Hydrophile-Lipophile Balance and Critical Micelle Concentration as Key Factors Influencing Surfactant Disruption of Mitochondrial Membranes*

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A systematic approach to selection of surfactants for disrupting biological membranes, for solubilizing their components, and for removing the surfactant by dialysis is described. The two relevant surfactant parameters were the critical micelle concentration (CMC) and the hydrophile-lipophile balance (HLB). Rat liver mitochondria were treated with two series of nonionic surfactants and the extent of extraction of total protein, total lipid, and six enzymes was determined. Within the homologous series of Triton surfactants, maximum protein and phospholipid extraction occurred at HLB values between 12.5 and 13.5. In addition, a single surfactant species solubilized more protein than a mixture of surfactants with the same mean HLB value.

In order to examine independently the effect of CMC and HLB on protein extraction, a specialty surfactant, S10-7, was prepared and compared with its structurally similar analog, Brij 56. Above a concentration of 0.35%, both Brij 56 and S10-7 extracted about 70% of the mitochondrial protein. Hence, for optimum extraction of mitochondrial protein and lipide the HLB must be about 13, and the surfactant concentration must be above the CMC. The S10-7 dialyzed almost as rapidly as cholate and far more rapidly than Brij 58 and Triton X-100. It therefore possesses the two most desirable surfactant properties for disruption of membranes, a high CMC for rapid dialysis, and an HLB value of 13.2.

In order to further understand the structure and function of biological membranes, membrane-bound proteins are being investigated in solubilized and purified form, either as unasociated molecules, or reassociated with purified lipids (1, 2). Techniques involving mechanical agitation, chaotropic salts, organic solvents, and surfactants have been utilized to effect solubilization of these membrane-bound proteins (3, 4).

Certain surfactants have also been found to induce cell fusion (6) and membrane mobility (6). The selection of surfactants for solubilizing membrane proteins or inducing membrane mobility has usually been empirical.

Although charged surfactants such as sodium dodecyl sulfate and cetyltrimethylammonium bromide are potent denaturing agents, nonionic surfactants often sustain the activity of enzymes they solubilize (7, 8). Nonionic surfactants are characterized by their critical micelle concentration, the concentration at which unassociated surfactant molecules aggregate into micelles (9), and by their hydrophile-lipophile balance, a number indicating the proportion of hydrophilic to lipophilic portions of the amphipathic molecule (10). The HLB* value is measured on a scale of 0 to 20; higher numbers correspond to greater water solubility. An HLB value between 12 and 14 was found to be optimal for solubilizing membrane proteins of Bacillus subtilis (11).

This communication describes the relationship between these key physical properties of surfactants, their capacity to extract proteins and phospholipids from rat liver mitochondria, and the ease of removal of the surfactant by dialysis. Correlations between HLB and extent of protein extraction have been established; rapid dialysis results from high CMC values. A rapidly dialyzable surfactant designated S10-7, in which these factors have been optimized, has been designed, prepared, and investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**

Triton surfactants were obtained from the Rohm & Haas Co., Philadelphia, Pa., while Brij 56 and Brij 58 were supplied by ICI United States Inc., Wilmington, Del.

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*The abbreviations used are: HLB, hydrophile-lipophile balance; CMC, critical micelle concentration.
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The surfactant S10-7 was prepared under the direction of Dr. George J. Stockburger at ICI United States (Batch number 2824-108) following a standard procedure (12). A total of 933.4 g of ethylene oxide was added gradually to a vacuum-stripped (100°) mixture of 475.7 g of decanol and 2.0 g of sodium hydroxide in a cooled 2-liter stainless steel autoclave. The reaction temperature was maintained at 150° and the maximum pressure was 65 p.s.i. After 4 hours the reaction was complete and the mixture was neutralized with phosphoric acid. The crude adduct was then vacuum-stripped for 15 min at 80° and filtered. The product had an acid number of 0.5, a sulfated ash content of 0.003%, and a hydroxyl number of 124. A hydroxyl number of 120 is expected for the heptaethoxy adduct.

