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Comparative study of the interaction of CHAPS and Triton X-100 with the erythrocyte membrane

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ABSTRACT

The zwitterionic detergent CHAPS, a derivative of the bile salts, is widely used in membrane protein solubilization. It is a "facial" detergent, having a hydrophilic side and a hydrophobic back. The objective of this work is to characterize the interaction of CHAPS with a cell membrane. To this aim, erythrocytes were incubated with a wide range of detergent concentrations in order to determine CHAPS partition behavior, and its effects on membrane lipid order, hemolytic effects, and the solubilization of membrane phospholipids and cholesterol. The results were compared with those obtained with the nonionic detergent Triton X-100. It was found that CHAPS has a low affinity for the erythrocyte membrane (partition coefficient $K = 0.06\ mM^{-1}$), and at sub-hemolytic concentrations it causes little effect on membrane lipid order. CHAPS hemolysis and phospholipid solubilization are closely correlated. On the other side, binding of Triton X-100 disorders the membrane at all levels, and has independent mechanisms for hemolysis and solubilization. Differential behavior was observed in the solubilization of phospholipids and cholesterol. Thus, the detergent resistant membranes (DRM) obtained with the two detergents will have different composition. The behaviors of the two detergents are related to the differences in their molecular structures, suggesting that CHAPS does not penetrate the lipid bilayer but binds in a flat position on the erythrocyte surface, both in intact and cholesterol depleted erythrocytes. A relevant result for Triton X-100 is that hemolysis is not directly correlated with the solubilization of membrane lipids, as it is usually assumed.

1. Introduction

Detergents are soluble amphiphilic molecules that are able to incorporate to liposomes and cell membranes [1,2]. They are essential tools for the study of biological membranes, as they are frequently used to solubilize membrane proteins and to investigate the interactions among membrane lipid components.

The classical description of the process of detergent solubilization of unilamellar liposomes involves three steps [2–4]. The first is the partition of detergent molecules between the aqueous medium and the membrane at low, sub-solubilizing concentrations, and can be characterized by a partition coefficient K. The second, initiated at a characteristic "saturating" detergent/lipid ratio R^{sat} and finished at R^{sol}, is the gradual incorporation of membrane lipids into detergent/lipid mixed micelles, and the third, when all the membrane lipids are solubilized into detergent micelles, is the gradual enrichment of mixed micelles in detergent, upon detergent increase. Further studies [5,6] pointed out that detergent cooperative binding in the membrane, rather than membrane saturation, is an essential step for solubilization. The models coincide in a proportionality between membrane bound and free detergent at low detergent concentrations [6]. Thus, it is relevant to determine the partition coefficient in order to know the amount of detergent molecules effectively incorporated to the membrane when equilibrium is established at each detergent concentration. The incorporation of detergent molecules to the membrane is expected to cause alterations in lipid order [7] more or less important depending on the molecular structure of the detergent and the membrane components.

A high amount of work has been devoted to study the interaction of detergents with model membranes [[3,4], and references therein]. However, the complex lipid composition of a biomembrane and the presence of proteins, are expected to increase the complexity of the process of detergent–membrane interaction [6].

The above remarks point to the need of precise information about the interaction of detergents with natural membranes that would be of direct application in improving methods for purification and reconstitution of membrane proteins.

Mammalian erythrocytes are frequently used in membrane studies, as they lack nuclei and organelles, having only the plasma membrane. Alterations in permeability are easy to detect colorimetrically through the presence of hemoglobin in the suspension medium. Preté et al. have recently characterized the different stages of the interaction of Triton X-100 with erythrocytes using spin label EPR spectroscopy [8]. In this and previous studies [9], the authors assume that hemolysis proceeds in parallel with membrane solubilization, and they estimate

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saturating detergent concentrations from the hemolysis curve. On the other side, in previous papers we have characterized the process of lipid solubilization by Triton X-100 in intact and cholesterol depleted human and bovine erythrocytes [10,11], but without analyzing sub-hemolytic effects.

We are now interested in describing the interaction of the synthetic zwitterionic detergent CHAPS with a biomembrane. To this aim, we carried out a study of membrane/water partition, alteration in lipid order, hemolytic behavior, and phospholipid and cholesterol solubilization of the membrane of human erythrocytes incubated with CHAPS. In order to compare these results, we also determine hemolytic behavior and alterations in lipid order under the action of the nonionic detergent Triton X-100 in identical conditions as those used with CHAPS. As erythrocyte membranes have cholesterol content higher than other cells, we also study the effect of CHAPS on cholesterol depleted erythrocytes.

CHAPS (Fig. 1A) is a peculiar detergent, with a ring steroid-type chemical structure similar to that of bile salts, having a hydrophilic side where three OH groups protrude, and a hydrophobic back [12–16]. It is very effective at breaking protein–protein interactions, disaggregating protein complexes without affecting secondary or tertiary structures [12,17]. In the interaction with model membranes composed of pure lipids, CHAPS has been characterized as a "weak" [18] and "non-disordering" [7] detergent. On the other side, the also synthetic nonionic detergent Triton X-100 (Fig. 1B) has polar and hydrophobic portions well defined along the molecule. It has been described as a "strong" [18] and "disordering" [7] detergent. Table 1 summarizes the main properties of the two detergents.

2. Materials and methods

2.1. Materials

The non-ionic detergent Triton X-100, the liposoluble spin labels n-doxyl stearic acid positional isomers (n-SASL, n = 5, 16), and methyl-β-cyclodextrin (MβCD) were from Sigma (St. Louis, USA), and 12-SASL from Toronto Research Chemicals (North York, ON, Canada). The zwitterionic detergent CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) was from AMRESCO LLC (Solon, USA). Solvents, inorganic salts and all other chemicals were of the highest available purity. A commercially available enzymatic assay (Wiener Lab, Rosario, Argentina) was used for cholesterol determinations.

