S) droplets are more likely to undergo exchange, leading to the separation of E and S, relative to their smaller counterparts.

The physical consequence of increasing ϕ^{aq}, therefore, is that to obtain the same rate, the substrate concentration must be increased to shift the E + S → E•S pre-equilibrium back to the right. Thus the K_m^m (the overall substrate concentration required to maintain [E•S] at 0.5[E]$_T$ increases. The slope of the plot of K_m^m v. $\phi^{aq}(R)$ is K_m^d, the maximum possible value of K_m^m, i.e. that in a $\phi^{aq} = 1$ microemulsion (maximum possible concentration of droplets of any given size, pp. 277–278) and this value is given. Since there is no analogous compartmentalization in aqueous solution kinetics, K_m^d cannot be compared directly with the aqueous solution Michaelis constant, K_m^w, but, on the basis of the unfavourable effect of compentmentalisation, it is expected to be larger, and the experimental data, indicating K_m^d:K_m^w ratios of 40–100, depending on the enzyme–substrate combination and microemulsion type (Oldfield *et al.*, 1992), are consistent with this result.

In the case of HLADH, a plot of $1/k_{cat}$ v. $1/\phi^{aq}(R)$ is also linear (equation 16; *Figure 20b*). This behaviour is expected for reactions in which the products are potential inhibitors of the enzyme, as is the case for HLADH and NADH or NAD$^+$, since then ($k_{-2}^d/N_A V_d$) is finite (*Figure 18*) and the binding of the product to the enzyme within droplet (E,P) may cause inhibition (ultimately alleviated by exchange, as shown in the last step of *Figure 18*). Reactions such as the hydrolysis of non-specific substrates by proteases, on the other hand, give products which are poor inhibitors of the enzymes and ($k_{-2}^d/N_A V_d \approx 0$). As an example, k_{cat} for the hydrolysis of *N*-benzoyl DL-arginine *p*-nitroanilide by trypsin is independent of $\phi^{aq}(R)$ (Martinek *et al.*, 1981).

No examples of enzymes obeying type (i) kinetics (the Michaelis complex forms within the transient dimer, p. 277) have yet been identified. This kinetic behaviour is characterized according to theory by a K_m^m which is independent of the droplet concentration, but which is proportional to the droplet size (i.e. to R) but, as discussed already (pp. 289–294), multiple factors operate to determine enzyme activity as a function of R and this property may not be obvious.

Some authors have dismissed the possibility that exchange can influence the reaction rate, citing the fact that $k_{ex} >> k_{cat}$ (Bru, Sanchez-Ferrar and García-Carmona, 1989a; Ruckenstein and Karpe, 1990). Equation (17) indicates that this is the case, since k_{ex} appears only in the k_{cat}/k_{ex} term. However, this ignores the compartmentalization and its effects on the (pre-)equilibrium position for bimolecular reactions. The importance of compartmentalization was also recognized by Verhaert *et al.* (1990a), who derived a similar rate equation for a slightly different kinetic scheme. Although there are some problems with this derivation (Oldfield *et al.*, 1992), the important term '$K_m^m \phi^{aq}$' does appear in the final rate equation. However, its significance with regard to the published studies already cited, indicating a dependence of K_m on $\phi^{aq}(R)$, and the possibility of systematically varying ϕ^{aq} as a test for the validity of their kinetic scheme, was not recognized. In subsequent studies Verhaert and co-workers assumed that k_{cat} and K_m^m are unchanged in the microemulsion environment (exactly the opposite conclusion to that of Oldfield, 1990) and the experimental data were fitted to the equations, primarily by manipulating the exchange rates (Tyrakowska *et al.*, 1990; Verhaert *et al.*, 1990b).

Enzyme inactivation

A number of studies have appeared which are concerned with the stability of enzymes in microemulsions relative to that in aqueous solution or in other non-aqueous solvent systems. Bearing in mind that the data set represents a fairly small number of enzymes, a number of general conclusions may be drawn, as follows:

1. Rates of loss of activity are dependent on the microemulsion type and its precise composition, and on the enzyme in question. Usually, the decay does not follow a simple kinetic (first- or second-order) pattern.
2. Loss of activity is associated with loss of active sites. This was demonstrated for LADH in an AOT microemulsion(Larsson, Oldfield and Freedman, 1989) by active-site titration and also seems to be the case for bilirubin oxidase in a CTAB microemulsion, where k_{cat} (for the oxidation of bilirubin) decreases, but K_m^m remains constant (Skrika-Alexopoulis, Muir and Freedman, 1993).
3. Enzyme stability under operational conditions, or in the presence of inhibitors or other ligands, is usually greater than under storage (no substrate) conditions. Examples include bilirubin oxidase (more stable in the presence of bilirubin; (Skrika-Alexopoulis, Muir and Freedman, 1993), lactate dehydrogenase (LDH; more stable in the presence of NADH or pyruvate; (Khmelnitsky *et al.*, 1993), HLADH (more stable in the presence of AMP, NAD^+ or NADH; (Samama, Lee and Biellmann, 1987; Larsson, Oldfield and Freedman, 1989)

and tryptophanase (more stable in the presence of indole; (Eggers and Blanch, 1988).

4. Choice of microemulsion components determines stability. For example, HLADH is more stable in CTAB microemulsions than in SDS microemulsions, and more stable in CTAB microemulsions with pentan-1-ol or hexan-1-ol, rather than butan-1-ol, as co-surfactant (Samama, Lee and Biellmann, 1987).

Only a few cases have been reported in which inactivation kinetics follows a fixed, simple order with respect to time. Examples are the first-order inactivation of Δ^5-ketosteroid isomerase in an AOT microemulsion (Levashov *et al.*, 1992), of cholesterol oxidases from several microbial sources, in CTAB/butanol microemulsions (Lee and Biellmann, 1986) and of HLADH in SDS/butanol microemulsions (Samama, Lee and Biellmann, 1987). In the latter case the inactivation was shown to be caused by the hydrogen peroxide produced during the oxidation of cholesterol to cholestenone, and could be considerably improved by addition of catalase.