Rates were obtained from the Charles River Breeding Labs, Wilmington, Mass. Unless specifically indicated, all other materials and chemicals were purchased from standard suppliers.

Methods

Surfactant Solutions—All the surfactants tested were soluble in water except Triton X-45, Triton X-114, and Brij 56. Although not totally soluble, Triton X-45 and Triton X-114 could be dispersed in water by prolonged stirring at a concentration of 2 g/100 ml. These suspensions were further agitated just prior to use. Stable aqueous dispersions of Brij 56 were prepared by heating the Brij 56-water mixture to form a clear solution at 42°, then cooling to room temperature.

Preparation of Mitochondria—Mitochondria were isolated from the livers of male Sprague Dawley rats according to the procedure of Schneider (13). After washing three times with 0.25M sucrose, the mitochondria were suspended at a concentration of 25 mg of protein/ml in 0.25M sucrose/10 mM Tris chloride at pH 7.4 (sucrose/Tris buffer).

Treatment of Mitochondria with Surfactants—To 2 ml of the ice-chilled mitochondrial stock suspension in sucrose/Tris buffer (25 mg of protein/ml) was added 0.25 ml of the surfactant solution at the desired stock concentration (between 1 and 8%). After stirring at 9° for 30 min, 2 ml of the treated mitochondrial suspension were centrifuged at 100,000 x g for 1 hour. The supernatant fraction was removed without disturbing the fluffy layer; the pellet and fluffy layers were then resuspended in about 4 ml of sucrose/Tris buffer. Following centrifugation of this suspension for an additional 30 min at 106,000 x g, the second supernatant fraction was removed and the pellet fraction resuspended in about 2 ml of the buffer. The volume and protein concentration of each fraction were determined and used to calculate the percentages of protein extracted and protein recovered. In all experiments, the average protein recovery was above 90%. Other components (i.e. enzymes) were assayed and related to these protein concentrations to assess their distribution among the fractions.

Protein Determination—The protein concentration of the various fractions was determined from a standard curve by the difference in absorbance between 215 and 225 nm (14, 15). Neither the sucrose/Tris buffer, the extent of dilution, nor added surfactant interfered with these determinations.

Phospholipid Determination—The phospholipids of mitochondria and surfactant-lysed mitochondrial fractions were extracted with chloroform/methanol (2/1) with the aid of homogenization and gentle warming. The organic fraction was removed, washed with water, and concentrated. The lipids were digested by heating with sulfuric acid and oxidized with hydrogen peroxide. Total phosphate was determined spectrophotometrically at 660 nm in an acid molybdate-Elon solution. The phospholipid content was calculated assuming a 4/4/1 ratio of phosphatidylcholine/phosphatidylethanolamine/cardiolipin.

Surface Tension Measurements—Surface tension measurements were performed at 25° using a Cenco-de Nouel interfacial tensiometer. The instrument was calibrated between 22 and 72 dynes/cm with water/methanol solutions. All surfactants were allowed to equilibrate with the tensiometer ring until three consecutive readings at 2-min intervals gave identical values.

Data of Surfactants—The 5 mM surfactant solutions (100-m1 volumes) were sealed into prewashed dialysis tubing (molecular weight cutoff 12,000). The bag was then suspended in 350 ml of water and dialyzed for 7 days at 4° with continual stirring. The solution outside the bag was sampled periodically and after 7 days the concentration of surfactant was measured by using a Waters 403 differential refractometer. Surfactant concentrations were established by differential refractive index measurements using a Waters 403 differential refractometer. Sucrose solutions of known refractive indices were used for calibration. A standard curve was then established for each surfactant using samples of known concentrations ranging from 0.1 to 1.5 mM.

The concentrations of dialyzed surfactant solutions were determined by comparison with these standard curves.

Enzyme assays—Table I shows the mitochondrial enzymes studied including the reference to the assay method. Listed in the fourth column are the averages of the enzyme activities determined for the surfactant-treated mitochondria prior to centrifugation at 106,000 x g. The enzyme activities for untreated mitochondria are also shown. The percentage recovery was established by comparing the activity of the surfactant-treated mitochondria to the total activity detected in the separated fractions (see below).