2.2. Erythrocyte separation

Fresh human blood was collected from healthy donors among laboratory personnel. Heparin was used as anticoagulant. Erythrocytes were separated from plasma and buffy coat by centrifugation at 1500 ×*g*, 5 min, and washed three times in isotonic Tris buffer saline (TBS, pH 7.4, 150 mM NaCl, 25 mM Tris).

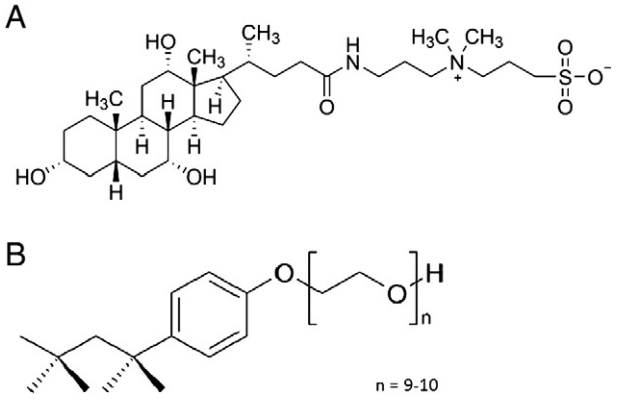

Fig. 1. Chemical structures of the zwitterionic detergent CHAPS and the non-ionic detergent Triton X-100.

Table 1
Main properties of the two detergents.

	CHAPS	Triton X-100
Molecular mass (g/mol)	614.9[a]	625[a]
CMC (mM)	3–10[b,c]	0.25[b]
Aggregation number	4–14[b]	75–165[b]

[a] Data from the providers (Section 2.1).
[b] [6].
[c] [16,17,19,20].

2.3. Detergent incubation of erythrocytes

One volume of packed washed erythrocytes, either intact or spin labeled, was incubated in 4 vol of a detergent solution in TBS (hematocrit 20%) at the desired concentration, on ice for 20 min (similar results were obtained in overnight incubations). In the experiments designed to study CHAPS membrane/water partition at sublytic detergent concentrations, 40% hematocrit was used in order to obtain a greater sensitivity in the color reaction, and samples were centrifuged at 1500 ×*g*, 4 °C for 30 min after incubation. The supernatant was reserved to determine the degree of hemolysis and the detergent concentration. In the experiments designed to measure the degree of lipid solubilization, after incubation the samples were centrifuged at 16,500 ×*g*, 4 °C for 30 min to pellet all the unsolubilized lipids, i.e., those not incorporated to detergent micelles.

2.4. Hemolysis degree

In order to determine the extent of hemolysis (hemoglobin loss from erythrocytes), the absorbance of the detergent incubation supernatants at 543 nm was compared with the absorbance of the supernatant of the incubation of erythrocytes at 20% hematocrit in distilled water (100% hemolysis).

2.5. Lipid extraction

Lipids were extracted from erythrocyte membranes and from the detergent insoluble pellets using organic solvents which were subsequently evaporated [21]. Briefly, 100 μl of packed erythrocytes were lysed in 140 μl distilled water. After 15-min incubation at room temperature, 1.32 ml of 2-propanol was added with vortexing. After 1 h of incubation, 0.84 ml of chloroform was added while vortexing, and after 1 h incubation, the suspension was centrifuged for 10 min at 2000 ×*g*, and the clear organic layer was separated. The organic solvent was evaporated, yielding a dry lipid extract. The detergent insoluble pellets were extracted by the same procedure, without the use of water.

2.6. Determination of phospholipid and cholesterol concentration in the lipid extracts

Phospholipids present in the dry lipid extract were quantified by inorganic phosphorus assay [22]. Briefly, perchloric acid was added to each sample and the tubes were heated until yellow color had disappeared. After adding water, molybdate solution, and ascorbic acid solution the tubes were placed in a boiling water bath. The absorbance of the samples was read at 800 nm.

Cholesterol was quantified with an enzymatic assay. One volume of isopropyl alcohol and cholesterol reagent was added to dry lipid extract of each sample. After that, the tubes were placed in a heated water bath. The absorbance was read at 500 nm. Assays were performed in triplicate.

The phosphorus content of the insoluble pellets was normalized to the phosphorus content of the corresponding original membranes, which was considered as 100%. This relative amount equals the relative phospholipid (PL) content of each sample. Cholesterol content was also informed as a percent content relative to that of the original membranes.

2.7. Measurement of CHAPS concentration in the incubation supernatants

Supernatants of CHAPS incubation of erythrocytes were reacted with concentrated sulfuric acid, and the absorbance at 389 nm, caused by sulfonation of the cholate ring, was measured [23]. A calibration curve obtained with CHAPS solutions of known concentrations was used to obtain the remaining CHAPS concentration in the supernatant $[Ch]_w$.

2.8. Erythrocyte spin labeling

The liposoluble spin labels n-SASL (n-doxyl stearic acid spin labels), which bear a stable free radical in a nitroxide moiety at the position n = 5, 12 or 16 of a stearic acid chain, and are able to sense lipid order/rigidity at different membrane depths, were incorporated by room temperature incubation to the erythrocyte membranes [24]. The final spin label/membrane lipid was less than 1%, in order to avoid line broadening effects in the EPR spectra. After 30 min incubation at room temperature, the samples were centrifuged and the pelleted erythrocytes were submitted to incubation with detergent at sub-hemolytic concentrations as described in Section 2.3.