More usually, denaturation kinetics are more complex and cannot be assigned a simple kinetic order. By far the most common pattern of behaviour involves an (apparently) biphasic decay of activity. Examples include LADH in several different systems (Larsson, Adlercreutz and Mattiasson, 1987; Lee and Biellmann, 1987), α-chymotrypsin and *Ch. viscosum* lipase (Fletcher *et al.*, 1984, 1985a) and for bilirubin oxidase (Skrika-Alexopoulis, Muir and Freedman, 1993). In the case of bilirubin oxidase it was shown that the denaturation kinetics could be described in terms of a double exponential decay equation, consistent with commonly observed patterns of enzyme inactivation in aqueous solutions under controlled conditions. These kinetics are consistent with unimolecular inactivation mechanisms of the type $N \leftrightarrow X \leftrightarrow D$, $X \leftrightarrow N \leftrightarrow D$ or $N \leftrightarrow D \leftrightarrow X$, where N and D are the native (active) and denatured (inactive) forms, and X is an on- or off-pathway intermediate (Ikai and Tanford, 1971; Hijazi and Laidler, 1972).

Nothing is known about the molecular mechanism of inactivation. Comparative studies (Lee and Biellman, 1986; Steinmann, Jäcle and Luisi, 1986; Samama, Lee and Biellmann, 1987; Schmidli and Luisi, 1990) indicate that surfactant type (non-ionic, cationic or anionic) is a critical factor. However, it is not clear whether protein 'binding' to the interfacial surfactant layer, or the formation of discrete protein–surfactant complexes, or both, is responsible for the denaturation.

Microemulsions as media for biotransformations

OVERVIEW

At the present time, most studies of enzyme behaviour in microemulsions have focused on fundamental issues, such as the factors that determine activity and stability. In this section, the applications of microemulsions are described in more detail, beginning with a summary of the properties of microemulsions which may be useful in process design (pp. 303–304). There is not a large literature concerning the development of microemulsion-based systems for specific biotransformations, although this situation is changing. Much recent attention has focused on the use of

microemulsions to facilitate lipase-mediated interconversions of lipids, and these are discussed on pp. 304–306. There are only few examples that demonstrate the potential of microemulsions as reaction media for other kinds of enzyme-catalysed reaction, and the more interesting of these are treated as casebook studies. This section concludes with a brief discussion of the application of Winsor II systems in protein purification.

GENERAL CONSIDERATIONS

From the foregoing, the unique properties of w/o microemulsions as reaction media for biotransformations can be summarized as follows:

1. compositional flexibility implies considerable potential for manipulating solvent properties to suit the reaction under consideration (a good example is the system for the production of tryptophan;
2. thermodynamic stability (spontaneous formation) implies none of the mechanical mixing problems normally associated with maintaining fine emulsion dispersions, and no scale-up problems;
3. very high surface area:volume ratios for the dispersed phase implies no phase-transfer problems, and also confers optical transparency, which permits facile monitoring of reaction progress using spectroscopic techniques;
4. manipulation of phase properties to facilitate product recovery.

Microemulsions provide an inherent flexibility in design by virtue of the fact that a large number of different oil and surfactant types, and even different polar materials, may be used. It has already been shown that microemulsion composition has a strong effect on activity and stability (pp. 288–301). In addition to those already discussed, some less obvious manipulations have turned up very interesting and useful results. For example, Yamada, Kuboi and Komasawa (1993) reported that the activity of *Ch. viscosum* lipase in AOT microemulsions can be increased by a factor of three by adding non-ionic surfactants of the Tween series, at AOT:Tween ratios of 16:1; Sebastiao, Cabral and Aires-Barros (1993) reported that the addition of hexanol (co-surfactant) to AOT microemulsions increases the stability (half-life) of a cutinase by a fctor of 45; and considerable improvements in the activity of lipoxygenase were obtained by optimizing the Nonidet P40:Triton X-35 ratio in a mixed microemulsion system (Piazza, 1992). Optimizations of this kind may well determine the economic viability of a process, and give a clear illustration of the importance of further basic research in understanding the behaviour of enzymes in these systems.

Methods for the recovery of products by taking advantage of phase-behaviour have already been outlined (pp. 263–270). The potential of this approach was clearly demonstrated by the work of Larsson, Adlercreutz and Mattiasson (1990), who showed that 4-methylcyclohexanol, produced by the horse-liver alcohol dehydrogenase (LADH)-catalysed reduction of 4-methylcyclohexanone could be recovered in the organic phase derived from AOT or by $C_{12}E_5$ w/o microemulsions by a temperature-induced Winsor IV $\rightarrow$ II shift. It was shown that multiple re-cycling of the aqueous phase, containing surfactant, enzyme and cofactors was possible with only marginal losses of enzyme activity at each step. The alternative use of ionic and non-

ionic surfactants demonstrated the opportunity for recovery of the oil-phase by either increasing or decreasing the temperature. This method of product recovery certainly deserves further attention. As discussed further below, alternative methods of product recovery involve the use of membranes to stabilize the interface between w/o microemulsion and conjugate oil phases, with product recovery by partitioning.

Cofactor recycling is an important issue in determining the economics of a process. Methods for the provision of reducing power (NADH) have been reviewed by Hilhorst (1989). Four basic methods of supplying reducing equivalents are outlined in *Figure 21*. In *Figure 21a* reducing equivalents are supplied by coupling the reduction of one alcohol to the oxidation of another in an equilibrium catalysed by horse-liver alcohol dehydrogenase (Larsson, Adlercreutz and Mattiasoon, 1987). In *Figure 21b* the photosensitized oxidation of an oil-soluble electron donor in a CTAB system is linked to the methylviologen-mediated reduction of enoic acid reductase. The process is most efficient with a photosensitizer which is located in the interphase, so establishing a vectorial electron transport chain (Verhaert *et al.*, 1989) (For a brief review of photoprocesses in microemulsion systems, see Luisi *et al.*, 1988). In *Figure 21c* the oxidation of hydrogen is linked to the lipoamide dehydrogenase (LipOH)-mediated reduction of NAD^+, which is utilized (in this example) by 20β-hydroxysteroid dehydrogenase, again in a CTAB microemulsion (Hilhorst, Laane and Veeger, 1983). A methylviologen acts as a mediator in the reduction of LipOH. A total turnover number (TTN = mol product formed/mol NAD^+ consumed) of the order of 10^2 was reported, which compares well with the 10^2–10^6 range required for an economically feasible cofactor regenerating system (Chenault, Simon and Whitesides, 1988). In *Figure 21d* hydrogen is replaced by an electrode. This last approach may not be very efficient due to the low electrical conductivity of oil-continuous microemulsions.

