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Biophysical Chemistry 122 (2006) 114–122

Biophysical Chemistry

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Detergent solubilization of bovine erythrocytes. Comparison between the insoluble material and the intact membrane

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Received 13 February 2006; received in revised form 14 March 2006; accepted 15 March 2006
Available online 31 March 2006

Abstract

Early works have shown that when biomembranes are extracted with the non-ionic detergent Triton X-100 at 4 °C, only a subset of the components is solubilized. The aim of this paper was to investigate the solubilization of a cell membrane at different Triton concentrations, and to compare the lipid composition and acyl chain order/mobility of the insoluble material with those of the original membrane. We choose bovine erythrocytes, because they have an uncommon composition, as they have a huge amount of sphingomyelin and phosphatidylcholine is almost absent. We determined the degree of order/mobility of the lipid acyl chains by EPR spectroscopy, using liposoluble spin labels. Incubation of bovine erythrocytes with increasing Triton X-100 concentrations yields decreasing amounts of insoluble material which is enriched in sphingomyelin and depleted in cholesterol. Complete lipid solubilization is achieved at a detergent/lipid ratio of about 60, which is much higher than the values reported for human erythrocytes, but is in line with results obtained in model systems. An insoluble pellet is still obtained at higher Triton concentrations, which seems to consist mainly of protein. A very high correlation is found between lipid chain mobility restrictions and sphingomyelin content in the lipid structures. The human erythrocyte membrane also fits well in this correlation, suggesting a significant role of sphingomyelin in determining acyl chain organization. The analogies and differences between our insoluble material and the detergent-resistant membranes (DRM) are discussed.

Keywords: Erythrocyte membrane; Lipid composition; Triton X-100 solubilization; EPR or ESR; Spin label; Detergent-resistant membranes

1. Introduction

It is well known that the lipid composition plays a central role in the function of biological membranes. Glycerophospholipids, sphingolipids, and sterols not only determine physical properties of the lipid bilayers as permeability or fluidity, they also interact with membrane proteins conditioning their biological activity [1]. However, the knowledge of the biological function of the numerous lipids composing cellular membranes is still limited. The recent discovery of lipid rafts, membrane microdomains enriched in sphingolipids and cholesterol, which have been implicated in a variety of sorting and signaling processes in cells [2–4], has shown the importance of a deeper understanding of the interactions among membrane lipids. One approach to investigate these interactions is to take profit from the selective solubilization of different lipids occurring when a biomembrane or a model system is submitted to the action of a non ionic detergent as Triton X-100, as early works have shown [5]. Vesicles composed of detergent-resistant lipids are recovered as detergent-insoluble material, and can be pelleted and analyzed for their lipid composition [5,6]. The comparison of lipid composition of the insoluble material with that of the original membrane is facilitated in the case of erythrocytes, which have only the plasma membrane.

In mammalian erythrocytes, half of the membrane mass is represented by protein, and the other half by lipid, mainly phospholipids and cholesterol (Chol), with a molar relation cholesterol to phospholipid by about 0.9 in humans [7]. While

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doi:10.1016/j.bpc.2006.03.005

phosphatidylcholine (PC) is the most abundant erythrocyte membrane phospholipid in humans, it is almost absent in bovines, which in turn contain high levels of sphingomyelin (SM) [8]. SM is, together with Chol, relevant in determining lipid conformational order and packing [3]. Natural forms of SM have long saturated hydrocarbon chains which are able to adopt the all-*trans* conformation and to interdigitate to the opposite hemilayer [9]. They also have both hydrogen bond donor and acceptor groups, which summed to the previous properties, promote the tight packing of SM molecules. There is strong evidence supporting preferential interactions between cholesterol and SM [9], which are relevant in determining detergent insolubility [6]. In model systems, detergent insolubility is a property of the liquid ordered phase [6], which is formed at high cholesterol concentrations [10]. In this 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.

A useful method to characterize the degree of order/mobility of the membrane lipids acyl chains is spin-label electron paramagnetic resonance (EPR) [11]. In previous works, we showed that EPR spectroscopy with liposoluble spin labels is useful to detect slight changes in lipid chain order caused by cholesterol modulation in human erythrocyte membranes [12]. We also studied insoluble material obtained from Triton X-100 incubation of intact or cholesterol depleted human erythrocytes [13], finding increased acyl chain order in the detergent-insoluble material when compared with the original erythrocyte membranes.

In the present work, we treat intact bovine erythrocytes with different concentrations of Triton X-100, characterize the lipid composition of the insoluble portions, and perform a comparison of the status of lipid chains at different levels of the lipid bilayer using EPR spectroscopy.

2. Materials and methods

2.1. Materials

Pure lipids were purchased from Sigma Chemical Co. (St. Louis, USA) and Avanti Polar Lipids (Alabaster, USA). The liposoluble spin labels 5-, 12-, and 16-SASL were from Sigma. Solvents, inorganic salts and all other chemicals were of the highest available purity. Thin layer chromatography plates were from Sigma.

