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ORIGINAL CONTRIBUTION

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Lecithin/propanol-based microemulsions used as media for a cholesterol oxidase-catalyzed reaction

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Abstract Reverse micelles, Winsor III and IV systems were examined as reaction media for the enzymatic conversion of cholesterol to cholestenone by cholesterol oxidase at 298.2 K. The micelles and the microemulsions, stabilized by soybean lecithin and ethanol or 1-propanol as cosolvent, were characterized with respect to phase behavior and distribution of 1-propanol between the phases of the Winsor III systems. The used oils were dodecane, tetradecane, and hexadecane. The Winsor IV systems and the surfactant-rich phase in the Winsor III systems exhibit bicontinuous structures. The reaction yield for the enzymatic conversion performed in a Winsor IV system was much higher than in a Winsor III system or in reverse micelles.

Key words Microemulsions – soybean lecithin – partial phase diagram – 1-propanol distribution – enzyme catalysis – cholesterol

Introduction

Microemulsions, systems of water, oil and surfactant forming optically isotropic, low viscous and thermodynamically stable solutions, possess several advantages as media for various chemical reactions, including enzyme catalyzed ones [1–4]. Microemulsions exhibit a large interface between oil and water and the water content can be varied within a large interval. This is of major importance, when water soluble enzymes acting on water insoluble reactants are used. The catalyzing effect is enhanced if the enzyme is surface active, i.e., if it is accumulated at the interface between oil and water [5–7].

In our laboratory, one- and three-phase microemulsion systems have been investigated as reaction media for the catalytic oxidation of 5-cholesten-3β-ol (cholesterol) to 4-cholesten-3-one (cholestenone). The yield was about 0.3 in a water-in-oil (W/O) microemulsion stabilized by the anionic surfactant sodium 1,4-bis(2-ethylhexyl) sulfosuccinate (AOT), while the yield was only 0.2 in a Winsor III system stabilized by the naturally occurring zwitterionic surfactant soybean lecithin [8, 9]. Since the latter surfactant is slightly too lipophilic to form microemulsions spontaneously in oil-water systems, a fourth component, i.e., ethanol was added to the system, where it acts as a cosolvent rather than as a cosurfactant [9–11]. The low yield in the Winsor III system was a result of unfavorable distributions of the enzyme cholesterol oxidase and the reactant cholesterol between the three phases. A high alcohol content can inactivate the enzyme [7] and, as less propanol than ethanol is needed to create a Winsor III system, propanol has been used in this work. On the other hand, the distribution of the enzyme and the substrate between the phases of the Winsor III system is still an insurmountable problem. Therefore, we also investigated a Winsor IV system as reaction medium for the enzymatic conversion of cholesterol to cholestenone. This system is a one-phase isotropic solution usually situated in the middle of a triangle diagram consisting of oil, water, and surfactant. To complete the picture, the enzymatic conversion of the same substrate has also been performed in reverse micelles stabilized by soybean lecithin and ethanol as cosolvent. These micelles consist of an aqueous core surrounded by surfactant molecules in an oil continuous medium, while a Winsor IV system has a bicontinuous structure of intertwined aqueous and oil channels separated by a surfactant film with low or zero curvature [12, 13]. Larsson [14] has shown that these different structures of the microemulsions had no impact on kinetic parameters and the variation of the reaction rate was explained by different substrate distributions between the microdomains in the microemulsions. During the course of reaction, formed products may, however, compete for a place at the interface between the aqueous and oil domains causing changes in the structure of the film and accordingly changes in the reaction rate [15]. Cholesterol is surface active and penetrates into the surfactant film. This penetration can, to some extent, be different at a curved or at a planar interface, which again can affect the reaction rate.

The different microemulsions have been characterized by partial phase diagrams determined at 298.2 K and the distribution of 1-propanol between the phases of the Winsor III systems. The used oils are dodecane, tetradecane, and hexadecane, respectively. Preparative conversions of cholesterol to cholestenone catalyzed by cholesterol oxidase have also been carried out by Khmelnitsky et al. [16] in detergentless "microemulsions", and by Lee and Biellmann [17] in W/O microemulsions stabilized by hexadecyltrimethylammonium bromide. In these studies the conversion was complete with a low initial concentration of cholesterol. In the present work both the initial substrate concentration and the enzyme content have been varied. All conversions were performed at 298.2 K and no buffer was added to the systems.