LIPASE-CATALYSED REACTIONS

Lipases are model examples of enzymes for cell-free biotransformations since they are catalytically efficient, stable and inexpensive enzymes which catalyse reactions of recognized industrial application, including the conversion of low- to high-grade fats by transesterification. Furthermore, triglycerides and carboxylic acids are highly soluble in AOT microemulsions and phospholipids themselves stabilize microemulsions (p. 273). The massive interfacial area provided by the microemulsion environment is conducive to extremely efficient transformation rates.

Several publications deal with the application of AOT microemulsions as solvent systems for lipase-catalysed ester hydrolysis or synthesis. Han and Rhee (1985) have described an efficient procedure for the batchwise hydrolysis of olive oil using *Candida rugosa* lipase. Kim and Cheung (1989) have similarly described methods for the hydrolysis of palm oil by *Rhizopus arrhizus* lipase and Derksen and Cuperus (1992) described the application of several lipases in the hydrolysis of Crambe oil. Stamatis *et al.* (1993a) have described the *Penicillin simplicissimum* lipase-catalysed esterification of *n*-alkanols with carboxylic acids and Hedström *et al.* (1992) reported the *C. rugosa* lipase-catalysed synthesis of cholesterol oleate by direct condensation, in AOT–iso-octane–water microemulsions. Singh and Shah (1993) have made a

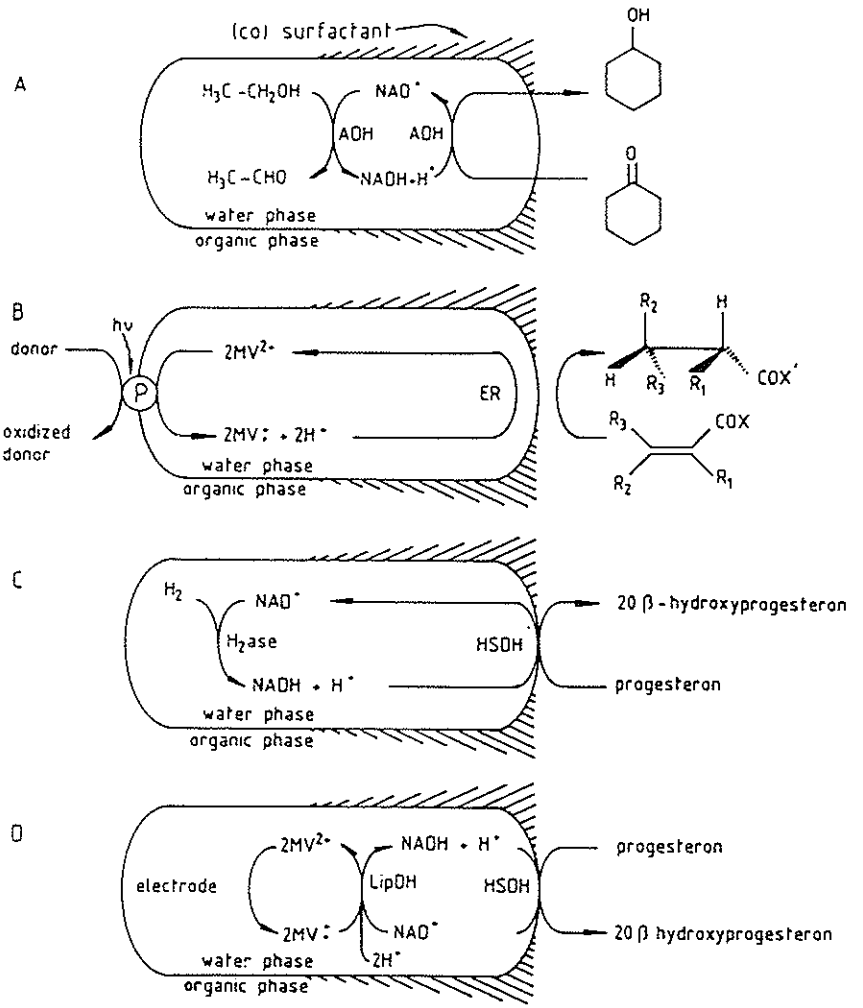


Figure 21. Enzyme-linked cofactor-regeneration in w/o-microemulsions. See text for details. (Reproduced from Hilhorst, R. (1989). In *Structure and Reactivity in Reverse Micelles* (M.P. Pileni, Ed.), pp. 323–340. Elsevier, Amsterdam, with permission.)

comparative study of the Lipozyme-catalysed esterification of glycerides in AOT-stabilized microemulsions and in lipid monolayers. Holmberg and co-workers have developed microemulsions based on AOT and other synthetic surfactants which support transesterification reactions catalysed by lipases (Holmberg and Osterberg, 1989; Osterberg, Blomstrom and Holmberg, 1989) and phospholipases A_2 (Na *et al.*, 1990).

There are also a few examples of the application of AOT-based microemulsions for the lipase-catalysed production of fine chemicals. For example Stamatis, Xenakis and Kolisis (1993) have studied the synthesis of (–)menthyl oleate from racemic menthol and medium- or long-chain carboxylic acids using *Penicillin simplissimum* lipase in AOT microemulsions. In an optimized system (iso-octane for the organic phase, R = 6), the esterification of the (–)enantiomer of menthol is made 6–8 times faster than

that of the (+)enantiomer, and yields of 75% (quoted with respect to conversion of the FA, present in limiting amounts) were obtained during a 45 min incubation. This is reportedly much faster than in organic solvent/enzyme suspensions. Hayes and Gulari (1992) have described a method for preparing polyol-carboxylate esters by direct condensation using *C. rugosa* and *R. delemar* lipases, in an AOT microemulsion system.