2.2. Separation of erythrocytes

Blood from venous puncture of Holstein calves (mean age: 2 months) was kindly collected by Vet. R. Cerutti and used before 48 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 isotonic phosphate buffered saline (PBS, pH 7.4).

2.3. Detergent extraction of bovine erythrocytes

One volume of packed washed bovine erythrocytes was incubated on ice for 45 min in 4 vol. of a PBS solution of the non-ionic detergent Triton X-100. The following detergent concentrations (w/v) were used for lipid and EPR studies: 1%, 2%, 3%, 4%, 6%, and 10%. A wider and more detailed range of concentrations was used to investigate the yield of insoluble material. Taking into account a total lipid content of 3.8 mg/ml of packed bovine erythrocytes [8], the molar ratio detergent/total lipids can be estimated around 10 for the 1% Triton solution, increasing proportionally at the different detergent concentrations. After incubation, samples were centrifuged in a microcentrifuge at 18 000×*g*, 4 °C for 30 min, and the insoluble pellet was washed at least 3 times in PBS and centrifuged as above. The final aspect of the pellet was that of a yellowish gel which volume decreased with detergent concentration. At visual inspection, its appearance was turbid for the lowest detergent concentrations, but turbidity decreased for increasing detergent, and the pellet obtained with 10% Triton was completely transparent.

The volume of the insoluble pellet obtained with 1% Triton was about 40% of the original packed bovine erythrocyte volume. By contrast, the volume yield when human erythrocytes were treated with 1% Triton in similar conditions was always less than 16% (data not shown). The high yield of insoluble material in bovine erythrocytes incubated with 1% Triton is in agreement with results obtained with other ruminant erythrocytes in Koumanov et al. [14].

2.4. Lipid quantification

Lipids from erythrocyte membranes or from the insoluble material were extracted by the method of Bligh and Dyer [15]. Total lipid phosphorus was quantified according to [16]. Thin layer chromatography (TLC) plates were pre-washed with chloroform/methanol/water 60 : 35 : 8, and afterwards activated at 100 °C during 30 min. Standard lipid mixtures containing 1, 3, 6 or 12 μg of DPPC, DPPE, SM and Chol were prepared from stock solutions. Samples and standards were deposited by duplicate and developed into a previously equilibrated closed tank containing chloroform/methanol/water 60 : 25 : 4. Plates were colored with Coomassie Brilliant Blue, scanned and analyzed by densitometry with the free software V-Scion Image (beta version).

2.5. 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 erythrocyte membranes or to the insoluble material. 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. The hyperfine parameter A_{max}, calculated from the EPR spectra as described in the forward sections, was used to compare, in an empirical way, the status of lipid chains in the different samples.

3. Results and discussion

3.1. Lipid composition of erythrocyte membranes and Triton X-100-insoluble material

Fig. 1 shows in a bar diagram the lipid composition of the membrane of intact bovine erythrocytes (BE) and of the insoluble material obtained by detergent extraction with different Triton X-100 concentrations up to 6%. The bars represent the percent mass of each lipid component, relative to the total mass of lipids considered here, i.e. SM + PC + PE + Chol. The pellet obtained with 10% Triton X-100, although extracted in the same way and deposited on the TLC plates in similar volumes as the insoluble material resulting from other detergent concentrations, gave very faint spots, which could not be adequately scanned on the TLC plate. Lipid phosphorus analysis confirmed that these samples contained undetectable levels of lipids, as will be shown below.

The lipid contents of intact human erythrocyte membranes (HE), obtained from [7], and calculated as a percentage of the total lipids quantified here, are also included in Fig. 1. Important differences in lipid composition between bovine and human

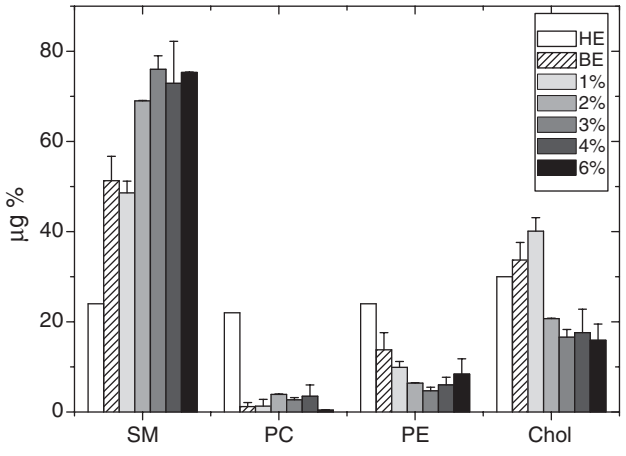

Fig. 1. Lipid composition of erythrocyte membranes and Triton X-100 insoluble material. Relative mass composition of polar lipids (means ± S.D., $n = 5$) evaluated after lipid extraction and TLC analysis of the membrane of intact bovine erythrocytes (BE) and the insoluble material obtained at 4 °C from bovine erythrocytes with the Triton X-100 concentrations listed in the label. SM = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; Chol = cholesterol. The 100% lipid mass corresponds to (SM + PC + PE + Chol). Values corresponding to human erythrocytes (HE) taken from Alberts et al. [7] are also included.