Materials and methods

Chemicals

Soybean lecithin (Epikuron 200), a zwitterionic surfactant with two head chains connected to a phosphatidylgroup, was a gift from Lucas Meyer Co., FRG. The distribution of fatty acids was according to the producer: palmitic and stearic acid 16–20%, oleic acid 8–12%, linoleic acid 62–66%, linolenic acid 6–8%. Hexadecane and tetradecane were from Sigma Chemicals, USA. Hexane, 1-propanol and 2-propanol were obtained from Merck, FRG, while ethanol was from Oy Alko Ab, Finland. They all had an estimated purity > 99%. Dodecane (90–95% purity) was from Fluka, Switzerland. The water was distilled and deionized immediately before use. Cholesterol oxidase (EC 1.1.3.6; *Brevibacterium sp.* 10.4 U/mg) was from Beckman, USA, and 5-cholesten-3β-ol, 4-cholesten-3-one and 5β-cholestan-3α-ol (Epicoprostanol) were from Sigma Chemicals. Bis(tri-methylsilyl)-trifluoroacetamide (BSTFA) used for silylation was also from Sigma Chemicals.

Phase behavior

The phase behavior was determined by preparing samples with fixed compositions. The samples were thoroughly shaken and then allowed to equilibrate in a thermostated bath at 298.2 K for 2 days.

Distribution of alcohol

The distribution of 1-propanol between the microemulsion phases, the excess organic and aqueous phases of the Winsor III systems was determined gas-chromatographically. The samples were injected into a Varian 3400 instrument equipped with a Quadrex column (Quadrex Scientific 25 m × 0.53 mm with a 5.0 μm film thickness).

Enzymatic reaction

Cholesterol and cholesterol oxidase were added to Winsor III, Winsor IV systems and to reverse micelles, respectively. Samples were taken both from the microemulsion and the excess organic phases after given time intervals for analysis. Water and a solution of hexane and 2-propanol were added to the samples. The resulting two-phase systems were thoroughly shaken and then centrifuged for 10 min at 1500 rpm. New samples from these organic phases were evaporated together with an internal standard. BSTFA was added and the samples were kept for 1 h at 343.2 K. The samples were then analyzed gas-chromatographically. The used column was a DB-5 column (J&W Scientific; 30 m × 0.252 mm with a 0.25 μm film thickness).

Results and discussion

Figure 1 shows partial phase diagrams determined at $\alpha_{\text{oil}} = m_{\text{oil}}/(m_{\text{water}} + m_{1-\text{propanol}}) = 1$ for water/1-propanol-lecithin-alkane systems, where the alkanes are dodecane, tetradecane or hexadecane. The phase diagram with hexadecane as the oil is in good agreement with the diagram for the corresponding system determined by Shinoda et al. [10, 11]. The same authors have also shown