Morita *et al.* (1984) were apparently the first to consider the use of water–oil microemulsions stabilized by phosphatidylcholines as media for lipid modifications. In their paper they described the synthesis of triglyceride (TG) from 1,2-diacylglycerol and free fatty acid (FFA) by *Rhizopus delemar* lipase in *n*-hexane–water microemulsions stabilized by soybean lecithin. Similarly Schmidli and Luisi (1990) reported the synthesis of tricaprylin from glycerol and caprylate, using the same enzyme, and Marangoni, McCurdy and Brown (1993) reported the *Rhizopus arrhizus* lipase-catalysed interesterification reaction between triolein and tripalmitin, leading to the formation of TG's of mixed FA composition. The kinetics of the esterification of oleate with ethanol, catalysed by *Mucor miehei* lipase in a lecithin–water–dodecane microemulsion, have been studied in some detail by Oliveira and Cabral (1992).

Gaathon, Groos and Rozhanski (1989) reported the use of a related enzyme, tannin acyl hydrolase, for the synthesis of propyl gallate, a food antioxidant, by transesterification of the tannic acid with propanol, in an AOT-stabilized system.

A number of studies have recently appeared which deal with the scale-up of these reactions. A good example of a simple continuous-flow membrane reactor is the continually stirred tank reactor (CSTR) system described by Chang, Rhee and Kim (1991) for the preparation of 1-mono-oleoyl glycerol by the continuous glycerolysis of triolein, catalysed by *Ch. viscosum* lipase in an AOT-iso-octane system with a glycerol–water mixture for the polar subphase. The microemulsion was contained within Amicon®-type ultrafiltration unit fitted with a hydrophobic (polysulphone) membrane. Substrate dissolved in iso-octane was delivered under pressure, which served to simultaneously ultrafilter the microemulsion and maintain volume. Under steady-state conditions the reactor (volume 20 ml), optimized for microemulsion composition and lipase type, delivered 1-mono-oleoyl glycerol at rate of 75 μmol h^{-1} for at least 6 days.

Prazeres *et al.* (1993a,b) have reported on the operational characteristics of a CSTR for the *Ch. viscosum* lipase-catalysed hydrolysis of triglycerides, operated either in batch mode or with circulation through a hollow-fibre system for the continuous removal of products (glycerol, free fatty acids). The hollow-fibre system is reported to be superior to the batch system due to the alleviation of product inhibition by free fatty acids. However, the microemulsion droplets may leak out of the hollow-fibre lumen into the permeate tube. In order to circumvent this generally recognized problem, Nakamura and Hakoda (1992) have proposed the use of a modified ultrafiltration in which an electrical potential is applied between the wall of the hollow fibre and a stainless-steel rod, passed through the centre of the lumen. The polarity of the electrical field is such that the droplets (which usually bear a net charge; Kim and Huang, 1986) are repelled from the wall.

TRYPTOPHAN PRODUCTION

Another example of a membrane reactor, which additionally illustrates the flexibility of design which microemulsions provide, was described by Eggers and Blanch (1988) for the synthesis of tryptophan from indole and serine, using tryptophanase (*Figure 22*). Here, the basic problem was to design a solvent system in which both indole and serine are soluble, and which also supports enzyme activity. The microemulsion selected consisted of cyclohexane, water, Brij 56 (surfactant) and Aliquat 336 (co-surfactant; commercial name for trioctylammonium bromide). The aqueous phase was buffered with potassium bis-tris-propane (BTP, pH9). This formulation was arrived at by choosing Aliquat 336 as an ion-pairing reagent for serine, so improving the solubility of the latter in the microemulsion. A non-ionic surfactant was selected on the basis that an anionic surfactant (or the counterion of a cationic surfactant) might ion-pair with the co-surfactant, leading to a complex phase-behaviour. The same considerations led to the choice of BTP as buffer; the cationic conjugate acid will not interact with the co-surfactant (the base is uncharged); an anionic buffer might ion-pair with the co-surfactant, leading to a change in pK_a, and hence pH, of unpredictable magnitude. The pH was selected to ensure stabilization of the Aliquat 336/serine ion-pair (the α-amino group, pK_a 9.1, must be protonated in order to form an ion-pair), noting that the pH optimum of the enzyme lies in the range 7.2–8.8. The potassium salt of BTP was selected since this metal has a stabilizing effect on the enzyme. The oil-soluble product was recovered by partitioning into a conjugate oil phase separated from the microemulsion phase by a hydrophobic membrane. The system was reported to yield about 10 g tryptophan/g enzyme/hour, from a 1 l reactor, compared with a reported value of 1.3 $g/g/h^{-1}$ for a batch reactor utilizing an aqueous micellar Triton X-100 system as a solvent for the indole. The authors concluded with the comment that the system generated better yields of tryptophan than those obtained in a conventional two-phase system (using recombinant *E. coli* as the biocatalyst), although the high price of indole renders the system economically non-viable at the present time.

DEGRADATION OF TOXIC ORGANOPHOSPHATES

Recent work by Russell and co-workers (Komives, Lilley and Russell, 1994; Komives, Osborne and Russell, 1994; Russel and Komives, 1994) exemplifies the use of microemulsions in the area of bioremediation. These workers have described a method for continuous enzymatic detoxification, based on the use of a centrifugal reactor in conjunction with a water–*n*-hexane–Tween 85 Winsor II system, the microemulsion phase of which acts as an enzyme-containing liquid membrane. The example given describes the design for a system to remove toxic organophosphates (residual pesticides) from soil, using the enzyme organophosphorus hydrolase from *Pseudomonas diminuta*. The soil is decontaminated as it passes under centripetal force through the microemulsion phase, and is recovered from the aqueous-phase sludge. It might be noted that the chosen enzyme is extremely active, with reported k_{cat} values for dephosphorylation of para-oxon of 1100 s^{-1}. Tween 85 (sorbitan trioleate) was chosen because it is biodegradable.

