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Factors determining detergent resistance of erythrocyte membranes

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ABSTRACT

The degree of detergent insolubility of cell membranes is a useful parameter to test the strength of lipid–lipid interactions relative to lipid–detergent interactions. Thus, solubility studies could give insights about lipid–lipid interactions relevant in domain formation. In this work we perform a detailed study of the solubilization of four different erythrocyte membrane systems: intact human and bovine erythrocytes, and human and bovine erythrocytes depleted in cholesterol with methyl-β-cyclodextrin. Each system was incubated with different concentrations of the non-ionic detergent Triton X-100, and the insoluble fraction was characterized by determining cholesterol and phosphorus content. A distinct solubilization behavior was obtained for the four systems, which was quantified by a "detergent resistance parameter" obtained from the fit of the solubility curves. In order to correlate these findings with membrane structural parameters, we quantify the degree of acyl chain order/rigidity of the original membranes by EPR spectroscopy, finding that detergent resistance is higher when acyl chains are more rigid. Regarding compositional properties, we found a good correlation between detergent resistance parameters and the total amount of cholesterol plus sphingomyelin in the original membranes. Our results suggest that a high degree of acyl chain packing is the determinant membrane factor for resistance to the action of Triton X-100 in erythrocytes.

1. Introduction

Many works support the existence of sphingolipid- and sterol-enriched microdomains, known as lipid rafts, in the plasma membrane [1–3]. They have been shown to be involved in a wide variety of cellular processes, but a clear characterization of the interactions involved in their formation is still lacking. On the other hand, experiments performed in several cell types have shown that an important fraction of the membranes (DRM, for Detergent Resistant Membranes) is resistant to the solubilization with non-ionic detergents as Triton X-100 [4]. Although it has been clearly established that DRM do not reflect membrane organization at steady state, and therefore cannot be directly equated with lipid rafts [5], the detailed study of the membrane solubilization process can give valuable information about lipid–lipid interactions governing raft structure. Detergent insoluble structures can be pelleted and analyzed for their lipid composition [6–9].

Lipid composition plays a central role in the function of cell membranes and governs physical membrane properties as permeability and fluidity. The activity of certain membrane proteins is also regulated by the interaction with lipids [1,2,10]. In mammal erythrocytes, half of the membrane mass is represented by proteins and the other half are lipids, mainly phospholipids and cholesterol (Chol) [11]. In model systems, the liquid ordered (lo) phase [7], which is formed at high cholesterol concentrations, presents a detergent resistance higher than the other phases. In the lo phase, a high lateral mobility (i.e. translational disorder) coexists with a high degree of acyl chain (conformational) order induced by the presence of the rigid plane of the Chol molecule [12].

Phosphatidylcholine is the more abundant phospholipid in the human erythrocyte membrane, but is almost absent in bovine erythrocytes, which in turn contain high levels of sphingomyelin (SM) [13]. SM is, together with Chol, relevant in determining lipid conformational order and packing [2]. Preferential interactions between SM and Chol have been reported [14] which could be relevant in determining detergent resistance of model systems [7].

Electron paramagnetic resonance spectroscopy (EPR) with the aid of spin labels is a useful method to characterize the degree of order/rigidity of the acyl chains of membrane lipids [15]. In previous works, we used EPR spectroscopy with liposoluble spin labels to detect slight changes in lipid chain order caused by cholesterol modulation in human erythrocyte membranes [16]. We have also studied the insoluble material obtained from Triton X-100 incubation of human or bovine erythrocytes [8,17], finding increased acyl chain order/rigidity in the detergent insoluble pellet when compared with the original erythrocyte membranes.

The aim of this work is to investigate in detail the solubilization process of human and bovine erythrocyte membranes, both intact and cholesterol depleted, under the action of Triton X-100, and to correlate their solubility behavior with structural and compositional parameters of the original membranes. We choose erythrocytes for two main reasons. First, they have only the plasma membrane, as they lack

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Table 1
Lipid percentage molar composition of the studied erythrocyte membranes

	IBE	CDBE	IHE	CDHE
SM	28	33	16	20
Chol	44	34	43	29
PC	3	3	17	21
PS	6	7	7	9
PI	1	2	1	1
PE	17	20	16	19
PA	1	1	1	2

Values calculated from data in refs. [11] and [13] considering approximate molecular masses, and taking into account the degree of cholesterol depletion of 45% in human erythrocytes and 36% in bovine ones (see Materials and methods). Glycolipids were not taken into account.
IBE, intact bovine; CDBE, cholesterol-depleted bovine; IHE, intact human; CDHE, cholesterol-depleted human erythrocyte membranes. Lipids: SM, sphingomyelin; Chol, cholesterol; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid.