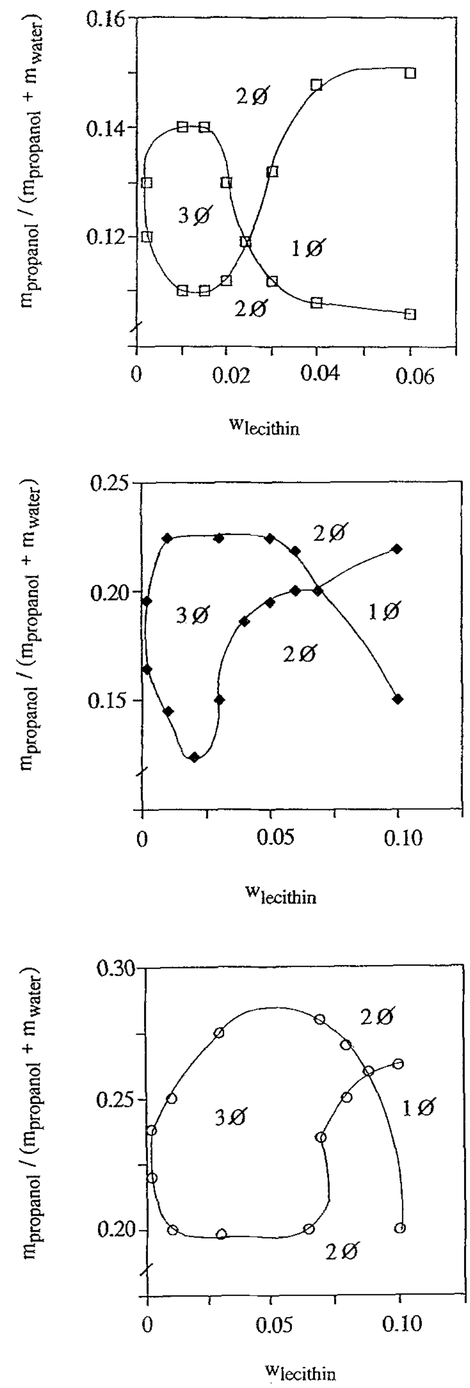
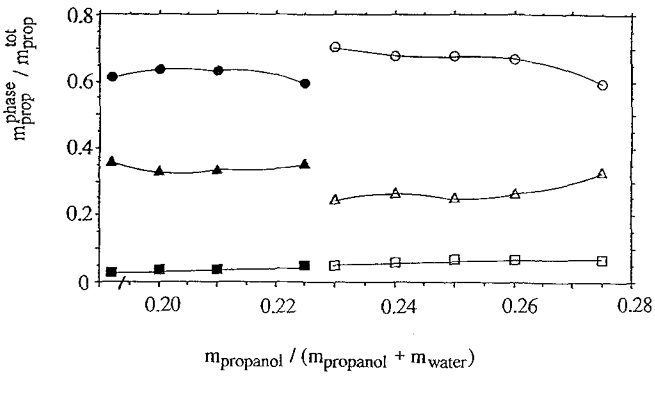


Fig. 1 Partial phase diagrams at 298.2 K for the systems soybean lecithin-water/1-propanol-hexadecane (a, □), tetradecane (b, ◆) and dodecane (c, ○) at $\alpha_{oil} = m_{oil}/(m_{water} + m_{propanol}) = 1$. NØ denotes number of phases and m_i and w_i are the mass and mass fraction of component *i*, respectively

that in this system 1-propanol acts as a cosolvent rather than as a cosurfactant. Propanol shifts the HLB balance in the system by making the water less hydrophilic. At low alcohol contents two-phase regions are present for all systems in Fig. 1, i.e., a microemulsion phase is in equilibrium with an aqueous propanol solution. The microemulsion phase consists of aqueous propanol droplets stabilized by lecithin in an oil continuous medium. Some propanol can also penetrate the stabilizing interface between the aqueous propanol and oil domains. The amount of propanol needed to transform these Winsor II systems to Winsor I systems via Winsor III systems increases in the order of hexadecane, tetradecane, and dodecane, i.e., with decreased size and decreased hydrophobicity of the alkanes. Hence, the smallest amount of 1-propanol is needed to destabilize the aqueous droplets in the hexadecane system. Shinoda et al. [10, 11] have deduced from molecular NMR selfdiffusion coefficients that the surfactant-rich phase of these Winsor III systems exhibits a bicontinuous structure. For the ionic surfactant sodium dodecyl benzene sulphonate, Ahsan et al. [2] have shown that more 1-butanol per surfactant molecule is required to invert the systems from Winsor I to III containing tetradecane compared to those containing decane or heptane. In these systems 1-butanol, however, acts as a cosurfactant. The explanation is that shorter alkanes penetrate more strongly than longer alkanes into the chain regions of surfactant monolayers both at planar and curved interfaces. Much more 1-butanol can thus be accumulated at the interface in the presence of longer alkanes.

Propanol is present in all three phases of the Winsor III systems, as can be seen in Fig. 2. The propanol content in the organic phase of the tetradecane system is smaller than in the dodecane system. However, these propanol contents are much smaller than the propanol contents in the aqueous phases for both systems. It can be pointed out that the distribution coefficient for 1-propanol between alkane and water decreases with increased number of carbon atoms in the hydrocarbon chain [18]. On the other

Fig. 2 Distribution of 1-propanol at varying propanol contents for the tetradecane system (closed symbols) and dodecane system (open symbols) at 298.2 K and $w_{lecithin} = 0.03$. (□) excess organic phase, (△) microemulsion phase, (○) excess aqueous phase



hand, the propanol content in the middle phase is larger for systems with longer alkanes, and most of the propanol is located in the aqueous channels, thereby making them less hydrophilic. This can, to some extent, explain why the transition from Winsor III to I occurs at lower propanol contents for the tetradecane system than for the dodecane system.