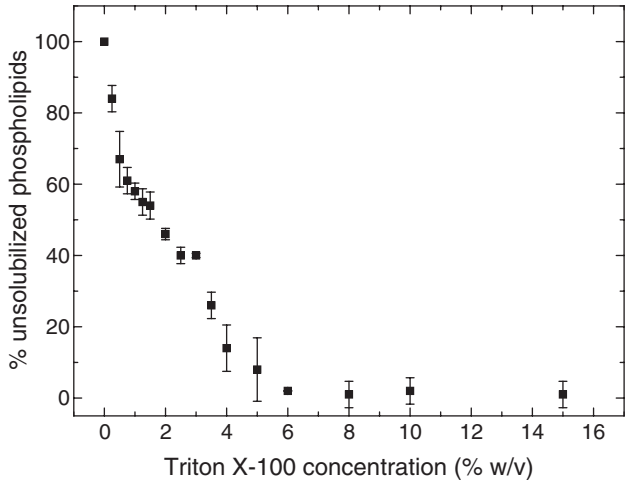

Fig. 2. Analysis of the solubilization of the bovine erythrocyte membrane at 4 °C under different Triton X-100 concentrations. Relative phosphorus content, indicative of residual phospholipid content in the insoluble pellets obtained after incubating bovine erythrocytes with Triton X-100 at different concentrations, for 45 min at 4 °C. The value of 100% corresponds to the phospholipid content of the intact membrane. Overnight incubations yield essentially the same results.

erythrocyte membranes can be clearly appreciated in the figure. While SM is the most important phospholipid of bovine erythrocytes, PC is nearly absent. The insoluble material is even more enriched in SM. Instead, in human erythrocytes, PC, PE, and SM contribute equally in weight to the membrane composition, having half the SM of bovine cells. Cholesterol content is similar in the intact erythrocyte membrane of both species, but decreases in the bovine-insoluble pellet for 2% Triton X-100 and above. As seen in Fig. 1, the lipid composition of the material extracted with 1% Triton is very similar to that of the intact bovine erythrocyte membrane. For 2% Triton X-100 concentrations and above, important differences are observed, because the insoluble pellets are enriched in SM and depleted in Chol compared with the original membrane, showing only slight variations in composition between 2% and 6% Triton. This fact suggests that in the bovine erythrocyte membrane, a certain proportion of cholesterol is solubilized by Triton X-100 more easily than sphingomyelin.

3.2. How much Triton X-100 is needed to achieve the complete solubilization of the bovine erythrocyte membrane?

Fig. 1 shows that for the lowest Triton concentration (1%, corresponding to an approximate detergent/lipid ratio of 10, as stated previously), the composition of the insoluble material is similar to that of the intact bovine erythrocyte membrane. This fact, together with the high yield of insoluble material (see Materials and methods), led us to investigate the total amount of insoluble lipids as a function of Triton concentration. Inorganic phosphorus analysis was performed after lipid extraction of the insoluble pellet with a large and more refined set of Triton X-100 concentrations, and the results were plotted in Fig. 2. It can be observed there that complete solubilization, i.e., undetectable phosphorus levels in the insoluble pellet, is achieved only for Triton concentrations higher than 6%. According to our

previous estimations, these concentrations correspond to detergent/lipid ratios higher than 60:1. As we are working with detergent concentrations much higher than Triton X-100 critical micellar concentration (0.25 mM or 0.02%, [17]), this detergent/lipid ratio is practically coincident with the parameter R_e^{sol} [18].

Our estimation of $R_e^{sol} \sim 60$ is much larger than the value 2.15 reported by Preté et al. [19] for the complete solubilization of human erythrocyte membranes. This discrepancy could be ascribed to the differences in lipid composition between the two species, but it must also be considered that the criterion used in their study [19] to assess complete membrane solubilization is the observation of complete hemolysis, without performing a search for insoluble vesicles. Regarding model systems, egg PC vesicles need an effective detergent/lipid molar relationship of 1.9 to be solubilized at 4 °C, but mixtures including SM and Chol have a different behavior [6]. Sot et al. have analyzed vesicles formed by egg PC/SM/Chol in proportions $N:1:1$. The amount of detergent needed to solubilize these vesicles increased when decreasing the PC proportion N, and for $N \leq 3$, the vesicles could not be solubilized even at a detergent/lipid ratio of 5 [6]. They also reported that the presence of both, SM and Chol, are needed to decrease the Triton solubility of systems containing PC. In a gross approximation, and taking into account the lipid composition displayed in Fig. 1, the molar relationship glycerophospholipid/SM/Chol in the bovine erythrocyte membrane can be estimated as 0.2:0.9:1.1. Considering the results of Sot et al. [6], the high resistance of this membrane to complete solubilization could be explained by its particular composition, i.e., a small content of glycerophospholipid, and a much higher amount of SM and Chol in similar proportions.