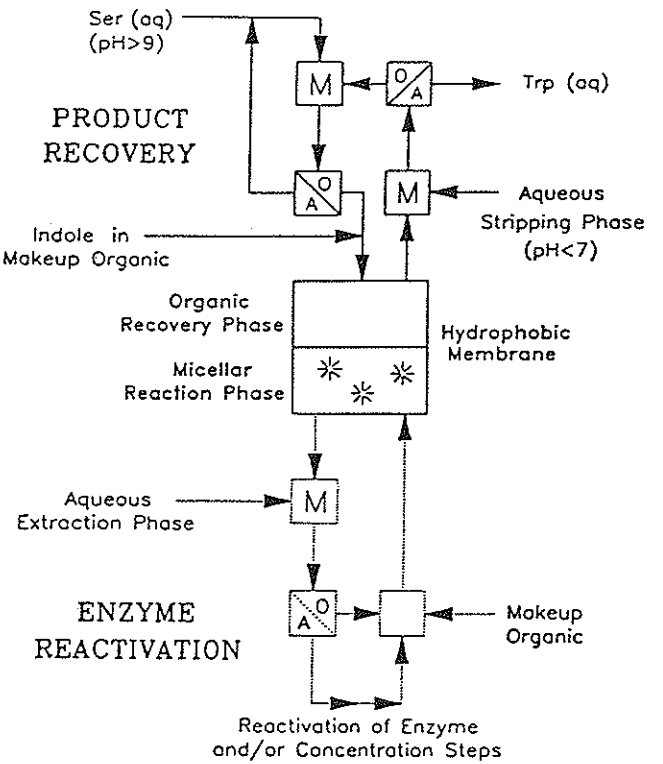


Figure 22. Reactor scheme for the production of trytophan from indole. (Reproduced from Eggers, D.K. and Blanch, H. (1988) *Bioprocess Engineering* 3, 83–91, with permission.)

POLYMER SYNTHESIS

Rao *et al.* (1993) have described the use of w/o microemulsions as media for the synthesis of polyethylphenols from 4-ethylphenol using horseradish peroxidase. Synthesis in water–AOT–iso-octane microemulsions was found to be superior to that in iso-octane or water alone, or in iso-octane-water two-phase systems, since the microemulsion is a better solvent for both substrate (monomer) and enzyme. In practice, the polymer precipitates on reaching a critical size, which is determined by the droplet concentration. Furthermore, the polydispersity index of the material is relatively low. The microemulsion phase can be recycled (more monomer added) after filtration to recover the polymer, so suggesting the possibility of a very simple batch process. Once again, a key factor in the commercial viability of a microemulsion-based process is the high activity of the enzyme (k_{cat} = 600 s^{-1}).

PROTEIN PURIFICATION BY LIQUID–LIQUID EXTRACTION

The use of w/o microemulsions for the extraction of solutes from a conjugate aqueous phase has received considerable attention. This application follows the discovery that water-soluble solutes (salts, amino acids, proteins) partition between the microemulsion and conjugate aqueous phases of a Winsor II system, that the distribution coefficients

differ for different compounds and, furthermore, that the distribution can be modulated by factors such as pH and electrolyte concentration. These observations find useful application in the purification of proteins (e.g. from culture-broths, cell-extracts) by selective extraction (Leodidis and Hatton, 1989).

At least for Winsor II systems prepared with ionic surfactants, a combination of electrostatic interactions, and the size of the protein, determine uptake. In the case of microemulsions stabilized by the cationic surfactant trioctyl ammonium chloride (TOMAC), for example, the optimal pH for phase transfer is given by the expression (Wolbert *et al.*, 1989),

$$pH_{OPT} - pI = 0.11 \times M_R - 0.97, \qquad \text{Eq. (19)}$$

where M_R is the molecular weight in kDa. This equation simply says that for good phase transfer, the pH must be about one unit above the iso-electric pH (so that the protein has a net negative charge) plus one more pH unit for each 10 kDa of molecular weight. Furthermore, there is a correlation between the charge distribution on the protein and the extent of partitioning (*Figure 23*) at the optimal pH; proteins with more asymmetric charge distributions partition more extensively into the microemulsion phase, presumably because those regions on the protein surface which bear a high degree of negative charge interact strongly with the surfactant monolayer at the microemulsion–water interface, the committing step in phase transfer.

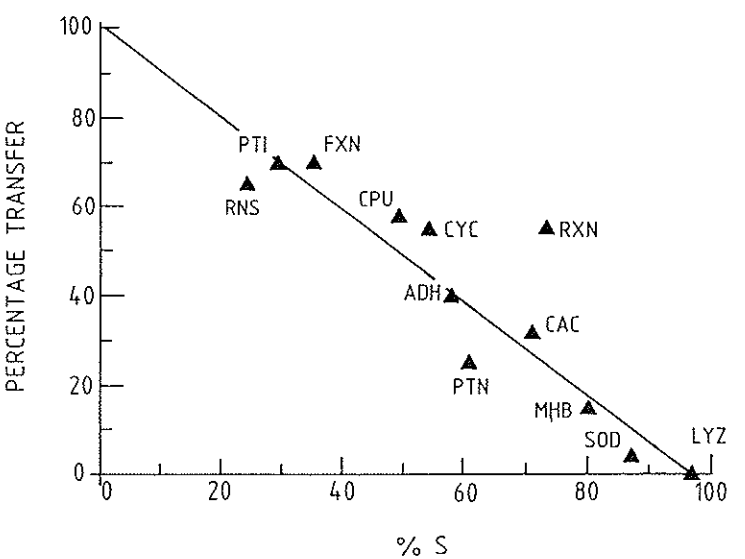


Figure 23. Dependence of degree of transfer to the microemulsion phase in a Winsor II system as a function of charge asymmetry for different proteins at their optimal extraction pH. Lower %S equates with greater charge asymmetry. Abbreviations: PTI, trypsin inhibitor; RNS, ribonuclease A; FXN, flavodoxin; CPU, parvalbumin; CYC, cytochrome *c*, ADH, liver alcohol dehydrogenase; PTN, trypsin, CAC, carbonic anhydrase; RXN, rubredoxin; MHB, haemoglobin; SOD, superoxide dismutase; LYZ, lysozyme. (Reproduced from Wolbert, R.B.G., Hilhorst, R., Voskuilen, G., Nachtegaal, H., Dekker, M., van't Riet, K. and Bijsterbosch, B.H. (1989). *European Journal of Biochemistry* **184**, 627–633, with permission.)