Very few enzymatic reactions have been performed in Winsor III systems [9, 19]. To attain good yields the enzyme should be surface active enough to penetrate into the chain regions between aqueous solution and oil in the surfactant-rich phase. Furthermore, most of the substrate should also be solubilized in this phase and, finally, the products should separate spontaneously into the excess phases [9, 19]. The low yield, about 0.26 in the hexadecane and about 0.20 in the dodecane systems clearly shows that a Winsor III system is of little use for the catalytic oxidation of cholesterol to cholestenone and H_2O_2. One reason is that the enzyme cholesterol oxidase is distributed between the aqueous channels in the surfactant-rich phase and the excess aqueous phase. The substrate cholesterol is again distributed between the oil channels, the aqueous/oil interface and the excess oil phase. This means that the enzyme in the aqueous phase has no substrate to catalyze and the cholesterol in the excess organic phase has no enzyme catalyzing it. The initial reaction rate is highest in the dodecane and lowest in the hexadecane system. The higher viscosity of the latter system can probably explain the low initial rate. On the other hand, the best yield was obtained in the hexadecane system and the less in the dodecane system. The mass fraction of lecithin was only one-third of the mass fractions of lecithin in the dodecane and tetradecane systems and according to McGuiness et al. [7] a too high surfactant content can abolish the enzyme activity. However, this cannot be the correct interpretation in our case because the yield in a Winsor IV system, where the lecithin content is higher than in the Winsor III system, is more than three times higher for the same enzyme activity, i.e., $2\,\mathrm{U\,cm^{-3}}$. A more favorable distribution of cholesterol to the surfactant-rich phase is a more probable explanation for the increased yield with increased hydrophobicity of the oil.

The results from the conversion of cholesterol to cholestenone at 298.2 K in Winsor IV systems can be seen in Fig. 3. The enzyme activity and the substrate concentration have in these experiments been kept at the same level as in the Winsor III systems and already after 10 h a better yield was received. Apparently, the best yield, i.e., 0.72, was reached in the dodecane system after 2 days. An increase of the substrate concentration from 5 to 15 mM resulted in a decreased yield, while an increase of the enzyme activity from 2 to 15 $\mathrm{U\,cm^{-3}}$ gave an almost complete conversion. The results from these experiments have been summarized in Fig. 4. For this purpose, we define an enzyme activity to substrate concentration ratio, $r^{\mathrm{enz}}_{\mathrm{subs}} = a_{\mathrm{enz}}/c_{\mathrm{subs}}$. Figure 4 clearly shows the mechanism of the conversion reaction. There is no mean to operate with $r^{\mathrm{enz}}_{\mathrm{subs}} < 1\ \mathrm{U}/\mu\mathrm{mol}$ in order to reach a high yield at 298.2 K in this lecithin stabilized Winsor IV system. Either a high enzyme activity or a low substrate concentration has to be used. The first alternative is an expensive solution and the latter moves the reaction apart from a real synthesis, and beside that, a Winsor IV system transforms easily into a multi-phase system at high substrate concentrations. The first problem can be solved by enzyme recovery by transforming the Winsor IV system to a Winsor I system. Lee and Biellmann [17] have pointed out that hydrogen peroxide formed as one of the products inactivates cholesterol oxidase and to protect this unwanted effect they introduced the enzyme catalase into the reaction system. Added catalase and decreased temperature increased the yield from 0.2 to 1.0 in detergentless "microemulsions" for the same reaction as in this work [16, 17]. On the other hand, the reaction time also increased from one day at 298.2 K to 35 days at 278.2 K. In a previous work [9], we have shown that an increase in the temperature decreases the reaction yield. To sum up: addition of catalase to the system and decreasing the temperature can shift the complete reaction yield towards lower $r^{\mathrm{enz}}_{\mathrm{subs}}$ (Fig. 4).