To discard the influence of kinetic factors (too short incubation times), we performed overnight Triton incubations of bovine erythrocytes, obtaining essentially the same results (data not shown). Thus, our results represent equilibrium states of the mixture Triton-bovine erythrocyte membrane.

3.3. Comparison of acyl chain order/mobility among the original membrane and the insoluble pellets

Fig. 3 shows the EPR spectra of the spin labels 5-, 12-, and 16-SASL in the membrane of intact human (HE) and bovine (BE) erythrocytes, and in the insoluble pellet obtained from bovine erythrocytes with different Triton X-100 concentrations. Although the intensity decreased for increasing Triton concentrations, all the spectra were normalized in this figure to unit peak-to-peak height, in order to facilitate the comparisons among them. A gradual change in the spectra can be observed from human to bovine erythrocytes, and from bovine erythrocytes to the insoluble material obtained with increasing Triton X-100 concentrations. The spectra corresponding to 2%

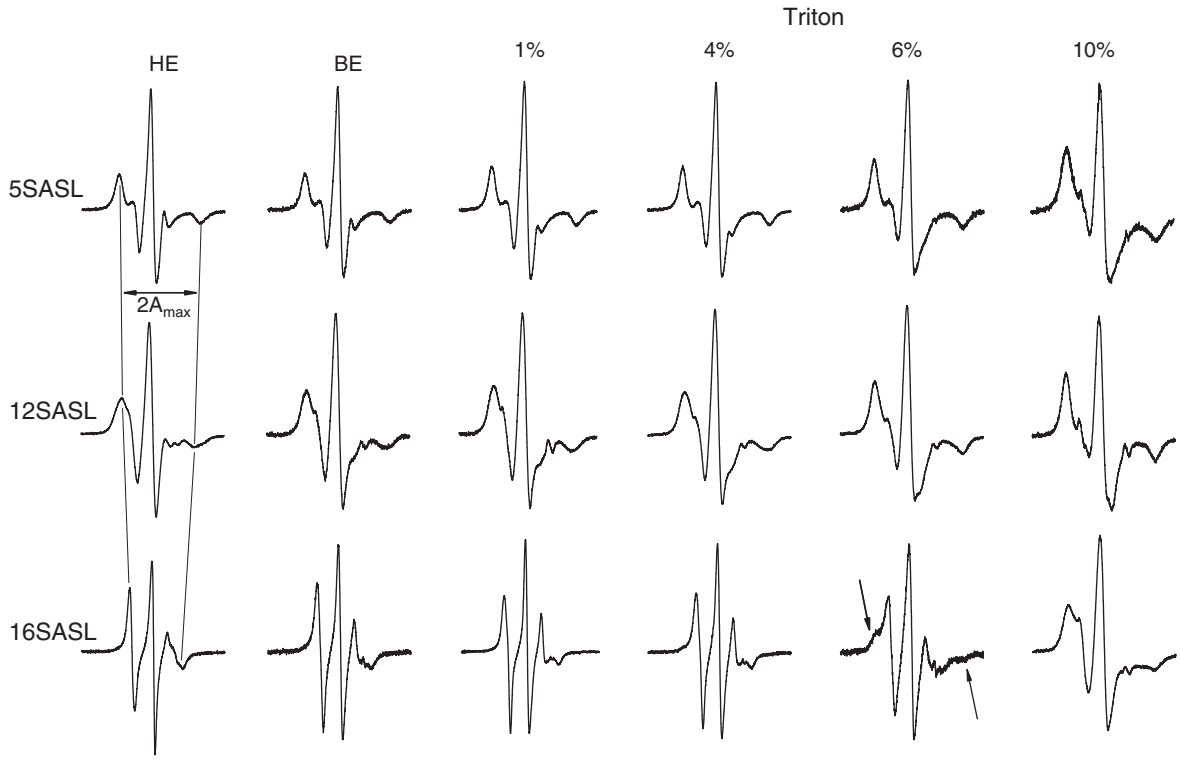

Fig. 3. EPR spectra. Electron paramagnetic resonance (EPR) spectra of liposoluble spin labels in the membranes of intact human (HE) and bovine (BE) erythrocytes, and in the insoluble material obtained from bovine erythrocytes with the indicated Triton X-100 concentrations. Temperature = 25 °C; microwave frequency = 9.75 GHz; magnetic field scan = 100 G. The spectra are normalized in amplitude to allow comparison among the different samples. Upper group: 5-SASL (labeled moiety at carbon 5, closest to polar head-groups); medium group: 12-SASL (labeled moiety at carbon 12); lower group: 16-SASL (labeled moiety at carbon 16, closest to the bilayer center). The lines point to the spectrum features whose magnetic field positions are used in the calculation of the hyperfine parameter A_{max}, indicative of acyl chain order/mobility. The arrows in the 6% Triton 16-SASL spectrum point to the spectral structures related to immobilized lipids.

and 3% Triton concentrations were not included in the figure for sake of clarity.