2.9. EPR (electron paramagnetic resonance) spectroscopy

Aliquots of the pelleted erythrocytes obtained after detergent incubation (usually 20 μl) were transferred into glass capillaries (1-mm i.d.), flame sealed and put into 4-mm quartz tubes. EPR spectra were acquired at 22 ± 1 °C at 9.8 GHz (X-band) in a Bruker EMX-Plus spectrometer. Field modulation frequency was 100 kHz. The hyperfine parameters A_{max} and A_{min} were determined from the spectra as described in [24], and the apparent order parameter S_{app}, a joint indicator of order/mobility restrictions, was calculated [25]. This parameter varies in the range of $0 < S_{app} < 1$. Values near 0 indicate high mobility in a completely disordered environment, while values near 1 correspond to highly ordered, low mobility environments [25].

2.10. Cholesterol depletion in erythrocyte membranes with methyl-β-cyclodextrin

Packed and washed erythrocytes were incubated at 5% concentration (v/v) in a 3 millimolar MβCD solution in TBS at 37 °C, with occasional mixing. Control erythrocytes were incubated in TBS in the same conditions. After 30 min of incubation, the samples were centrifuged for 3 min at 1500 ×*g*, and the pelleted erythrocytes were washed with TBS. The incubation with MβCD in these conditions caused a Chol reduction of 45%, without modifications in the phospholipid content.

3. Results and discussion

3.1. Membrane/water partition of the detergents at low concentrations

In order to determine the amount of CHAPS effectively bound to erythrocyte membranes upon detergent incubation at sub-hemolytic concentrations, we quantitated the molar concentration of CHAPS remaining in the aqueous supernatant, $[Ch]_w$, by the colorimetric assay described in Section 2.7. The resulting values are plotted in Fig. 2 as a function of the total CHAPS concentration ($[Ch]_t$) in the incubation solution, showing a linear dependence in the measured range. The full line represents the best fit of the data, given by the equation $[Ch]_w = 0.86 \times [Ch]_t (R^2 = 0.9967)$. The slope of the fitted line (0.86) indicates that, in the present incubation conditions (4 °C, 40% hematocrit), 86% of the CHAPS molecules remain in the aqueous solution, and thus only 14% of the CHAPS molecules bind to the erythrocyte membranes.

In order to proceed with the calculation of a partition constant, we need to quantitate the molar concentration of membrane lipids [L] (referred to the aqueous volume) corresponding to these incubation conditions. To this aim, we used the data of Preté et al. (2002) [9] relating erythrocyte lipid concentration to hematocrit, and correcting it to refer the concentration to the aqueous volume, we obtain [L] = 5.67 mM at 40% hematocrit.

We assume a simple partition equilibrium [18] in which $K = [Ch]_b / ([L] \times [Ch]_w)$, where K is the membrane/water partition coefficient, and $[Ch]_b$ is the membrane bound detergent concentration. Taking into account that $[Ch]_t = [Ch]_w + [Ch]_b$, it is easy to deduce the following equation that would apply to the data in Fig. 2:

$$[Ch]_w = \frac{1}{1 + K[L]} [Ch]_t. \quad (1)$$

From the comparison to the equation fitted to the data of Fig. 2, it should be $1 / (1 + K[L]) = 0.86$, showing the validity of assuming a constant K in the studied range. Replacing [L] = 5.67 mM, the membrane/water partition coefficient for CHAPS can be evaluated as $K = 0.03\ mM^{-1}$. However, Heerklotz and Seelig (2000) [18] argue that CHAPS is not able to flip to the inner lipid hemilayer, incorporating only to the outer hemilayer. Adopting this criterion, we obtain a CHAPS partition coefficient $K = 0.06\ mM^{-1}$.

In model systems, partition coefficients of CHAPS reported by Schurholz [26] range between 0.3 and 0.6 mM^{-1} in fluid membranes, and 0.12 mM^{-1} for DMPC in the gel phase. Schurholz found that K decreases in cholesterol containing and similar condensed bilayers, and proposed that CHAPS does not penetrate the bilayer in those cases. Viriyaroj et al. [27] found $K = 0.024\ mM^{-1}$ for egg PC vesicles at very low CHAPS concentrations. K increased with increasing CHAPS concentration, being about 0.1 mM^{-1} at saturating conditions [27]. However, the comparison with our results is not straightforward, as a slightly different definition of K was used by Viriyaroj et al. [18,27], and they do not assume the partition of CHAPS only into the outer hemilayer.

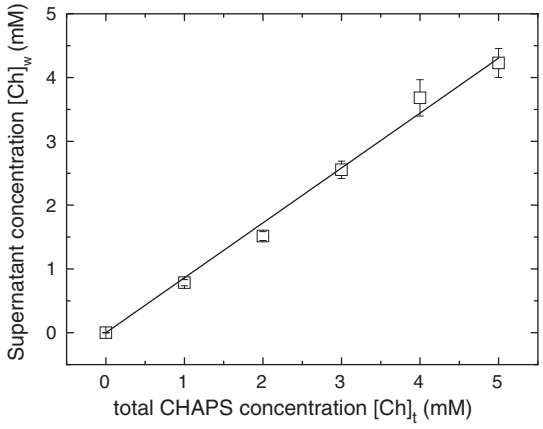

Fig. 2. Membrane/water partition of CHAPS. Squares represent the measured values of $[Ch]_w$, the mole CHAPS concentration remaining in the supernatant after erythrocyte incubation at 4 °C, 40% hematocrit, plotted as a function of total CHAPS concentration in the incubation solutions. Full line is the least squares fit of the data.