the nucleus and organelles. Second, by studying human and bovine erythrocytes, we have two membrane systems with similar protein and lipid components, whose main difference is the amount of SM. In both cells, Chol content can be depleted by using methyl-β-cyclodextrin. Thus, we have four natural membrane systems with varying amounts of SM and Chol, which are the lipids known to govern lipid packing [2]. In Table 1 we show the approximate molar percent composition of each of the membrane systems estimated from data in references [11] and [13].

It is interesting to remark that our procedures allow us to study the whole intact cells, without the need of submitting membranes to hypotonic stress in order to obtain ghosts.

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, 12 and 16), and methyl-β-cyclodextrin (MβCD) were from Sigma (St. Louis, 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. Separation of erythrocytes

Blood from Argentine Holstein bovines was obtained by venous puncture and used before 72 h of extraction. Fresh human blood was collected from healthy donors among laboratory personnel. In both cases, 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 Tris buffer saline (TBS, pH 7.4).

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

Methyl-β-cyclodextrin (MβCD) is an efficient agent for removing cholesterol from culture and suspended cells [17–20]. It has been proved that MβCD does not incorporate into the treated membranes [19]. Packed and washed human or bovine erythrocytes were incubated at 5% concentration (v/v) in a 3 mM 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 tubes 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 46% in human erythrocytes and of 35% in bovine erythrocytes, without modifications in the phospholipid content.

2.4. Detergent solubility analysis

One volume of packed washed erythrocytes, either intact or cholesterol depleted, was incubated on ice for 20 min in 4 vol of a TBS solution of the non-ionic detergent Triton X-100 of concentrations varying between 0.5% and 5% (w/v). After incubation, samples were centrifuged at 16,500 ×g, 4 °C for 30 min, and the insoluble pellet was washed at least 2 times in TBS and centrifuged as above.

2.5. Lipid extraction

Lipids were extracted from erythrocyte membranes and from the insoluble material 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 for 10 min, 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 membrane phospholipid concentration

Phospholipids present in the dry lipid extract were quantified by phosphorus assay [22]. Briefly, one volume of perchloric acid was added to each sample and the tubes were heated until yellow color had disappeared. After adding one volume of 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. Assays were performed in triplicate. The phosphorus content of the detergent resistant pellets was normalized to the phosphorus content of the corresponding original membranes, which was considered as 100%. This relative amount equals the relative phospholipid content of each sample.

2.7. Determination of membrane cholesterol concentration

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.

2.8. EPR experiments and data analysis

The liposoluble *n*-doxyl-stearic acid spin labels (*n*-SASL), which bear a stable free radical in a nitroxide moiety at the position *n*=5, 12 or 16 of a stearic acid chain, were incorporated by room temperature incubation to the membranes of whole erythrocytes. The final spin label/membrane lipid molar ratio was less than 1%, in order to avoid line broadening effects in the EPR spectra. After 30 min room temperature incubation, the labeled samples were washed, pelleted, and transferred (usually 20 μl) into glass capillaries (1-mm i.d.), which were flame sealed and put into 4-mm quartz tubes. The EPR spectra were recorded at (25±1) °C and 9.8 GHz (X Band) in an ER-200 spectrometer (Bruker Analytische Messtechnik GMbH, Karlsruhe, Germany). Field modulation frequency was 100 kHz, and modulation amplitude was well below 30% of the minor line widths, in order to avoid spectral shape distortions.

3. Results and analysis

Intact human erythrocytes (IHE), intact bovine erythrocytes (IBE), cholesterol-depleted human erythrocytes (CDHE), and cholesterol-