The spectral features pointed out with lines on the EPR spectra in Fig. 3 are part of the hyperfine structure due to the interaction between the radical unpaired electron and the ^{14}N nucleus [20]. The magnetic field separation between these structures, named $2A_{max}$, can be used to characterize the rotational disorder and rotational rates of the spin labeled lipid chain segments. As order and mobility effects are coupled when slow motions are present, it is not correct to attribute the observed changes between the different spectra only to ordering effects [20], but this parameter is a useful indicator to compare segmental mobility in a series of similar samples [21].

Fig. 4 shows the "flexibility gradient", i.e., the hyperfine parameter A_{max} calculated from the spectra of Fig. 3, 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). The possible values of A_{max} range between the isotropic limit of about 16 G, expected for a spin-label performing unrestricted fast reorientation in an isotropic (completely disordered) environment, and the rigid limit value of about 32 G [20], corresponding to an immobilized spin label. Thus, the plot of Fig. 4 constitutes a depth profiling of the lipid chain mobility 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 [12,21]. The observation of A_{max} values near the rigid limit for the three spin labels used would indicate that the environment is immobilized in the EPR time-scale.

As seen in Fig. 4, the spin labels in the membrane of intact bovine erythrocytes have higher A_{max} values than in human

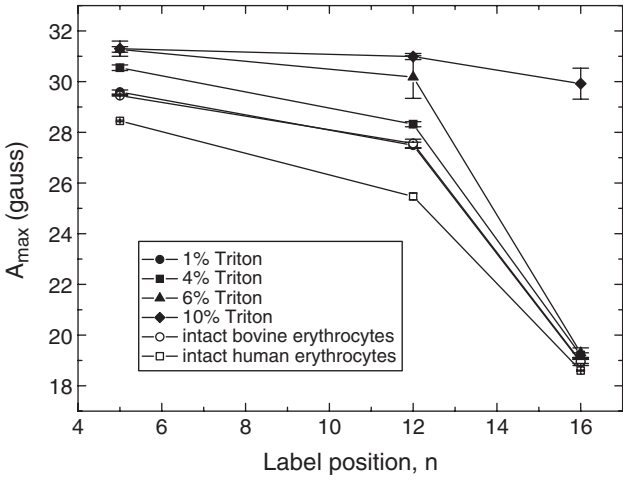

Fig. 4. Flexibility gradient. Hyperfine parameter A_{max}, obtained from the EPR spectra of Fig. 3, as a function of the position n of the labeled moiety on the stearic acid chain. Data from human and bovine intact erythrocyte membranes, and from the insoluble pellets obtained after incubation of bovine erythrocytes with Triton X-100 at the indicated concentrations. Decreasing A_{max} values correspond to decreasing lipid segmental mobility restrictions, indicative of increased fluidity. Note that data corresponding to intact bovine erythrocytes cannot be resolved from those corresponding to the insoluble material obtained with 1% Triton. Note also that the flexibility gradient is abolished for the insoluble pellet obtained with 10% Triton, indicating a rigid environment (bars indicate S.D. values for $n=3$).

ones, at all levels of the lipid bilayer, indicating that segmental motions of the acyl chains are more restricted, resulting in less fluidity, in the bovine membrane. The insoluble material extracted with 1% Triton shows A_{max} values coincident with those of bovine erythrocytes. For higher Triton concentrations up to 6%, A_{max} increases for the three labels, indicating increasing mobility restrictions (decreasing fluidity), and keeping the characteristic shape of the flexibility gradient corresponding to a bilayer environment. (A_{max} values corresponding to the insoluble pellet for 2% and 3% Triton concentrations are not included for sake of clarity, but they are intermediate between those corresponding to 1% and 4% Triton.) However, for 10% Triton, the flexibility gradient is nearly absent, and A_{max} is almost constant (and near its maximum value) at the three levels sensed by the spin labels. This fact is indicative that the spin labels in the insoluble material obtained with 10% Triton are not in a bilayer environment. The origin of this kind of spectrum will be discussed in the following sections.

3.4. Influence of SM and Chol on acyl chain order/mobility

The results of the previous sections showed that human and bovine erythrocyte membranes, and the insoluble material obtained from the extraction of bovine erythrocytes with Triton X-100 concentrations up to 6%, are membrane systems with a broad range of SM concentrations, differing in their acyl chain order/mobility. In the present section, we want to focus on the relationship between acyl chain order/mobility and lipid composition. It is well known that Chol and SM are lipids that promote acyl chain stretching [3,9], which will restrict segmental mobility. We plotted in Fig. 5(a) the values of A_{max} as a function of the relative Chol content of the corresponding sample. The three panels correspond to the three spin labels. It can be seen that the variation of A_{max} is not monotonous, and a negative correlation between A_{max} and Chol content can be inferred, which at a first glance is striking, taking into account that decreasing A_{max} values represent increasing fluidity. In Fig. 5(b), the values of A_{max} were plotted as a function of the SM content of each sample. A high positive linear correlation among A_{max} values and SM content was obtained in this case at the three depth levels within the membrane, with the best correlation and regular behavior corresponding to 16-SASL. Human erythrocytes also fit well in this correlated behavior.

Thus, in our membrane systems, SM seems to be the main regulator of acyl chain fluidity, and its ordering effect is strong enough to overcome the effects of a cholesterol reduction.