The mole ratio of membrane bound detergent to lipid $R_{bCh} = [Ch]_b / [L]$ at the working condition of 20% hematocrit, can be determined as:

$$R_{bCh} = \frac{K}{1 + K[L]}[Ch]_t = 0.05 mM^{-1}[Ch]_t. \quad (2)$$

In order to compare the behavior of CHAPS to that of the more conventional detergent Triton X-100 (Tx), we assumed that the simple partition behavior is also valid for this detergent, and use the value of $K = 2.4\ mM^{-1}$ reported by Pantaler et al. [1] for Triton X-100 membrane/water partition in erythrocytes. Using Eq. (2), we can calculate that at 20% hematocrit the mole ratio of membrane bound detergent to lipid is $R_{bTx} = 0.4\ mM^{-1}\ [Tx]_t$.

3.2. Effects of detergent binding on membrane lipid order

The next step was to investigate how detergent incorporation affects lipid order of erythrocyte membranes. To this aim, erythrocytes previously spin labeled with 5-, 12- or 16-SASL were incubated with each one of the detergents at different sub-hemolytic concentrations, and after incubation, their EPR spectra were obtained. Apparent order parameters S_{app}, indicative of order/rigidity of lipid chains [25], were calculated from the spectra. In order to obtain a better insight of the results, we analyzed them not as a function of the total detergent concentration, but as a function of R_b, the bound detergent/lipid ratio, calculated for each detergent as described in Section 3.1. Fig. 3 shows the apparent order parameters plotted as a function of R_b.

The possible values of S_{app} range between 0, expected for a spin label performing unrestricted fast reorientations in an isotropic (completely disordered) environment, and the rigid limit value of 1, corresponding to an immobilized spin label [25]. Thus, the highest values of S_{app} are expected to be obtained with 5-SASL, sensing the upper part of the bilayer, close to the lipid polar heads and with restricted movements, while the lowest values are expected for 16-SASL, sensing the center of the bilayer, where acyl chains have a high mobility. These tendencies are observed in the panels of Fig. 3. Considering now the values of S_{app} for Triton X-100 incubated erythrocytes (black symbols), it is observed that in the three panels the values of S_{app} decrease with R_b, showing that increased binding of Triton X-100 to the membrane causes a gradual disorder of the acyl chains at all levels in the lipid bilayer. On the other side, the data for CHAPS show a decrease in S_{app} only for 16-SASL, indicating that the effect of CHAPS binding is manifested as a slight increase of disorder/mobility only in the bilayer center, having little to no effects on lipid order at the upper levels of the membrane.

These different behaviors of the two detergents can be interpreted taking into account their different molecular structures (Fig. 1). CHAPS is a "facial" detergent [28], with a rigid ring system having a concave polar side bearing three OH groups, and a hydrophobic convex side. It has also a tail bearing two charged groups with zero net charge. Lunkenheimer et al. [14] have shown that CHAPS is capable of acquiring diverse molecular orientations in the air–water interface, being in a flat orientation along the interface at low concentrations. CHAPS has a high CMC (Table 1), and according to our results, a low affinity for erythrocyte membranes. According to these results, we propose that CHAPS molecules are incorporated to the surface of erythrocyte membranes lying on their hydrophobic side, and leaving the OH groups of the opposite side in contact with the aqueous solution. If this picture is valid (note the similarity with the "carpet" mechanism proposed for peptide/membrane interaction [29]), CHAPS would not penetrate deeply into the bilayer, and lipid order alterations would be minimal. On the other side, Triton X-100 is more similar to a "classical" detergent, having a conical molecular shape due to its large polar moiety and its short hydrophobic tail. It is expected to penetrate the bilayer causing a local curvature increase and promoting disorder [7]. Pantaler et al. [1] found that Triton X-100 has a strong membrane perturbing effect, attributed to its bulky polar moiety.

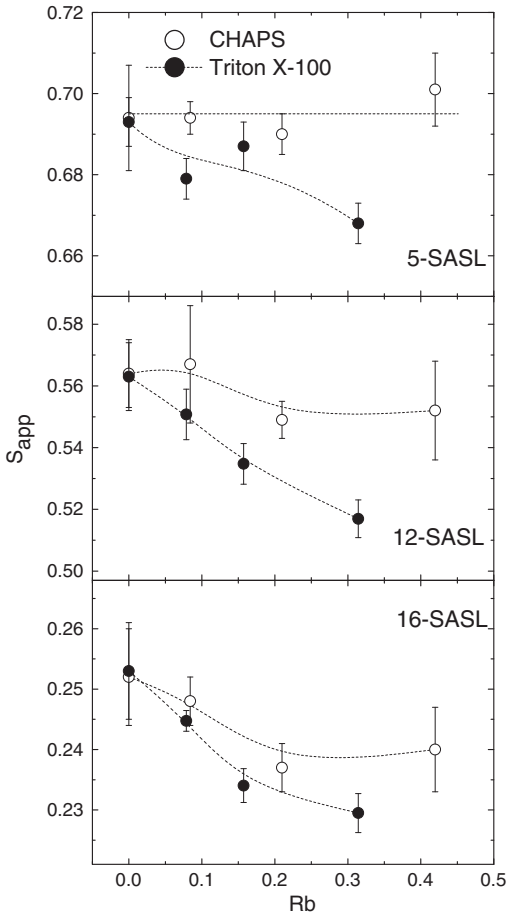

Fig. 3. Detergent effects on membrane lipid order. Apparent order parameter S_{app} evaluated from the room temperature EPR spectra of erythrocytes labeled with 5-, 12- and 16-SASL and subsequently submitted to detergent incubation at 20% hematocrit, 4 °C. White symbols, incubation with CHAPS; black symbols, incubation with Triton X-100. S_{app} is an estimator of order/rigidity of lipid acyl chains. 5-SASL senses the bilayer near the polar heads, 12-SASL at the middle chain level, and 16-SASL near the bilayer center. The data are plotted as a function of the mole ratio of membrane bound detergent to lipid, R_b. The dotted lines are only a guide to the eye.

