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# Action of Detergents on Membranes: Differences between Lipid Extracted from Red Cell Ghosts and from Red Cell Lipid Vesicles by Triton X-100†

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ABSTRACT: A comparative study was undertaken of the solubilization of red cell ghosts and red cell lipid vesicles by Triton X-100 (Triton) to assess the influence of ghost membrane proteins on the amounts and on head-group and fatty acyl compositions of phospholipids removed from both types of membranes. Similar amounts of phospholipid and of cholesterol were solubilized from ghosts and liposomes at a Triton/lipid mole ratio of 1:1, but ~1.5–2 times as much phospholipid and cholesterol was released from ghosts as from liposomes at higher Triton/lipid ratios. Both ghosts and liposomes were less readily solubilized than vesicles of red cell lipid from which cholesterol had been removed. No differences between phospholipid compositions of Triton-extracted lipid from ghosts and liposomes were demonstrable at a Triton/lipid ratio of 1:1, which releases almost no protein from ghosts. At higher Triton/lipid ratios, which release nearly all intrinsic membrane protein from ghosts, however, more phosphatidylserine was removed from ghosts than from liposomes. The latter difference was apparent, regardless of whether ghosts and liposomes were subjected to the same level of Triton or whether ghosts and liposomes were subjected to different levels so as to release the same amounts of lipid from both types of membranes. The selective cosolubilization of phosphatidylserine and certain membrane proteins from ghosts but not from liposomes indicates that phosphatidylserine and at least one of those membrane proteins are associated in untreated ghost membranes. This protein-dependent, selective solubilization of ghost lipid by Triton is distinguishable from a protein-independent, selective solubilization of both ghost and liposome lipids by Triton, the soluble lipid from both membranes having a lower content of sphingomyelin relative to the total amount of lipid extracted.

The mechanism by which detergents solubilize membranes is of interest because of their usefulness not only for the isolation and purification of membrane proteins but also for the resolution of membrane structure. On exposure to various mild detergents, some proteins are released from their membrane sites in association with specific phospholipid(s) not representative of the total membrane lipid, e.g., glycophorin (Armitage et al., 1977; van Zoelen et al., 1977; Buckley, 1978), the erythrocyte anion channel or band III (Ross & McConnell, 1978), and $Na^+,K^+$-ATPase (Hokin & Hexum, 1972). In addition to demonstrations of coisolation of individual proteins with specific phospholipid(s), intrinsic membrane proteins are also released from erythrocyte ghosts along with a mixture of phospholipids, the composition of which differs from that of total red cell lipid (Yu et al., 1973; Kirkpatrick et al., 1974; Coleman et al., 1976). These findings could signify interactions in the intact membrane between the extracted protein(s) and lipid(s) and/or between the nonextracted protein(s) and lipid(s), which would have survived exposure to detergent. Alternatively, a cosolubilization of specific lipids with certain proteins could reflect a similar affinity of detergent for those proteins and lipids, which would enter the same micelle independently of each other.

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The likelihood of a detergent-independent, lipid–protein association should be inferable from a comparison of lipid solubilization by detergent from a native cell membrane with the solubilization by detergent of liposomes composed solely of lipid previously extracted from the same type of cell membrane. The processes chosen for this study were the solubilization of lipid by Triton[1] from the red cell ghost and Triton solubilization of liposomes composed solely of red cell lipids. Whereas Triton does preferentially solubilize certain lipids in the absence of membrane proteins, i.e., from liposomes, results reported here indicate that membrane proteins can influence the amounts and kinds of phospholipids solubilized by Triton.

## Experimental Procedures

*Membrane Preparation.* Immediately prior to detergent treatment, human red cell ghosts were prepared by a modification of the procedure of Dodge et al. (1963) from whole blood which was less than a week old and had been obtained on the day of withdrawal from a local clinical laboratory. Red cell lipid for preparation of liposomes was extracted according to a modification of the Bligh & Dyer (1959) procedure from isotonic saline washed red cells, received in the form of outdated, whole blood from a blood bank. The lipid-containing phase was brought to dryness in a rotary evaporator, suspended in chloroform–methanol, 2:1, filtered through glass wool, and stored as small aliquots under $N_2$ at approximately −20 °C. Liposome membranes were formed by drying aliquots of the red cell lipid under $N_2$, hydrating the dried lipid film in cold 0.01 M Tris-HCl, pH 7.6, and vortexing the suspension until all the lipid had been resuspended and no large particles could be seen.