RESULTS

Surfactant Structure and Properties—The physical properties and chemical characteristics of the surfactants are listed in Table II. Each is composed of a hydrocarbon moiety coupled to an ethylene oxide polymer. The number of ethoxy groups and the molecular weight are number averages (see "Materials").

Two general types of surfactant were studied, one with the octylphenyl group as the hydrophobic moiety (Triton series) and the other with straight chain alkyl groups (Brij 56 and S10-7). The Triton series provided a chemically homologous set of surfactants of graded properties; their CMC and HLB values are proportional since both are determined by the length of the ethoxy moiety. With an increase in the number of monomer units from 5 units for Triton X-45 to 30 units for Triton X-305, the HLB value increased from 10.4 to 17.3 and the CMC increased in proportion from 100 to 650 μM. On the other hand, the specialty surfactant, S10-7, was designed (see below) to leave an HLB value nearly identical with that of Brij 56, but with a much higher CMC.
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Effect of Surfactant Concentration on Extraction of Mitochondrial Protein—Mitochondria at 25 mg of protein/ml were treated with concentrations of 0.11 to 0.66% of Triton X-45, Triton X-114, Triton X-102, Brij 56, and S10-7. From these measurements, the surfactant level required to reach a maximum in protein extraction was determined.

As shown in Fig. 2, Triton X-45, Triton X-114, and Brij 56 reached their maximum protein-solubilizing capacity at 0.15% and only nominal increases in protein solubilization were achieved by further increasing surfactant concentration. On the other hand, Triton X-102 at levels up to 0.33% did not show a plateau. However, above 0.22%, Triton X-45 and Triton X-102 yielded large fluffy layers, rendering measurements of protein extraction unreliable. S10-7 was the most potent solubilizer at concentrations above 0.4%, although at 0.2% it was only moderately effective. Only nominal fluffy layers were generated by this surfactant.

Extraction of Mitochondrial Enzymes—In addition to measurements on the extraction of total mitochondrial protein as described above, the effectiveness of the surfactants in extraction of several specific mitochondrial enzymes was also studied. Monoamine oxidase is characteristic of outer membrane proteins whereas succinic dehydrogenase, $\beta$-hydroxybutyrate dehydrogenase, and cytochrome oxidase are firmly associated with the inner membrane. $\alpha$-Glycerol phosphate dehydrogenase is a loosely bound inner membrane enzyme, whereas malic dehydrogenase is a matrix enzyme. Mitochondria were treated with 0.22% concentrations of Triton X-45, Triton X-114, Triton X-100, Triton X-102, and Triton X-165 and each of these enzymes was assayed as described under "Methods."

As found previously for total protein, each enzyme was extracted most effectively by Triton X-100 (HLB 13.5) and least effectively by Triton X-45 and Triton X-165 while Triton X-114 and Triton X-165 gave intermediate values. Similar profiles would result from plotting against the CMC values of these Tritons. A maximum of 92% of the malic dehydrogenase was extracted by Triton X-100, followed by $\alpha$-glycerol phosphate dehydrogenase (70%), succinic dehydrogenase (8%) monoamine oxidase (67%), and cytochrome oxidase and $\beta$-hydroxybutyrate dehydrogenase (6%).

Extraction of Mitochondrial Protein by Mixed Surfactants—To determine whether the solubilizing capacity of surfactant mixtures was as effective as single surfactants,
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The extraction of mitochondria with surfactants and the determination of protein distribution are described in the legend to Fig. 1. The concentration of the stock surfactant solution was varied to give the final concentrations as indicated.

two sets of mixed surfactants with mean HLB values of 13.5 were compared to Triton X-100. One mixture was composed of Triton X-102 and Triton X-114 while the other contained Triton X-165 and Triton X-45. Mitochondria were extracted with 5 mM concentrations of these mixtures using the techniques described under "Methods."