Viriyaroj et al. [27] performed spin label EPR experiments in egg PC vesicles incubated with CHAPS, finding no changes in order with 5-SASL, but an increased order with 16-SASL. However, taking into account the results of Schurholz in cholesterol containing membranes [26], it is probable that CHAPS penetrates the bilayer when binding to egg PC, in a different behavior to the one we are proposing for the case of erythrocytes.

In a recent paper analyzing the disorder effects of several detergents in model POPC membranes using fluorescence spectroscopy [7], CHAPS was classified as a heterogeneously membrane-perturbing surfactant which reaches the onset of solubilization with little membrane disordering. On the other side, in [7] it is also shown that Triton X-100 destroys the membrane after reaching a critical extent of disordering. Our results, obtained in a real biomembrane, seem to be consistent with this work.

Decreased order in the upper level of erythrocyte membranes upon Triton X-100 incubation has also been reported by Preté et al. [9], as sensed by the spin label 5-SASL, although it was not related to the amount of detergent effectively bound to the membrane.

The lack of effects of CHAPS incubation in lipid order at the level sensed by 5-SASL is apparently inconsistent with our proposal that CHAPS incorporates to the membranes in a flat position, lying on its

hydrophobic back. However, this inconsistency could be explained if we take into account that the apparent order parameter S_{app} is a joint estimator of order/rigidity of the lipid chains [25]. In this way, the vicinity of CHAPS molecules to the polar heads can cause a decrease in order (decreasing S_{app}) but at the same time they can hinder the mobility (increasing S_{app}), leading to a null effect. On the other side, CHAPS effects in the hydrophobic portion of the membrane (12- and 16-SASL) should be interpreted as decreasing order/increasing mobility of the acyl chains, more noticeable in the center of the bilayer. This effect can be caused by a looser packing of the acyl chains due to a spacer effect of CHAPS binding.

3.3. Hemolytic effects

Direct evidence of damages due to the action of detergents on erythrocyte membranes can be obtained by measuring the extent of hemolysis, i.e. the leakage of hemoglobin from the cytoplasm, upon detergent incubation. Fig. 4 presents the data of percent hemolysis for the two detergents determined as described in Section 2.4 (open circles, CHAPS, black circles, Triton X-100) as a function of total detergent concentration. Triangles correspond to data of phospholipid solubilization, which will be discussed in Section 3.4. (Please note the logarithmic scale in the concentration axis).

The fits of the hemolysis curves with a sigmoid of equation

$$H\% = \frac{100}{1 + \exp\left(\frac{D - D_{50}}{\Delta D}\right)}$$

where D is the detergent concentration, are shown in dotted lines in Fig. 4. These fits allowed us to determine that D_{50}, the concentration causing 50% hemolysis, is 15.5 mM for CHAPS, and 1.28 mM for Triton X-100. The corresponding values of the width of the sigmoid transition (ΔD) are 3.2 mM and 0.25 mM. By extrapolating to the lower concentrations the straight portion of the curves, we can also evaluate a characteristic detergent concentration D_h^{on} corresponding to the onset of hemolysis. The values of D_h^{on} are 10 mM for CHAPS and 0.8 mM for Triton X-100. The comparison of D_{50} or D_h^{on} values would indicate that CHAPS is less efficient than Triton X-100 in causing membrane damages, as total CHAPS concentrations more than 10 times those of Triton X-100 are needed in order to cause a similar degree of hemolysis. However, taking into account the differences in partition behavior analyzed in Section 3.1, we can calculate, using Eq. (2), what is the amount of detergent effectively incorporated to the membranes in each case. At the onset of hemolysis, the ratio bound detergent/lipid is

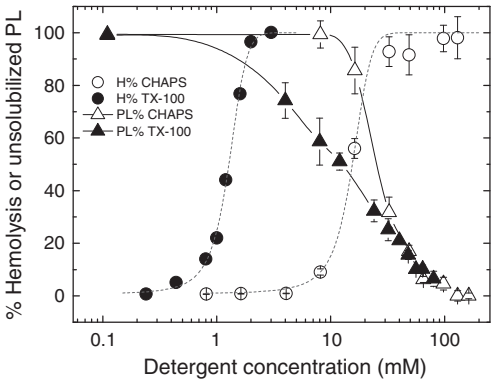

Fig. 4. Hemolysis and phospholipid solubilization. Percent hemolysis after incubation of erythrocytes with CHAPS (white circles) or Triton X-100 (black circles). Percent of unsolubilized phospholipids (PL) after incubation of erythrocytes with CHAPS (white triangles) or Triton X-100 (black triangles). Dotted lines are the fits of the hemolysis data to a sigmoid equation as described in Section 3.3. The lines joining solubilization data are only aids to the eye. Please note the logarithmic scale for detergent concentration. Incubation conditions, 20% hematocrit, 4 °C.

$R_{bCh} = 0.5$ for CHAPS, and $R_{bTx} = 0.3$ for Triton X-100. Thus, the striking differences in total detergent concentration needed for hemolysis are mainly due to the unfavorable partition of CHAPS into erythrocyte membranes, and are reduced when taking into account the detergent effectively incorporated to the membrane.

3.4. Phospholipid solubilization

We now want to analyze the evolution of phospholipid solubilization as a function of detergent concentration. To this aim, erythrocytes were incubated with a broad range of detergent concentrations, and the percent phospholipid (PL) amount in the insoluble pellets was determined after lipid extraction as described in Sections 2.5 and 2.6. Fig. 4 shows the amount of unsolubilized phospholipids (triangles) together with the hemolysis curves, plotted as a function of total detergent concentration.