3.5. Evidence of an immobilized lipid environment in the insoluble pellets

When analyzing the EPR spectra of the insoluble material obtained with increasing Triton concentrations, we obtained evidences of the coexistence of a highly immobilized environment with a more or less fluid membrane environment. One of these evidences is the appearance of additional spectral structures, pointed out with arrows in Fig. 3, in the outer wings

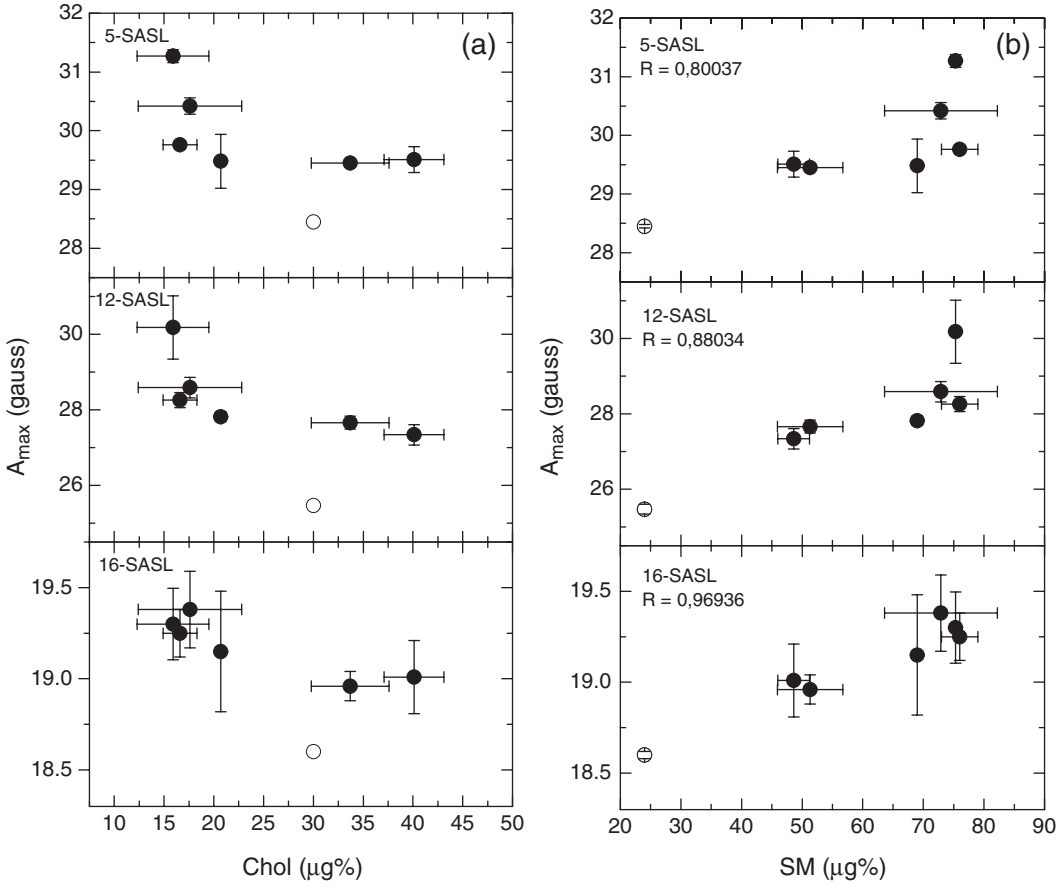

Fig. 5. Influence of cholesterol and sphingomyelin on lipid chain segmental mobility at different depths. Hyperfine parameter A_{max}, indicative of segmental chain mobility restrictions, for the membrane of bovine erythrocytes and its insoluble material, plotted as a function of (a) the relative cholesterol content of each sample, and (b) the relative sphingomyelin content of each sample, for the three spin labels used in this work. The values corresponding to intact human erythrocytes are also included (open symbols). Lipid contents correspond to the data displayed in Fig. 1. A_{max} values were evaluated from the EPR spectra. The insert values are linear correlation coefficients. Error bars indicate S.D. values.

of the 16-SASL spectrum of the sample obtained with 6% Triton (vide infra). Also, it can be seen in Fig. 4 that the A_{max} values of the most delipidated 10% Triton samples do not show the characteristic fluidity gradient associated to a bilayer environment. The value of A_{max} in the 16-SASL spectrum is near the rigid limit, indicative of immobilized spin labels. These facts, together with the visual appearance of the insoluble pellet (see Materials and methods), suggest that the insoluble material resulting from the incubation of bovine erythrocytes with Triton X-100 contains lipid and protein in different proportions. Preliminary results of electrophoresis (data not shown) indicate that several proteins of the erythrocyte membrane, including high molecular weight proteins that can be ascribed to spectrin, are present in the insoluble pellet at all Triton concentrations. Spectrin is the main component of the cytoskeleton underlying the erythrocyte lipid bilayer. Connections between spectrin and integral membrane proteins provide the linkage between the cytoskeleton and the bilayer [22]. Several papers reported the recovery of cytoskeleton material in Triton X-100-insoluble residues [5,23,24]. In a recent paper, Ciana et al. [25] reported that attachment to the cytoskeleton made it difficult the isolation of erythrocyte DRM (detergent-resistant membranes) by ultracentrifugation in sucrose gradients.