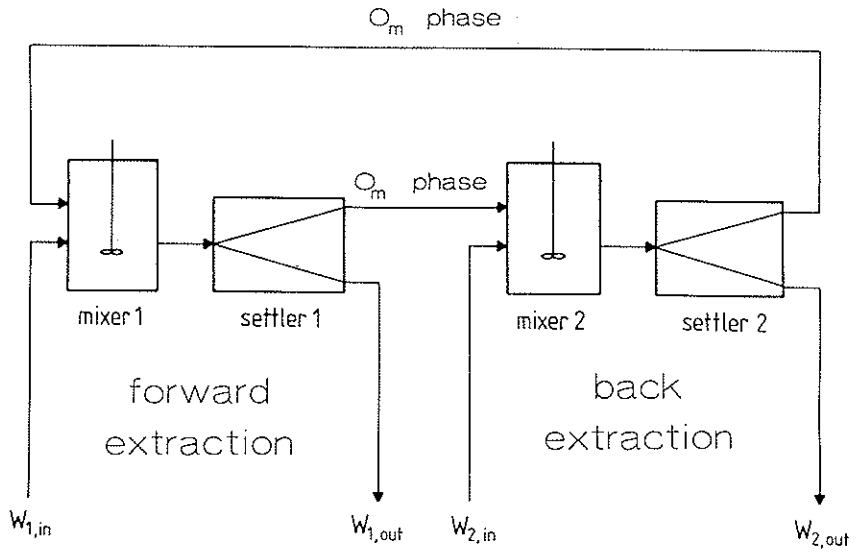


Figure 24. Reactor scheme for extraction and recovery of α-amylase. (Modified from Dekker, M., Van't Riet, K., Weijers, S.R., Baltussen, J.W.A., Laane, C. and Bijsterboch, B.H. (1986). *Chemical Engineering Journal* **33**, B27–B33, with permission.)

A fair qualitative understanding of the relation between protein charge and size and the distribution coefficient has developed (Leodidis and Hatton, 1989). The dependence on size suggests an entropy effect, the coalescence of several small droplets to house large protein molecules being unfavourable. However, a negative entropy contribution to the free-energy of phase transfer may be compensated for by a sufficiently large negative enthalpic term, arising (at least for systems containing charged surfactants) by favourable electrostatic interactions (formation of salt-links) between the protein and the interphase. The required charge on the protein is obtained by appropriate adjustment of the pH. The corollary of this argument – supported by several studies – is that extraction selectivity can be modulated either by altering the droplet size or by altering the pH. The droplet size can be altered independently of the ionic strength by changing the electrolyte type (changing the concentration leads to changes in both droplet size and, through screening effects, electrostatic interactions). For example, the droplets formed in Winsor II systems stabilized by AOT are much smaller when KCl is used instead of NaCl at the same concentration (Leodidis and Hatton, 1989). It is also possible to change the surfactant counterion, since this also strongly affects the droplet size (Eastoe *et al.*, 1992). This can be understood in terms of *Sp* (equation 2). Different counterions have different screening efficiencies and hence determine different values for a_0.

The application of Winsor II systems for large-scale protein extraction has been demonstrated for several specific proteins. *Figure 24* shows the design for a reactor system for the extraction of α-amylase from a commercially available crude enzyme preparation (Dekker *et al.*, 1986). Again, a TOMAC microemulsion system is used. The forward transfer takes place in the first CSTR (the aqueous solution contains 0.5 M Tris–HCl, pH 9.85). The equilibrated mixture then passes to the first settler unit where the microemulsion and aqueous phases separate out. The aqueous phase is

discarded and the microemulsion phase passes to mixer 2, together with a fresh aqueous phase set at pH 6.9 (1.0 M tris–HCl) which permits back-extraction. After transfer of the protein to the aqueous phase the microemulsion phase is recycled. An eight-fold purification of α-amylase and 70% recovery were reported. Similarly, Barthomeuf, Régaret and Pourrat (1994) have reported a purification of tannase from fungal mycelia by partial enzymatic hydrolysis of the mycelium followed by liquid–liquid extraction using a CTAB microemulsion system. A 2.5-fold purification and 43% recovery were reported.

A variation on this theme involves disrupting cells by solubilizing them in microemulsions. After removal of cell debris e.g. by low-speed centrifugation, enzymes may be selectively back-extracted into an aqueous phase (Giovenco, Verheggen and Laane, 1987). Lee *et al.* (1989) have shown that squalene epoxide hydrolase can be extracted from rat-liver microsomes using this approach.

In a few cases, back-extraction procedures involving changing the electrolyte concentration, pH and temperature have proven to be ineffective. For example, cytochrome b_5 cannot be back-extracted from CTAB microemulsions (Pires and Cabral, 1993). A different approach to back extraction involves recovering the aqueous phase by treating the microemulsion with silica at pH 8 (Leser, Mrkoci and Luisi, 1993). This treatment afforded good recovery (yield and activity) of trypsin and α-chymotrypsin from an AOT microemulsion phase.