Triton X-100 tested alone extracted 70% of the total protein, 8% superior to the Triton X-102/114 mixture, and 63% superior to the Triton X-165/45 mixture. Although less effective than Triton X-100, the Triton X-102/114 mixture extracted 12% more protein than expected based on the individual HLB values while the Triton X-165/45 mixture extracted 66% more. These expected extraction values were based on the effectiveness of the individual surfactants (Fig. 1).

Effect of Surfactants on Mitochondrial Phospholipids—Mitochondria were treated with Tritons X-45, X-114, X-102, and X-165 at equal concentrations by weight. The sedimentable and nonsedimentable fractions were assayed for phospholipid and protein content.

Fig. 3 shows that a maximum phospholipid extraction of 19%, together with a maximum protein extraction of 65%, occurred with Triton X-114 (HLB = 12.4). The patterns of both phospholipid and protein extraction were qualitatively similar. The weight ratio of phospholipid to protein in the starting intact mitochondria was 0.14 while the average for the surfactant-extracted mitochondria prior to separating the fractions by centrifugation was 0.15, indicating that surfactant had no influence on phospholipid determinations.

Critical Micelle Concentrations (CMC)—The variation of surface tension with surfactant concentration for aqueous solutions of Triton X-100, Brij 56, and S10-7 is shown in Fig. 4. The CMC is the surfactant concentration at the intersection of the two lines.

Triton X-100, studied as a control, gave a CMC of 250 µM, in good agreement with the previously reported value of 300 µM (23). The value for Brij 56 was 40 µM, again similar to the reported value of 35 µM for a similar surfactant (25). Since a value of 2.1 µM has been reported for the CMC of Brij 56 using prolonged equilibration periods (26), 35 µM may be an upper limit. The surfactant S10-7 gave a CMC value of 1000 µM, many times higher than Brij 56 and similar to the value of 1300 µM obtained for the 9 ethoxy adduct of decanol (28).
equally effective. Both extracted a maximum of 70 to 74% of the protein.

**Dialysis Rate of S10-7**—In order to establish the relative dialysis rate of S10-7, dialysis rates were determined for 5 mM solutions of a series of water-soluble surfactants. The order of dialysis rates was: sodium cholate > S10-7 > Triton X-100 > Brij 58 (Table III). This order held for both 20 and 168 hours of dialysis. As expected, these dialysis rates increased with increasing CMC.

**DISCUSSION**

This communication examines the relationships between two fundamental properties of surfactants, the hydrophilic-lipophile balance (HLB) and the critical micelle concentration (CMC), and protein extraction from mitochondria, to provide a more rational basis for the use of surfactants in disrupting and dispersing components of biological membranes in general. The HLB value describes the relative ratio of hydrophilic to hydrophobic regions within a surfactant molecule (10). Measured on a scale of 0 to 20, higher HLB values correspond to molecules having more hydrophilic character. Both HLB and CMC values are readily available for a large variety of surfactants (29, 30).

The importance of surfactant choice in membrane protein solubilization and stabilization has been shown for the membrane-bound ATP-ADP translocase of mitochondria (31). Although membrane-associated enzymes are most effectively extracted from Bacillus subtilis by surfactants with HLB values between 12 and 14 (11), in that study the roles of HLB, CMC, and chemical structure were not assessed independently. In many other studies of the extraction of enzymes from membranes the choice of surfactant has been largely empirical and made without regard to its specific physical properties.

The influence of surfactants on intramembrane protein mobility and cell fusion associated with intermembrane phospholipid exchange have come under investigation. Cell fusion has been induced by lysophospholipids and fatty acids (5, 32) and intramembrane protein mobility has been stimulated by synthetic ethoxylated surfactants (6). Their capacity to induce these phenomena is probably related to the same surfactant properties as membrane disruption, and it seems likely that the same criteria of surfactant selection would apply.

Since mitochondria contain characteristic marker proteins with varying degrees of membrane association they provided an excellent system for examining the solubilizing and disrupting effect of surfactants on membranes. Thus, it has been possible to follow the effect of the various surfactants on release of a soluble matrix enzyme, a rather loosely bound inner membrane enzyme, tightly bound inner membrane enzymes, an outer membrane enzyme, membrane phospholipids, and total proteins. It seems likely that the principles described herein for extraction of mitochondria could be applied generally to extraction and disruption of biological membranes.