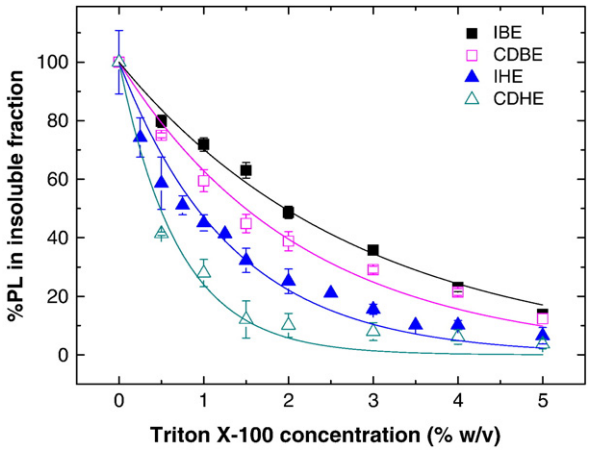

Fig. 1. Percentage of phospholipids (PL) in the detergent resistant material relative to the PL content of the original membranes submitted to Triton X-100 incubation. The four systems under study were incubated in variable amounts of Triton X-100 at 4 °C for 30 min. The phosphorus content of each sample was evaluated colorimetrically and normalized to the phosphorus content of the corresponding original membrane, which was considered as 100%. This relative amount equals the relative phospholipid content of each sample. Full lines correspond to fits to exponential functions, as explained in the text. Each point corresponds to the average of three independent experiments, and the bars represent the standard deviations.

depleted bovine erythrocytes (CDBE), were incubated in Triton X-100 solutions of different concentrations and subsequently centrifuged. The relative amount of phospholipids (PL) in the insoluble pellets of the four membrane systems was quantified and plotted as a function of the Triton X-100 concentration used to incubate the cells. The results are shown in Fig. 1. A distinctive behavior can be observed for bovine and human membranes, and also between intact and cholesterol-depleted membranes of each species. Full lines in Fig. 1 correspond to fits to decreasing exponential functions, as will be discussed below.

The relative amount of cholesterol in the insoluble pellets was also quantified and plotted in Fig. 2 as a function of Triton X-100 concentration. Besides a distinct Chol solubilization behavior for human and bovine erythrocytes, the comparison of Figs. 1 and 2 shows that in each system cholesterol and phospholipids have different susceptibility to detergent, as for each kind of membrane, the shape of the PL and Chol solubilization curves are different. For instance, in the case of intact bovine erythrocytes, at the lowest Triton X-100 concentrations the PL content is significantly affected by the detergent, while Chol content is slightly affected. Instead, in the case of cholesterol-depleted

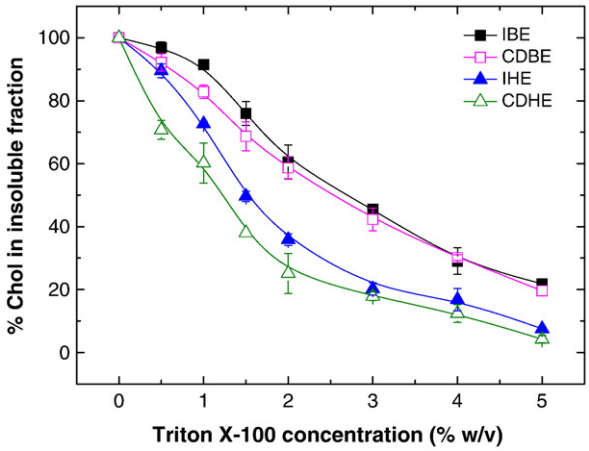

Fig. 2. Percentage of cholesterol in the detergent resistant material obtained from the four systems under study, as a function of the detergent concentration used to incubate the samples. The cholesterol content in the original membrane is considered the 100% for each case. Each point corresponds to the average of three independent experiments, and the bars represent the standard deviations. Full lines are only an aid to the eye.

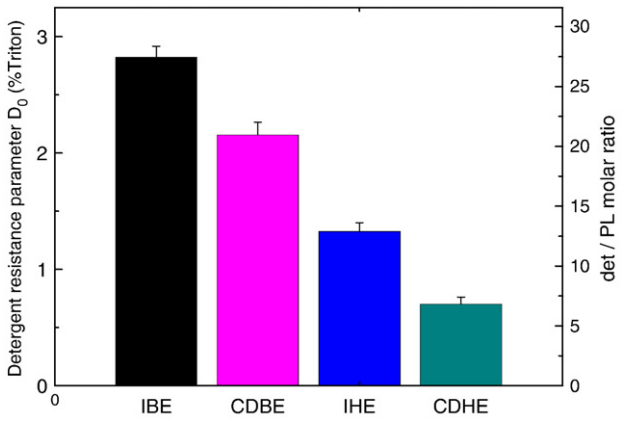

Fig. 3. Detergent resistant parameter D_0, indicative of the concentration of Triton X-100 needed to solubilize 63% of the phospholipids of the original membranes at 4 °C, obtained from the fit of the solubility curves of Fig. 1.

human erythrocytes, both PL and Chol show significant decreases at the lowest detergent concentrations.