In order to estimate the amount of the different components in the composite spectrum obtained with 16-SASL in the 6% Triton sample, we performed the following assumptions: (a) the spectrum of the insoluble material obtained with 10% Triton corresponds to a 100% spin label population strongly immobilized (probably in a "belt" around the hydrophobic domains of membrane proteins); (b) the spectrum of the insoluble material obtained with 2% Triton corresponds to spin label in almost a 100% membrane-like environment (this assumption is justified as no additional structures are observed in this case, and the amount of unsolubilized lipids in this sample is more than 20 times as much as that of 6% Triton sample, as it can be seen in Fig. 2); (c) the spectrum of the insoluble material obtained with 6% Triton contains contributions from both components; (d) the spin label partitions with equal probability in both environments. With these assumptions, spectral addition of double integrated normalized spectra allowed us to estimate that in the insoluble pellet obtained with 6% Triton, 70% of the lipids are immobilized in the boundary of integral membrane proteins, while the remaining 30% corresponds to lipids in a normal membrane environment. The results of this decomposition are shown in Fig. 6.

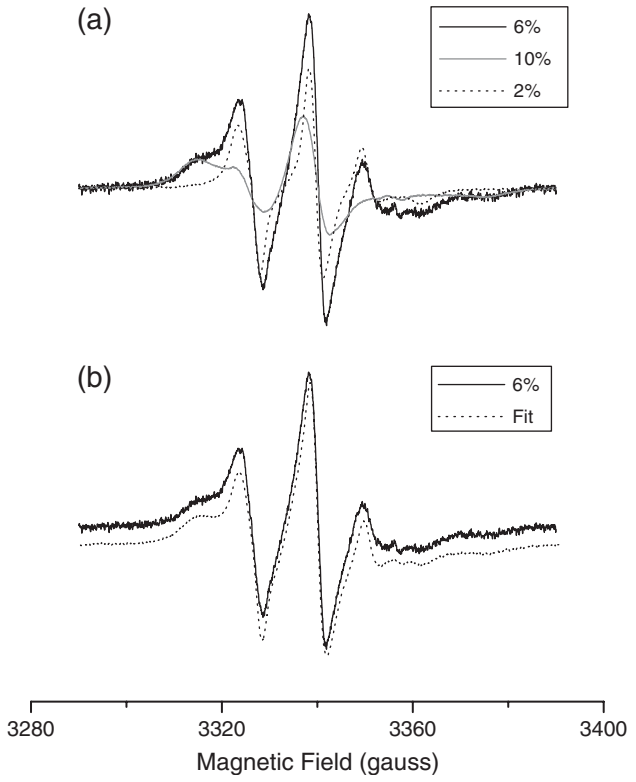

Fig. 6. Coexistence of membrane-like and immobilized lipid environments in the insoluble pellets. Analysis of the composite EPR spectrum of 16-SASL in the insoluble pellet obtained with 6% Triton. It was assumed that the 10% Triton spectrum corresponded only to immobilized lipids in close vicinity of integral proteins, and the 2% Triton spectrum corresponded only to lipids in a membrane-like environment. It is shown that the 6% spectrum can be reproduced by a linear combination of the above mentioned spectra. (a) Double integrated normalized spectra. The spectrum corresponding to 2% Triton is affected by a factor of 0.3, and the spectrum of 10% Triton is affected by a factor 0.7. (b) Sum of the weighted spectra (dotted line) in comparison with the 6% Triton spectrum (full line).

Similar spectra were obtained by Marsh and Horvath in several works studying proteoliposomes with high protein/lipid content (reviewed in [11]). They showed the existence of two spin-labeled lipid populations, the mobility of one of which was severely hindered by direct interaction with the hydrophobic domain of proteins. These effects could be clearly seen when the spin labeled moiety was close to the terminal methyl end of the chain, because in that case the EPR spectra features of fluid lipid bilayer membranes are relatively narrow, allowing the detection of superimposed spectra of immobilized labels, which appear at the outer wings [11].Chachaty et al. [26] and Veiga et al. [21] reported two component EPR spectra when studying codispersions of egg sphingomyelin–cholesterol. They assigned the more immobilized component to segregated SM–Chol complexes, and their results correlated well with the different compositions of their model systems. In our case, the samples obtained with 2% and 6% Triton show well-differentiated 16-SASL EPR spectra, being evident an immobilized component only for the largely delipidated 6% Triton sample. As the lipid composition of the two samples is similar (Fig. 1), the observation of an immobilized component in the 6% sample spectrum should not be related to the segregation of SM–Chol complexes, but to the increased relative weight of the proteins in the insoluble pellet.