3.4.1. Comparison of the solubilization and hemolysis processes of each detergent

As can be seen in Fig. 4, the completion of hemolysis is reached at about 30 mM CHAPS (hollow circles), and at this concentration, about 30% of the phospholipids remain unsolubilized (hollow triangles) as they are recovered in the pellet. On the other side, for Triton X-100 (full circles) hemolysis is complete at about 2.5 mM, but at this detergent concentration, about 80% of the PL still remains unsolubilized. This means that Triton X-100 causes membrane permeation leading to complete hemolysis (probably by the formation of pores) with the solubilization of a small portion of the bilayer lipids. On the other side, the processes of hemolysis and solubilization are closely correlated for CHAPS, leading us to propose that in this case, hemolysis does not proceed by pore formation, but directly by membrane disruption. This behavior is consistent with the molecular differences between CHAPS and Triton X-100, leading to differences in their interaction with the erythrocyte membrane.

Triton X-100 is expected to incorporate to the membranes causing a local curvature increase which could favor the formation of membrane pores leading to hemolysis [7]. It is also expected to segregate from cholesterol [30], as will be discussed in Section 4. Ahyayauch et al. [31] recently published a study involving several surfactants (not including CHAPS) acting on egg PC membranes and on erythrocytes, showing that flip-flop, leakage, and lysis/reassembly are independent from membrane solubilization, and appear in different sequences for each detergent. Thus, these processes do not appear to be part of a unified overall event, and a characteristic mechanism for each surfactant should be elucidated. Our results for Triton X-100 are consistent with this study. However, in the case of CHAPS we have shown that lysis and solubilization seem to be part of a unified process.

A clear conclusion from this section is that the hemolysis curve should not be used to obtain estimations for the parameters D^{sat} and D^{sol}, related to lipid solubilization, especially in the case of Triton X-100 [8,9].

3.4.2. Comparison of the solubilization curves of the two detergents

The detergent concentrations at which the membrane is saturated (D^{sat}) and completely solubilized (D^{sol}) [2], can be calculated from the PL solubilization data as follows. As D^{sat} corresponds to the onset of membrane lipid solubilization [2–4], we adopt the approach of extrapolating the straight portion of the solubilization curves in Fig. 4 to the 100% ordinate value, assigning the value of D^{sat} to the corresponding abscissa value of detergent concentration. D^{sol} values were obtained from the extrapolation of the straight portion to the 0% ordinate. The obtained values are listed in Table 2. (Figs. 1S and 2S in the Supplementary Material show graphically the values of D^{sat} and D^{sol}). It is interesting to observe that $D^{sat}_{CHAPS} > D^{sat}_{TritonX\text{-}100}$, but the opposite is valid for the solubilizing concentrations, as $D^{sol}_{CHAPS} < D^{sol}_{TritonX\text{-}100}$. Thus, although starting at a higher detergent concentration, the process of erythrocyte PL solubilization by CHAPS is completed in a narrower

Table 2
Summary of the parameters describing the interaction of CHAPS and Triton X-100 with erythrocyte membranes.

	K (mM^{-1})	D_h^{on} (mM)	D^{sat} (mM)	D^{sol} (mM)	R_b^{sat}
CHAPS	0.06*	10	12	50	0.6
Triton X-100	2.4 [a]	0.8	1.4	90	0.56

(*) Considering that CHAPS only partitions in the outer membrane hemilayer [18] (See Section 3.1).
([a]) data from Pantaler et al. [1].
D_h^{on}: total detergent concentration at the onset of hemolysis (Fig. 4).
D^{sat}: total detergent concentration at membrane saturation (onset of PL solubilization, Fig. 4).
D^{sol}: total detergent concentration required for complete PL solubilization of erythrocyte membranes (Fig. 4).
$R_b^{sat} = D_b^{sat}/[L]$: bound detergent/lipid ratio (D_b^{sat}: molar concentration of detergent bound to the membrane at the onset of PL solubilization, see Section 3.4).
R_b^{sat} must be considered only as approximate, as it is estimated assuming that the partition behavior of Section 3.1 is valid up to D^{sat}.
(Details on the estimation of D^{sat} and D^{sol} can be found in the Supplementary Material).

range of detergent concentrations than in the case of Triton X-100. This fact will be discussed in the context of the data presented in Section 4.

If we assume that the membrane/water partition behavior reported in Section 3.1 can be extrapolated up to the saturating detergent concentration D^{sat}, we can also give a gross estimation of the corresponding bound detergent/lipid ratios R_b^{sat}. These estimations are included in Table 2. Note that our estimation of R_b^{sat} could be considered as an upper limit for the "effective" detergent/lipid ratio R_e^{sat} usually determined from the slope of the boundary between the pure membrane and the membrane + micelle phases in a detergent–lipid phase diagram [2]. This is so because when the membrane begins to be saturated with detergent, it is reasonable to expect a deviation from the linear dependence of R_b on total detergent concentration, yielding a slower than linear increase of R_b [3,6]. Note that the total concentration D^{sat} needed to saturate erythrocyte membranes in our experimental conditions is about an order of magnitude larger for CHAPS than for Triton X-100, but for R_b^{sat} values this difference is reduced due to the lower affinity of CHAPS for membranes, as determined in Section 3.1.