In order to characterize quantitatively the solubilization process of the four membrane systems, the insoluble phospholipid data (%PL) as a function of detergent concentration (D) were fitted to the simple decreasing function

$$\%\mathrm{PL} = 100.e^{-D/D_0}$$

where D_0 represents the concentration of Triton X-100 for which %PL = 37%, meaning that 63% of the original membrane phospholipids are solubilized. As higher D_0 values would correspond to membranes needing more detergent to be solubilized, D_0 constitutes a useful parameter to quantify the degree of resistance to solubilization, and we call it the "detergent resistance parameter".

Full lines in Fig. 1 represent the best fits of the PL solubilization behavior of each membrane to the proposed function, and the resulting detergent resistance parameters D_0 are plotted in Fig. 3. The characteristic behavior of each membrane system under solubilization is reflected by the striking differences in D_0 values, decreasing in the sequence IBE>CDBE>IHE>CDHE. The right axis in Fig. 3 corresponds to detergent/lipid molar ratio.

Structural properties of the original membranes were evaluated by EPR spectroscopy, using liposoluble spin labels which were incorporated to the membrane of whole erythrocytes, either intact or cholesterol

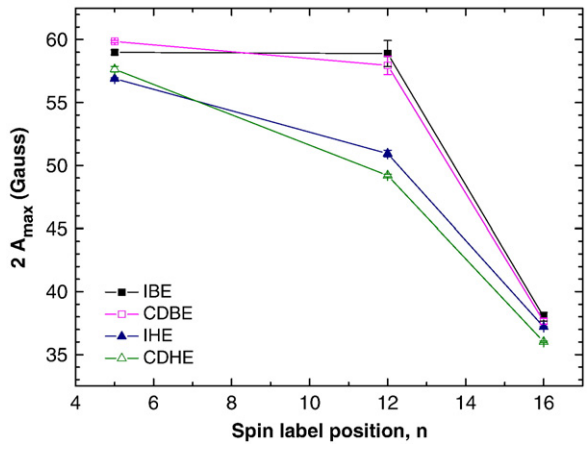

Fig. 4. "Flexibility gradient", i.e., hyperfine parameter $2A_{max}$ calculated from the EPR spectra of the spin labels 5-, 12- and 16-SASL in the original membranes, plotted as a function of the position of the nitroxide moiety along the stearic acid carbon chain (depth of the spin label inside the bilayer). Higher $2A_{max}$ values are indicative of higher order/rigidity of the acyl chains at the corresponding bilayer depth.

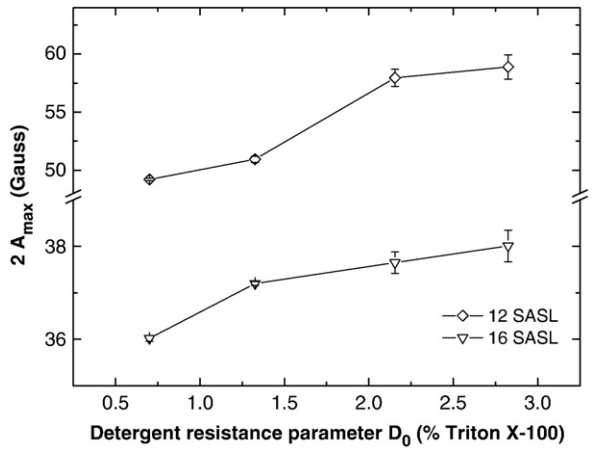

Fig. 5. Hyperfine parameters $2A_{max}$, indicative of acyl chain order/rigidity as sensed by spin labels at different bilayer depths, plotted against the detergent resistance parameter D_0 of each membrane system, in order to show their positive correlation.

depleted, by incubation at room temperature. The EPR spectrum of these labels is sensitive to the degree of acyl chain mobility at different depths inside the lipid bilayer. The hyperfine parameter $2A_{max}$, representative of acyl chain order/rigidity, was evaluated from each spectrum as described in [8], and the results are shown in Fig. 4 as a function of the depth of the probe inside the lipid bilayer.