3.6. Origin of the membrane-like EPR spectra in the insoluble pellets

In the previous sections we have presented experimental results showing that the insoluble material obtained when submitting bovine erythrocytes to the action of Triton X-100 contains lipids in a membrane-like environment, together with strongly immobilized lipids in a non-bilayer environment, probably the hydrophobic belt of integral proteins which remained attached to the cytoskeleton. We have also shown that acyl chain ordering/mobility in the membrane-like environment is strongly correlated to SM content and not to Chol content.

The following question is about the identity of the membrane-like structures in the insoluble material. As they were obtained by centrifugation, they are expected to be closed lipid vesicles. It would be interesting to determine if these structures resemble the detergent-resistant membranes (DRM), operationally defined as membrane fractions which resist solubilization by Triton X-100 at 4 °C and float after ultracentrifugation in the light zone of sucrose gradients [2–4,27,28]. In canonical DRM extraction procedures [4,28,29], the whole volume of detergent extraction is submitted to equilibrium ultracentrifugation in a sucrose gradient, without a previous pelleting of the insoluble material (an exception to this procedure can be found in Salzer and Prohaska [27]). In our procedure, the 30-min microcentrifugation used to pellet the insoluble material is probably not capable of sedimenting small unilamellar vesicles. Thus, a certain amount of light vesicles formed by insoluble lipids could be washed out in our procedure. However, our pelleted vesicles, although mixed with integral proteins attached to cytoskeleton debris, are expected to have the same lipid composition as the DRM, as they were recovered as detergent-insoluble lipid structures.

A great amount of experimental data indicates that DRM are enriched both in Chol and SM [2–4]. In our work, the insoluble pellet is enriched in SM but depleted in Chol when compared to the original membrane. However, from the lipid composition data displayed in Fig. 1, and considering typical values of lipid molecular masses, the molar ratio SM/glycerophospholipid/Chol can be estimated roughly as 60:10:30 (for Triton concentrations of 2% and above). When these values are located in the phase diagrams of ternary systems SM/POPC/Chol [3], they correspond to the coexistence of liquid ordered and solid ordered phases. Thus, although Chol depleted in comparison with the original membrane, our insoluble vesicles seem to have an adequate composition to be detergent-resistant membranes.

Regarding the physiological significance of our results, there is presently a hot discussion calling into question the resemblance of DRM to lipid rafts existing in the membranes prior to detergent treatment [6,30,31], as it have been demonstrated that Triton is per se able to induce the formation

of lipid domains [32]. If the detergent-insoluble material resembled any pre-existent structure of the erythrocyte membrane, our results regarding lipid organization would be relevant, because our rapid and simple procedures allow us to obtain the insoluble pellet with a minimum degree of perturbation of the lipid structure. The interference in the EPR spectra of insoluble proteins pelleted along with the lipid vesicles could be filtered by spectral analysis, allowing a precise determination of lipid fluidity as a function of composition.

4. Conclusions

We have shown that the bovine erythrocyte membranes have a high resistance to solubilization in cold Triton X-100, needing more than 6% Triton to reach complete solubilization. This represents a detergent/lipid ratio of about 60, a value much higher than those reported for human erythrocytes, but in line with results obtained in lipid mixtures of adequate compositions.

Lipid and proteins are present in the insoluble pellet. At the lowest detergent concentrations, the lipid composition of the insoluble material is indistinguishable from the original membrane. For Triton concentrations between 2% and 6%, the decreasing amounts of insoluble lipid show increased SM and decreased Chol content. EPR spectroscopy with stearic acid spin labels allowed us to discriminate protein and lipid contributions to the spectra when the labeled moiety is close to the terminal methyl end (16-SASL). For Triton 10%, the pellet contains predominantly insoluble proteins.

Lipid chain order is increased and/or mobility is decreased in the bilayer portion of the insoluble pellet in comparison with the original bovine erythrocyte membrane. This occurs in spite of the fact that the partial cholesterol content is lower than in the intact membrane. Our results suggest that sphingomyelin is the main responsible for the differences in membrane fluidity between human and bovine erythrocyte membranes, and also between the Triton X-100-insoluble material and the original membrane. Comparison with model systems also suggests that the high resistance to Triton solubilization shown by bovine erythrocytes could be attributed to molecular interactions between sphingomyelin and cholesterol, together with the low glycerophospholipid content. In order to confirm these assumptions, we plan to investigate in detail the solubilization of human erythrocytes and model systems of adequate compositions.

The particular lipid composition of the bovine erythrocyte membrane suggests that liquid ordered phases could be present. We plan to perform EPR experiments as a function of temperature to gather information about the phase state of the lipids in the original membrane and in the insoluble pellets. Our results could be physiologically relevant in the case that the detergent-resistant material resembled any structure originally present in the intact cell membrane.

Acknowledgements

This work was supported by ANPCyT and Universidad Nacional del Litoral (Grants PICTO-UNL 03-13214 and CAI+D 19-130), and by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Grants PIP 2559 and 5370), Argentina. We thank Vet. R. Cerutti for gently providing the bovine blood samples and helpful advice. AMG is a member, and PMR is a fellow of CONICET, Argentina.

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