Apart from control of pH and electrolyte concentration, opportunities for modulation of extraction specificity include changing the surfactant type. Goto and Nakashio (1992) have reported on the extraction specificity of several different proteins with microemulsions prepared using AOT and several other surfactants. More sophisticated methods for controlling extraction specificity involve the use of affinity ligands. An elegant demonstration of this approach has been described by Paradkar and Dordick (1991, 1993), for glycoproteins such as horseradish and soybean peroxidase (pIs in the range 3.5–4.5), which do not extract into AOT microemulsions at pH>5 due to charge–repulsion. It was found that these enzymes could be extracted after complexation with concanavalin A. The lectin has a high pI (>8) and extracts readily at pH 5, as does the lectin/glycoprotein complex, presumably because the positive charge on the lectin moiety compensates for the unfavourable negative charge on the target protein. Back-extraction was achieved by equilibrating the microemulsion phase with fresh pH 5.0 buffer containing mannose (which displaces the glycoprotein ligand from the lectin). The free lectin was separated from the enzyme with a final extraction using fresh microemulsion phase (pH 5.0, 25°C), whereupon the lectin/mannose complex alone re-extracts into the microemulsion phase. Using this approach the authors reported a 30-fold purification and 30% recovery of soybean peroxidase from a commercial source of soybean hulls. In principle, the method can be extended to use antibodies for even more specific affinity-based microemulsion extraction procedures.

In an interesting elaboration on the liquid–liquid extraction theme, Hatton and coworkers have described the use of microemulsion extraction to renature proteins (Hagen, Hatton and Wang, 1990a,b). The process involves:

1. extraction of denatured proteins into the microemulsion from aqueous guanidine hydrochloride;

2. selective back-extraction of GuHCl, leaving the enzyme to refold in the microemulsion phase (reduced glutathione was addded to facitate disulphide bond formation during refolding); and
3. back-extraction to recover the enzyme from the microemulsion phase.

This procedure was successfully applied to ribonuclease, but was not successful in the case of γ-interferon, a more hydrophobic protein. It was suggested that this is because hydrophobic proteins interact much more strongly with the surfactant layer. Garza-Ramos *et al.* (1992) have described a similar approach in which GuHCl-denatured triosphosphate isomerase was renatured by injecting the protein solution into a CTAB microemulsion.

Special topics

MICROEMULSION-BASED GELS AND NANOGELS

Overview

Microemulsion-based gels (MBGs) is a generic term for systems which have the combined properties of gels (semisolid in nature; deformable with a high shear viscosity) and liquid microemulsions (usually transparent, optically isotropic, and thermodynamically stable). These gels are considered to have potential as a new generation of immobilized biocatalyst supports. Two important types are being studied at the present time: lecithin gels and gelatin gels (see below). Nanogels are prepared by polymerization of water-soluble monomers using the microemulsion as a polymerization medium and the gel may be removed from the microemulsion. Once again, nanogel-entrapped enzyme is considered to have potential as an immobilized biocatalyst. Additionally, there is some interest in the use of nanogel particles as drug-delivery systems.

Lecithin gels

First described by Scartazzini and Luisi (1988), lecithin microgels, rather than liquid microemulsions, form spontaneously in oils such as decane, hexane and cyclohexane, but not in benzene or diethylether, above a critical (water+lecithin) volume-fraction, as discussed on pp. 273–274. These gels, like droplets-type microemulsions, are optically transparent, and again the progress of chemical reactions can be followed spectroscopically. There seems to be only one report so far concerning lecithin gel-entrapped enzymes. In this example the *C. rugosa* lipase was shown to hydrolyse tricaprylin. No hydrolysis of the lecithin occurred (Scartazzini and Luisi, 1990).

Gelatin gels

Liquid microemulsions may be induced to gel by the addition of relatively large amounts of gelatin. Again the gels, prepared by mixing the microemulsion with gelatin at 55°C and cooling, are essentially transparent, isotropic and stable. Phase diagrams for the AOT–water–*n*-heptane–gelatin system are given in Atkinson *et al.*

(1991). Neutron-scattering studies suggest that gels consist of an interlaced network of surfactant-coated gelatin gel rods, co-existing with microemulsion droplets (*Figure 25*) (Atkinson *et al.*, 1989). *Chromobacterium viscosum* lipase immobilized within the gels is active, shows good stability properties, and has been shown to catalyse the condensation of a variety of alcohol/carboxylic acid combinations. The gels can be sectioned and packed in columns to make continuous-flow reactors for biotransformations (Jenta, Robinson and Thomson, 1991; Rees *et al.*, 1991). The properties and applications of gelatin-based microgels have recently been reviewed by Rees and Robinson (1993).

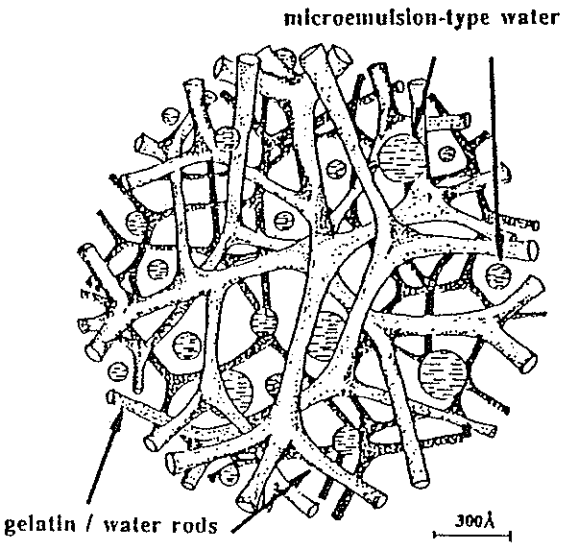


Figure 25. Proposed structure of the AOT–water–*n*-heptane–gelatine gel system. (Reproduced from Atkinson, P.J., Robinson, B.H., Howe, A.M. and Heenan, R.K. (1991). *Journal of the Chemical Society – Faraday Transactions* **87**, 3389–3397, with permission.)

Nanogels

A different kind of microemulsion gel involves the chemical or photo-polymerization of water-soluble monomers (e.g. acrylamide/bis-acrylamide, polyvinylpyrrolidone, methylcyanoacrylates), within the aqueous subphase. One can envision the creation of a range of gel structures, depending on the composition of the original microemulsion and on the rate of polymerization, from 'nanogels', generated by rapid polymerization in droplets-type systems, to extended sponge-like networks arising from slower polymerization in bicontinuous systems. The gels may be recovered from the parent microemulsion by precipitation, filtration or centrifugation.