Surfactants with HLB values between 12.5 and 13.5 caused the most extensive solubilization of overall mitochondrial proteins and phospholipids. This correlation also held for each of the six individual enzymes tested. The most effective surfactants were S10-7 (the heptaethoxy adduct of decanol), Triton X-100, Triton X-114, and Brij 56. Although the Tritons provided a homologous series of surfactants with several HLB values, HLB and CMC could not be varied independently. Consequently, the second set of surfactants, Brij 56 (HLB 12.9 and CMC 40 μM) and S10-7 (HLB 13.2 and CMC 1000 μM) were examined to vary CMC at constant HLB value. Within both sets of surfactants chemical composition remained the same. Taken together, these experiments demonstrated that HLB value was the principle surfactant parameter determining the extent of extraction of the mitochondrial components.

As expected, the extent of solubilization of each specific enzyme varied inversely with its degree of membrane association. Moreover, the mitochondrial enzymes tested varied from water-soluble (malic dehydrogenase) to tightly membrane-associated (cytochrome oxidase), all were extracted at the same relative proportions regardless of the HLB value of the surfactant. Consequently, it must be concluded that surfactants cannot be selected on the basis of HLB values to extract greater proportions of hydrophilic or of hydrophobic membrane-associated proteins. Furthermore, low levels of membrane-bound enzymes were consistently extracted along with matrix enzymes indicating a correlation between membrane protein extraction and membrane leakage.

Although above a concentration of 0.35%, Brij 56 and S10-7 were equally effective and extracted about 70% of the mitochondrial protein. At lower concentrations Brij 56 was significantly more potent than S10-7. At 0.22%, S10-7 would be only 4 times above its CMC in water while Brij 56 is at least 67 times greater. The actual surfactant concentration in the mitochondrial suspensions could have been diminished by binding to macromolecules; in the case of 0.22% S10-7 the actual surfactant concentration in this suspension may be close to its CMC. Hence, for optimum protein extraction, the surfactant concentration should be well above the CMC. These requirements emphasize that membrane disruption results from penetration of surfactant molecules into the hydrophobic core of the membrane.

Membrane-bound enzymes when removed from their protective hydrophobic environment are frequently inactive. However, in some instances, synthetic surfactants reverse or prevent this inactivation and thus may simulate the hydrophobic environment normally provided by the nonpolar domain of the lipid bilayer (8, 11). The precise structure of surfactant-protein aggregates is not known, although complexes of Triton X-100 with human low density lipoprotein, human erythrocyte stroma (33), a hydrophobic fragment from cytochrome b₅ (34), and with bovine serum albumin (35) have been studied in some detail.

Phospholipid extraction paralleled protein extraction for the Triton surfactants (Fig. 3). Consequently, at least a portion of the solubilized membrane proteins may be in the form of lipid-protein complexes, very small membrane fragments, or resealed vesicles which do not sediment at 106,000 × g in 1
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hour. Further work, possibly by gel filtration procedures, will be required to determine to what extent any given solubilized protein occurs as a true molecular species, as a lipid-protein complex, or as a membrane fragment containing several different membrane proteins.

Following solubilization of a membrane-bound enzyme, it is usually desirable to remove the surfactant, most simply by exhaustive dialysis. Indeed, the wide use of cholate as a solubilizing agent is based partly on its ease of dialysis (1, 4). As shown in Table III, S1O-7 dialyzed almost as rapidly as sodium cholate; with exhaustive dialysis both would be expected to dialyze much more rapidly.

S1O-7 thus has an HLD that is optimal for solubilization of membrane components as well as a high CMC that favors fast dialysis. Further experience with S1O-7 will be required to determine whether its actual usefulness in solubilization and purification of membranes lives up to predictions based on the two parameters of hydrophile-lipophile balance and critical micelle concentration.

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