The possible values of $2A_{max}$ range between the isotropic limit of about 32 G, expected for a spin label performing unrestricted fast reorientation in an isotropic (completely disordered) environment, and the rigid limit value of about 64 G [23], corresponding to an immobilized spin label. Thus, the plots of Fig. 4 constitute a depth profiling of the lipid chain order/rigidity down the bilayer interior. The gradient of increasing flexibility toward the terminal methyl ends, due to the accumulation of *trans-gauche* isomerizations, is a clear signature of bilayer-like structures [8,16,24]. As seen in Fig. 4, the spin labels in the membrane of intact bovine erythrocytes have higher $2A_{max}$ values than in intact human ones, at all levels of the lipid bilayer, indicating that segmental motions of the acyl chains are more restricted, resulting in higher order/rigidity and more dense chain packing in the bovine membranes. This fact can be attributed to the higher SM content in bovine erythrocytes. Cholesterol depletion lowers $2A_{max}$ in both species for 12- and 16-SASL, indicating a reduced rigidity and looser acyl chain packing in the hydrophobic region of the bilayer. The opposite behavior is found for 5-SASL, i.e. in the region closer to the lipid headgroups. This result has been reported and discussed in a previous paper [16]. As the hyperfine parameter at this level is governed by the proximity of polar heads and not by the packing of the acyl chains [16], the behavior of $2A_{max}$ for 5-SASL will not be considered in our discussion.

In order to relate the different solubilization behaviors to the structural properties of the original membranes, the values of $2A_{max}$ for 12- and 16-SASL were plotted in Fig. 5 against the detergent resistance parameter D_0 of the corresponding membrane. Both curves show a monotonous increase, indicating that D_0 is positively correlated with acyl chain packing at the hydrophobic region (12th and 16th carbons).

Regarding compositional parameters of the original membranes, it could be expected that the molar amount of SM and Chol, which are the lipids governing lipid ordering and packing, would influence detergent solubility. To test this hypothesis, the detergent resistance parameter D_0 was plotted in Fig. 6 as a function of the percentage molar contents of SM and Chol taken from the data in Table 1. It can be seen that D_0 does not have a good correlation with the molar amount of Chol (first panel) or with that of SM (second panel), but when plotted against (Chol+SM) (third panel), D_0 displays a nice regular behavior, increasing monotonously with the summed lipid content.

4. Discussion

The above presented results show that membranes of human and bovine erythrocytes have different resistance when submitted to the action of the non-ionic detergent Triton X-100. Cholesterol depletion increases the solubility in both cases, and the four systems under study have detergent resistance parameters D_0 in the following order: IBE>CDBE>IHE>CDHE.

We wanted to relate this differential behavior with structural and compositional properties of the original membranes. Our results displayed in Figs. 5 and 6 show that detergent resistance increases monotonously with acyl chain order/rigidity in the hydrophobic region and with (Chol+SM) membrane molar content.

In order to perform comparisons with the behavior of model systems, we will consider the molar detergent/lipid ratios (R) corresponding to each of the values of D_0 appearing on the right axis of Fig. 3 (as we are working with detergent concentrations much higher than Triton X-100 critical micellar concentration (0.25 mM or 0.02%), this detergent/lipid ratio is practically coincident with the "effective" Triton concentration [7]). We can see that roughly 7 Triton molecules per lipid molecule are needed to solubilize 63% of the cholesterol-depleted human erythrocyte membrane, while 28 Triton molecules per lipid are needed for the intact bovine erythrocyte membrane. In