The application sof these systems as vehicles for controlled drug-release have long been recognized (Speiser, 1984). The possibilities for gel-entrapment of enzymes were first described by Khmelnitsky *et al.* (1989b). Polymerization of acrylamide/bisacrylamide in AOT microemulsions also containing α-chymotrypsin covalently modified with a detergent, Pluronic F-108 (a co-polymer with the formula $HO(CH_2\text{-}CH_2\text{-}O)_{150}\text{-}(CH(CH_3)\text{-}CH_2\text{-}O\text{-})_{55}\text{-}(CH_2\text{-}CH_2\text{-}O)150\text{-}OH$) generated nanodroplets of approximate radius 10–60 nm, which could be removed from the microemulsion by

precipitation with acetonitrile. Covalent modification was used simply to increase the hydrodynamic volume of the enzyme in order to prevent it from diffusing out of the gel. There are also opportunities for covalent attachment of the enzyme in order to prevent it from diffusing out of the gel-bead. The enzyme was reported to retain only about 10% of its activity after modification/entrapment, but was stable for several weeks. The gels were reported to be soluble in benzene, with retention of enzyme activity as demonstrated by the esterification *N*-acetyltyrosine with ethanol (resembling, in this respect, the PEG-modified enzymes described by Inada *et al.*, 1986).

DETERGENTLESS MICROEMULSIONS

Several enzymes have been studied in the hexane–isopropanol–water system (*Figure 8*), including trypsin (Khmelnitsky *et al.*, 1986), cholesterol oxidase (Khmelnitsky, Hilhorst and Veeger, 1988) and mushroom polyphenol oxidase (Vulfson *et al.*, 1991). All three enzymes are reported to be maximally active within the microemulsion region (C; *Figure 8*). Cholesterol oxidase is apparently much less stable than trypsin. No stability data were reported for the polyphenoloxidase. *Caldariomyces fumago* chloroperoxidase is reported to be active in toluene/isopropanol/water microemulsions (Fedorak *et al.*, 1993). This model system for the demetallation of asphaltenes (the enzyme cleaves porphyrin groups, resulting in the release of the metal ion) is reported to be superior for this purpose compared with a two-phase system.

MICROEMULSIONS AS MEMBRANE-MIMETIC SYSTEMS

The application of microemulsions as membrane-mimetic systems, duplicating the lipid–aqueous interface of the living cell, has been discussed by Martinek *et al.* (1986, 1989). In the cell many enzymes are intrinsic or peripheral membrane proteins and their activity and stability characteristics may well be determined in greater or lesser degree by the nature of the membrane association and by the properties of the interfacial water. A number of recent papers have dealt with the properties of membrane-associated proteins in phosphatidylcholine-based microemulsions. Examples include enzymes of the mitochondrial respiratory electron transfer chain (Escamilla *et al.*, 1989; Escobar and Escamilla, 1992) and F_1-ATPase in the phospholipid–toluene–water system (Garza-Ramos *et al.*, 1989). These studies, which may be regarded as preliminary, indicate that the intact respiratory chain can be made to function in microemulsions.

Conclusion: trends and future prospects

The goal is to make enzymes powerful and commercially viable tools in the synthesis of useful compounds such as medicines, food additives and novel polymeric materials, and in essential processes such as decontamination (bioremediation, biodetoxification). Microemulsions have been shown to have considerable potential as solvents for many of these reactions, arising from their unique solubility characteristics for apolar, polar or charged, and surface-active compounds.

Microemulsions-based enzymology is still a relatively young field and the majority of studies have focused on the fundamentals of enzyme behaviour in these systems. Although there are still many questions to be answered, there are the beginnings of an

understanding of the factors that influence reaction rates and equilibria and enzyme stability. Useful methods for product recovery, cofactor regeneration and recycling of essential components have been described. The applications detailed above show how this potential can be realized to great advantage in several different areas of bioengineering. Nearly all of these studies have been published in the past couple of years, and it is to be taken as an indication that the field has come of age that the transition from the basic to the applied is now being made.

Microemulsions-based cell-free technology, like all cell-free technologies, is nevertheless circumscribed at the present time by the relatively high cost of enzymes and cofactors, which often render them economically non-viable option compared with whole-cell biotransformations. It is reasonable to expect that, as costs come down and cell-free reactions become more competitive, more opportunities for exploiting microemulsions-based technology will appear.

The future should bring some exciting developments in the field of microemulsions prepared with biological surfactants and other novel, biodegradable surfactants. It is anticipated that microemulsions will find broader application, not only as media for the synthesis of fine chemicals (this application is heavily emphasized in the current literature), but also in bioremediation (as, for example, in the decontamination of organophosphorus-contaminated soils) and in the biopurification of petrochemicals (exemplified by use of a detergentless microemulsion as a model system for the demetallation of asphaltenes).

The further application of microemulsions for the controlled synthesis of polymers, both as added-values products and in the continued development of nanogel-encapsulated enzyme technology is anticipated. The concept of enzymes entrapped within extremely small acrylamide gel particles (good surface area:volume properties) which are active in variety of solvents and which can be stored in dry form with a long shelf-life, is one that should be actively pursued in the interests of improving the quality of off-the-shelf enzymes for use in biotransformations.

Of equal importance is the requirement for more basic research to underpin the applied effort. Good systematic characterization of the activity and stability of enzymes in different microemulsion systems would provide a useful database for applications purposes. In particular it is important to understand why certain enzymes become superactive in the microemulsion environment. Since improvements of the order described for HRP, tyrosinase and acid phosphatase can turn around the economics of a process. There is a case for the introduction and further characterization of new microemulsion systems, utilizing different surfactants (including a greater emphasis on the non-ionics); the literature bias towards AOT is understandable, but it is becoming clear that other systems have properties that make them more useful in applications. The possibility that from an holistic point of view, AOT is not the best-choice microemulsion surfactant is illustrated by the fact that only a few of the applications described above utilize this surfactant.

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