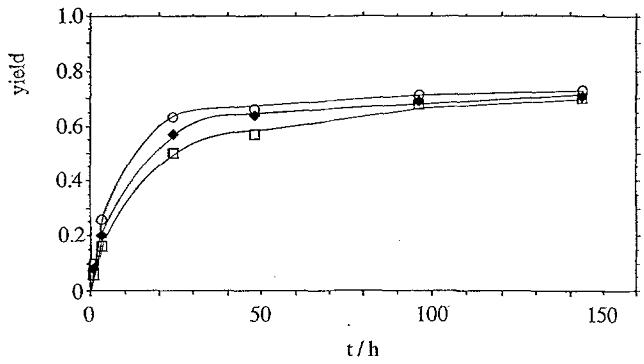


Fig. 3 The cholestenone yield for the enzymatic conversion of cholesterol at 298.2 as function of time in Winsor IV systems containing soybean lecithin-alkane-1-propanol-water. The symbols are the same as in Fig. 1. Enzyme activity $a = 2\,\mathrm{U\,cm^{-3}}$ and $c_{\mathrm{subs}} = 5$ mM

Addition of very small amounts of water to soybean lecithin-organic solvent mixtures transforms the solutions into transparent gels [20]. The number of molecules per surfactant molecule needed is in the range 1–12 depending on the organic solvent. Such organogels could not be detected in the reversed micellar phase L_2 of the system ethanol/water-soybean lecithin-hexadecane at 298.2 K [21]. The microstructure of this L_2 phase has been used as reaction medium for a comparison between the efficiency of ordinary W/O microemulsions and the bicontinuous

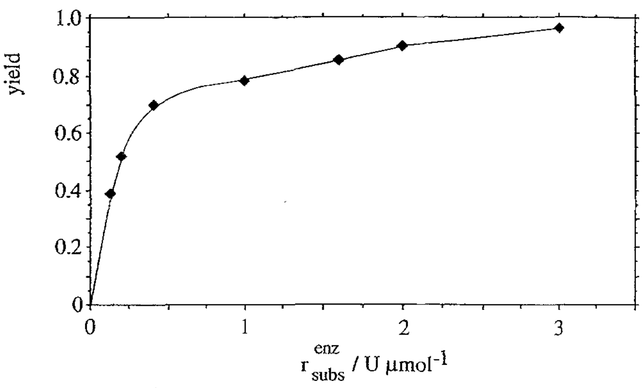


Fig. 4 The cholestenone yield for the enzymatic conversion of cholesterol at 298.2 K as function of enzyme activity to substrate concentration ratio in Winsor IV systems containing soybean lecithin-tetradecane-1-propanol-water

ones with respect to conversion and yield. The number of water molecules per lecithin molecule for the used reverse micellar system is only 2 and, according to Xenakis et al. [22], an optimal value lies between 14 and 16. This explains why no conversion occurs and the yield is poor. To summarize, the Winsor IV system gives the highest yield followed by W/O microemulsions [8] and the Winsor III system, while reverse micelles are a poor reaction medium for the conversion of cholesterol to cholestenone.

Concluding remarks

Addition of short-chain alcohols to water-alkane-soybean lecithin systems brings about microemulsion inversion, i.e., Winsor II → Winsor III → Winsor I transitions are possible at low lecithin contents. Depending on the oil, one-phase Winsor IV systems are also formed at higher lecithin contents. The surfactant-rich phase of the Winsor III systems and the Winsor IV systems are isotropic solutions with a bicontinuous structure.

The Winsor IV system is a much better reaction medium for the enzymatic conversion of cholesterol to cholestenone than the Winsor III system. The yield of the product cholestenone in the Winsor III system is as low as in a W/O microemulsion or in a detergentless "microemulsion" at room temperature. In a Winsor IV system the reaction is almost complete after one day if the enzyme activity to substrate concentration ratio is high enough. The same yield can be reached in a shorter time in Winsor IV systems as in cationic W/O microemulsions or in detergentless "microemulsions" at reduced temperatures to which also the enzyme catalase has been introduced. The enzymatic conversion in Winsor IV systems described here is thus characterized by high yields, room temperature, no additional enzymes and reasonable reaction times.

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