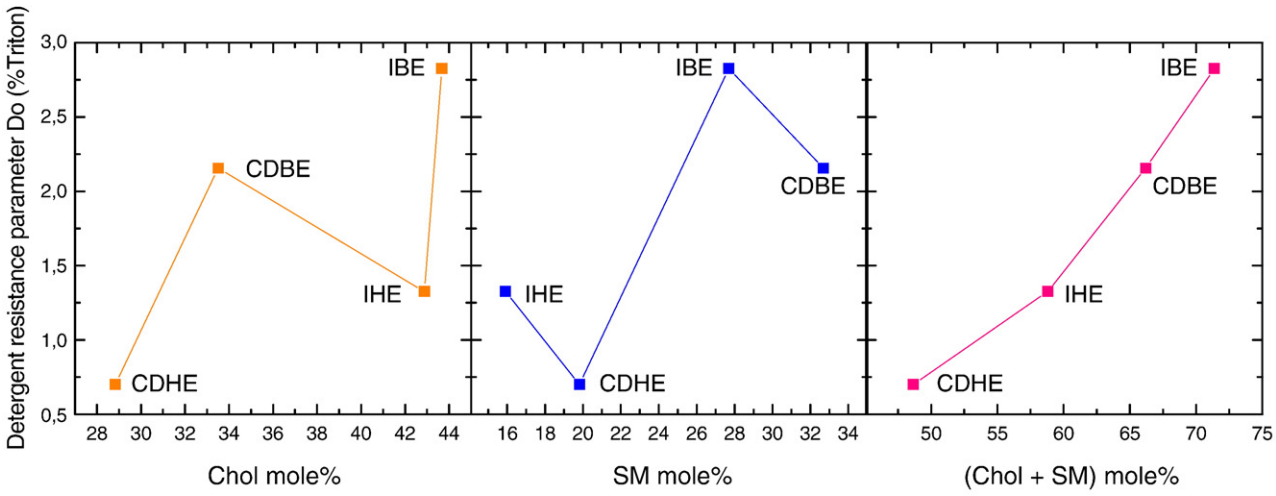

Fig. 6. Detergent resistance parameter D_0 plotted as a function of the percent molar contents of Chol (first panel), SM (second panel), and the joint (Chol+SM) content (third panel) in the different membrane systems.

comparison, a detergent/lipid ratio $R=2$ causes complete solubilization of egg PC liposomes at 4 °C [7], but model systems including SM and Chol have a very different behavior. Sot et al. [7] have studied eggPC/SM/Chol mixtures in proportions N:1:1, showing that very high values of R are needed to solubilize them when $N<3$. They also reported that the joint presence of SM and Chol is needed to decrease the solubility of model systems containing egg PC, and obtained evidence from FTIR studies of the formation of hydrogen bridges between SM and Chol, whose number is reduced by increasing amounts of egg PC [7]. (Molecular dynamics calculations [25] also show a preferential Chol–SM interaction, but interactions other than direct H-bonding are suggested to be responsible for this fact). A gross estimation of the molar ratio glycerophospholipids/SM/Chol in our systems, performed on the basis of the data in Table 1, indicates that the ratio glycerophospholipid/Chol (N) is always less than 3, and SM and Chol molar contents are roughly of the same order of magnitude. Thus, the lipid composition of our membranes resembles that of the model systems studied in [7], and the mechanisms governing detergent resistance could be related to selective interactions SM/Chol.

It is interesting to remark that in the systems studied in [7], no clear connections were observed between detergent insolubility and membrane fluidity. However, in our case we found a positive correlation between D_0 and membrane order/rigidity (Fig. 5), implying a higher detergent resistance parameter for the membranes having the lower fluidity. At this stage, it seems clear that detergent insolubility in erythrocyte membranes is directly related to acyl chain packing. The monotonous increase of D_0 with the summed amount (Chol+SM) (Fig. 6, third panel) can be rationalized by considering recent molecular dynamics simulations in Chol/SM/POPC systems [25]. In this paper it is showed that just one Chol neighbor is enough to promote the increase of acyl chain order in a SM molecule, and that having just one SM neighbor significantly reduces the tilt of Chol in the bilayer. In this sense, it can be expected that partial Chol depletion would cause a slighter decrease in acyl chain packing in a system with a high amount of SM compared to one with lower SM content, which is precisely what our experimental data report: bovine erythrocytes, having a higher SM content, have a slighter decrease in acyl chain order/rigidity upon Chol depletion than human ones (Fig. 5). The dependence of the detergent resistance parameter D_0 with the joint amount of (Chol+SM) is also in line with these considerations.

We can compare our results to those obtained by Ahyayauch et al. [26] using large unilamellar vesicles formed with different phosphatidylcholines in the fluid state. They found that in these systems, hydrocarbon chain molecular order, as measured by DPH fluorescence polarization, facilitates solubilization. If comparison with our more complex systems were valid, the opposite behavior obtained in our case would suggest that the phase state of an important portion of the lipids in the erythrocyte membrane would not correspond to the fluid liquid crystalline, but would more probably be similar to the liquid ordered state. The high content of SM and Chol also supports this hypothesis.

Besides the complexity in lipid composition of a natural biomembrane, a relevant difference with model systems is the presence of proteins, both integral and peripheric. Among the latter, erythrocytes have a complex cytoskeleton composed mainly by spectrin, which forms a network underlying the cytoplasmic face of the lipid bilayer, anchored through interactions with integral proteins [27]. Several papers reported the presence of cytoskeleton components in Triton X-100 insoluble residues [6,8,28]. An interesting issue is to determine if the structuration provided by this protein network influences the solubility of the attached lipid bilayer. This issue would be faced in future works.

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