4. Differential solubilization of cholesterol and phospholipids

As cholesterol is an essential component of lipid rafts, it is interesting to determine if the studied detergents have any kind of selectivity in the solubilization of cholesterol relative to phospholipids. To this aim, we measured the cholesterol content in the insoluble pellet after incubating erythrocytes at several detergent concentrations. The results are shown in Fig. 5, where the data of cholesterol solubilization are shown together with those of PL solubilization. In the upper panel (data for CHAPS), we observe that cholesterol content in the insoluble pellet is similar or slightly higher than PL content, up to 60 mM CHAPS. Thus, cholesterol is slightly more resistant than PL to CHAPS solubilization. In the case of Triton X-100 (lower panel), a distinct behavior is seen, as cholesterol is definitely more resistant to solubilization than PL, specially at low Triton concentrations. This means that the insoluble membranes obtained after CHAPS incubation will have a ratio chol/PL similar or slightly higher than that of the original membrane, while the insoluble membranes obtained after incubation with Triton X-100 will be clearly cholesterol enriched. As instance, upon incubation with 16 mM Triton X-100 (1% w/v, the commonly used concentration for the isolation of detergent resistant membranes (DRM) [32,33]) the insoluble membranes will have 46% of the original PL, but 72% of the original cholesterol. Instead, in the case of incubation with CHAPS 1% w/v (16.26 mM), the insoluble membranes will have 84% PL and 92% cholesterol. These results could help to understand the differences in composition of the DRM obtained with different detergents, not necessarily correlated

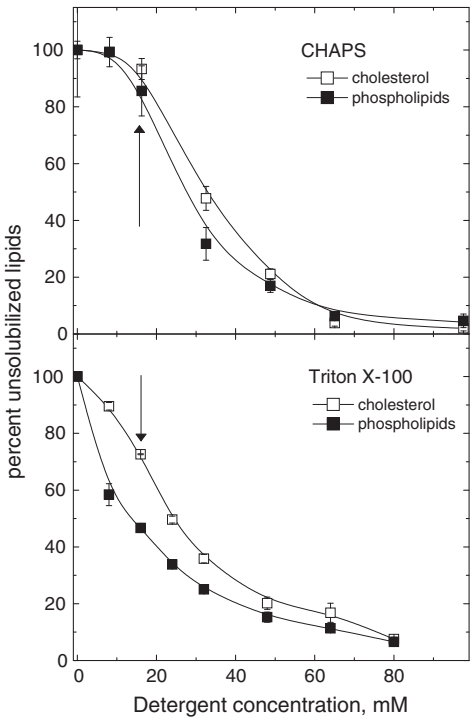

Fig. 5. Comparison of the solubilization process of cholesterol and phospholipids. Upper panel, percent content of cholesterol and phospholipids (PL) in the insoluble pellet obtained after incubation of human erythrocytes at 20% hematocrit, 4 °C in CHAPS solutions of different concentrations. Lower panel, the same data for incubation with Triton X-100. Abscissa represents total detergent concentrations. Data for PL solubilization are the same reported in Fig. 4, and are included here (in linear scales) in order to visualize the differences with cholesterol solubilization. In each case, 100% corresponds to the lipid content in the original erythrocyte sample submitted to detergent incubation. The arrows correspond to the concentration of 1% (w/v) commonly used for the isolation of DRM.

with the presence of different kinds of lipid domains in the original membranes.

The behavior observed for Triton is in line with the results of Tsamaloukas et al. [30], who studied model systems composed by POPC, cholesterol and sphingomyelin (SM). They found that Triton X-100/cholesterol contacts are unfavorable, while SM/cholesterol contacts are favorable. They conclude that Triton promotes the separation of SM/cholesterol rich domains from PC/Triton rich domains if the initial mixture is homogeneous. In the case of preexistent lateral domains in the bilayer, Triton prefently partitions into cholesterol poor domains. The latter are readily solubilized in the form of mixed micelles, while SM/cholesterol rich domains need higher detergent concentrations to be solubilized [30]. This behavior explains the wider range of Triton X-100 concentrations needed to achieve the full solubilization of phospholipids (which include SM, as they are quantitated through inorganic phosphorus), clearly observed in the logarithmic plot of Fig. 4 and in the data of Table 2. We must assume that the phospholipids solubilized at low detergent concentrations are mainly phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine; and those solubilized at the higher Triton concentrations are mainly SM, which would be solubilized along with cholesterol due to their favorable interaction. In support of these assumptions, note in Fig. 5, lower panel, that for Triton concentrations of 45 mM and higher, PL and cholesterol are solubilized in parallel. The behavior of Triton X-100 as a "segregating surfactant" has been reported by Nazari et al. [7] in model systems containing cholesterol. These results also agree with the conclusions of Ahyayauch et al. (2009) [32].

The differential solubilization of cholesterol and phospholipids observed for Triton X-100 also explains the lack of correlation between the hemolysis and solubilization curves of Triton X-100 shown in Fig. 4 (black symbols). In fact, the occurrence of hemolysis at low

concentrations of Triton X-100 can be attributed to the rapid segregation of the detergent molecules to cholesterol poor membrane zones, to avoid unfavorable contacts. The subsequent local solubilization of the fluid phospholipids in mixed micelles could cause holes in the membrane leading to hemoglobin leakage, while the majority of the membrane lipids are still unsolubilized (note that the onset of hemolysis is beyond Triton X-100 CMC).

In the case of CHAPS, the onset of hemolysis is very close to the onset of solubilization (Table 2 and Fig. 4), and the two processes are well correlated. This fact is consistent with the similar degree of solubilization of cholesterol and phospholipids, suggesting that once a solubilization threshold has been reached, all kind of lipids are equally solubilized in mixed micelles, causing hemolysis in parallel to a gradual disruption of the membrane. Also in this case, the onset of hemolysis/solubilization is at a detergent concentration higher than the CMC.

5. Interaction of CHAPS with cholesterol depleted erythrocytes

Mammalian erythrocyte membranes are peculiar in the sense that they have a huge amount of cholesterol (23% in mass related to total lipids, equivalent to a mole fraction 0.36 considering only phospholipids and cholesterol) [34]. In this sense, it is interesting to analyze how the reported effects change when cholesterol content is decreased. Cholesterol depletion was achieved by incubating the intact cells with methyl-beta-cyclodextrin, as detailed in Section 2.10. In this way, cells with a 45% cholesterol reduction and no changes in PL content were obtained, yielding membranes with a cholesterol mole fraction of 0.24, more similar to that of the plasma membranes of other mammalian cells.

We have reported in a previous paper [11] that cholesterol-depleted erythrocytes have increased susceptibility to solubilization with Triton X-100. In this paper, we studied the effects of cholesterol depletion on the interaction of CHAPS with the erythrocyte membrane.

Regarding the partition behavior, we found that it is similar to that of intact erythrocytes, yielding indistinguishable changes in the partition constant.

5.1. Hemolysis and PL solubilization

Fig. 6 shows the hemolysis and PL solubilization curves of cholesterol-depleted erythrocytes under the action of CHAPS, in comparison with those of intact erythrocytes. It can be seen that cholesterol depletion renders the membrane more prone to hemolysis and PL solubilization, but the two processes are still closely correlated. The fit of the hemolysis of depleted erythrocytes with a sigmoid (Section 3.3) gives for this case D_{50} = 10.2 mM, ΔD = 1.8 mM.

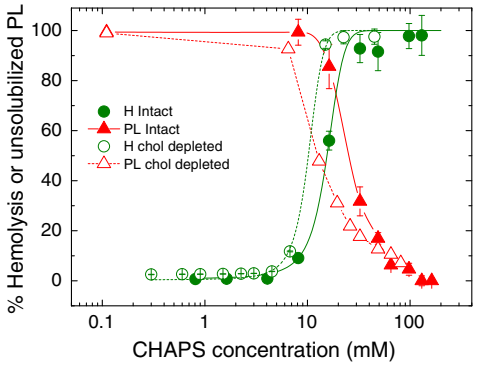

Fig. 6. Effects of CHAPS on cholesterol-depleted erythrocytes: hemolysis and PL solubilization. Percent hemolysis after incubation of cholesterol-depleted erythrocytes with CHAPS, open circles. Percent unsolubilized PL, open triangles. Full symbols correspond to the same data for intact erythrocytes (Fig. 4), and are included here for sake of comparison. The lines joining hemolysis data are fits with sigmoid equations (see Section 5.1). The lines along PL data are only a guide to the eyes.

Comparing with the results for intact erythrocytes of Section 3.3 (D_{50} = 15.5 mM, ΔD = 3.2 mM), we can state that CHAPS hemolysis of cholesterol-depleted erythrocytes occurs at a lower detergent concentration and is more abrupt than in intact erythrocytes. We have also obtained the curves for cholesterol solubilization. Fig. 3S (Supplementary Material) compares the detergent concentrations causing the solubilization of 50% of PL and cholesterol for intact and cholesterol depleted erythrocytes, and for the two detergents.

5.2. Alterations in lipid order

Cholesterol-depleted erythrocytes were spin labeled, submitted to incubation with CHAPS at sub-hemolytic concentrations (20% hematocrit), and subsequently pelleted and measured by EPR spectroscopy. Fig. 7 shows the resulting order parameters, compared to those of intact erythrocytes.

The changes in order parameter observed at 0 mM CHAPS are consistent with our previous results [24]: cholesterol depletion in erythrocytes decreases lipid order in the hydrophobic portion of the membrane (as sensed by 12- and 16-SASL), and causes negligible changes near the polar heads (as sensed by 5-SASL). As in the case of intact erythrocytes, incubation with CHAPS decreases lipid order mainly in the bilayer center (16-SASL), with a slight decrease also at the level of 12-SASL. Thus, the effects of increasing CHAPS concentrations in lipid order are similar for intact and cholesterol depleted erythrocytes. This fact leads us to think that cholesterol reduction does not change the mechanism by which CHAPS interacts with erythrocytes. It is expected that this mechanism will apply to the interaction of CHAPS with other mammalian cell membranes, incorporating in a flat position on the membrane surface.

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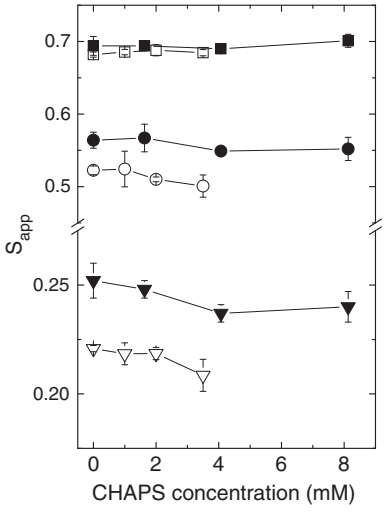

Fig. 7. Effects of CHAPS on cholesterol-depleted erythrocytes: membrane lipid order. Apparent order parameter evaluated from the EPR spectra of spin labeled cholesterol-depleted erythrocytes, subsequently submitted to incubation with CHAPS at sublytic concentrations (open symbols). Full symbols are the results obtained in intact erythrocytes and are included here for sake of comparison. Lines are only an aid to the eye. Squares, 5 SASL; circles, 12 SASL; triangles, 16 SASL. The data are plotted as a function of total CHAPS concentration because the partition constants are identical for intact and cholesterol-depleted erythrocytes.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.11.006.

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