*Lipid Solubilization by Triton Treatment.* According to a modification of the procedure of Yu et al. (1973), red cell ghosts and resuspended red cell lipid vesicles were exposed to appropriate concentrations (1:100 v/v) of Triton X-100 (Sigma Chemical Co., St. Louis, MO) in 0.01 M Tris-HCl + 0.05 M sodium borate, pH 8, and kept in the cold for 30 min with intermittent mixing. Insoluble or bilayer lipid was separated from soluble or micellar lipid by centrifugation in a Spinco SW 50.1 rotor at 210000*g* for 30 min. The cholesterol in each fraction was determined by a combination of the procedures of Courchaine et al. (1959) and Zlatkis et al. (1963), as described by Kates (1972). The phospholipid content of each fraction was determined by assaying inorganic phosphorus according to a modification of the procedures of Fiske & SubbaRow (1925) and Bartlett (1959), as described by Johnston (1971) and by Peterson (1978). The phospholipid and cholesterol contents of ghost and liposome lipid agreed with literature values (van Deenen & de Gier, 1974).

*Thin-Layer Chromatography.* Supernatant and resuspended pellet fractions obtained by Triton treatment of ghosts and liposomes were deproteinized by the procedure of Folch et al. (1957). Phospholipid recoveries ranged from 86.3 ± 5.3 to 100.6 ± 21.4%. Lipid extracts were layered onto silica gel (Unisil, 100–200 mesh; Clarkson Chemical Co., Inc., Williamsport, PA) columns, which were washed with chloroform–methanol, 19:1, to elute Triton and neutral lipid and with methanol to elute phospholipid. Aliquots of the concentrated methanol eluants were spotted on plastic sheets coated with silica gel (Sil N-HR; Macherly-Nagel and Co., W. Germany) and separated by one-dimensional thin-layer chromatography in chloroform–methanol–acetic acid–$H_2O$, 50:25:7:3, as described by Skipski et al. (1964). The comigration of phosphatidylserine and phosphatidylinositol under these conditions was acceptable since phosphatidylinositol comprises only 0.6%, whereas phosphatidylserine comprises ~15% of the total red cell lipid (Dodge & Phillips, 1967). Pieces of silica gel sheet with $I_2$-stained spots were soaked in methanol, which was then transferred to another tube and evaporated, and the residue was analyzed for inorganic phosphorus. Phospholipid compositions agreed with literature values (van Deenen & de Gier, 1974).

*Gas–Liquid Chromatography.* Phospholipid samples from which Triton and cholesterol had been removed were treated with methanolic HCl (Applied Science Laboratories, Inc., State College, PA) according to Kent et al. (1974) to generate fatty acid methyl esters. After incubation overnight at 75 °C, fatty acid methyl esters were extracted into ether. The ether extracts were washed with sodium bicarbonate, brought to dryness, and dissolved in hexane. Fatty acid methyl esters in hexane were separated on a 6 ft × 4 mm glass column, packed with 10% phenylcyanoalkyl-substituted polysiloxane on Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories, Inc., State College, PA), and installed in a Beckman GC 65 instrument with a flame ionization detector. The temperature program of Kent et al. (1974) was followed. Peaks were quantitated by calculating their areas.

## Results

*Phospholipid Solubilized as a Function of Triton/Lipid Mole Ratio.* At the outset it was necessary to find detergent/lipid mole ratios which would provide the basis of a valid comparison between lipid solubilization of ghosts and of liposomes. Since a selective solubilization of nearly all of the major intrinsic membrane proteins had been observed at a Triton concentration of 0.5% (Yu et al., 1973), increasing amounts of both ghost and liposome membranes were treated with that concentration such that Triton/lipid mole ratios ranged from 2.9:1 to 25:1. The amounts of phospholipid solubilized from ghosts and from liposomes plotted against the amounts of total phospholipid treated with Triton closely described least-squares regression lines with slopes of less than 1 (Figure 1, open circles and filled circles, respectively). The incomplete solubilization of ghosts and liposomes is probably due to the cholesterol in these membranes, as Triton treatment of cholesterol-free, red cell lipid vesicles under the same conditions brought about complete solubilization, i.e., a straight line with a slope of 1 (Figure 1, filled triangles). The relative resistance of cholesterol-containing red cell lipid membranes to Triton solubilization appears to be mitigated somewhat by one or more of the membrane proteins present in ghosts, as the slope of the line describing red cell ghost extraction is less than 1 (Figure 1, open circles) but greater than that describing red cell lipid vesicle extraction (Figure 1, filled circles). In agreement with Dennis & Owens (1973) and Hertz & Barenholz (1977), a Triton/lipid ratio near 2:1 was found minimally necessary to completely solubilize liposomes composed of egg lecithin (not shown).

*Geometric Considerations.* The greater extractability of ghost, as opposed to liposome, phospholipid depicted in Figure 1 gave rise to two concerns. (1) Since the liposomes were multilamellar, unlike the unilamellar ghost membranes, the time required for equilibration of Triton with the liposomes could have exceeded the time allowed prior to centrifugation of insoluble, bilayer lipid from soluble, micellar lipid. If so, the experimental conditions would have favored lipid solubilization from ghosts over lipid solubilization from liposomes. It seemed likely that equilibration of Triton with even the

[1] Abbreviations used: Triton, Triton X-100; FAME, fatty acid methyl ester.

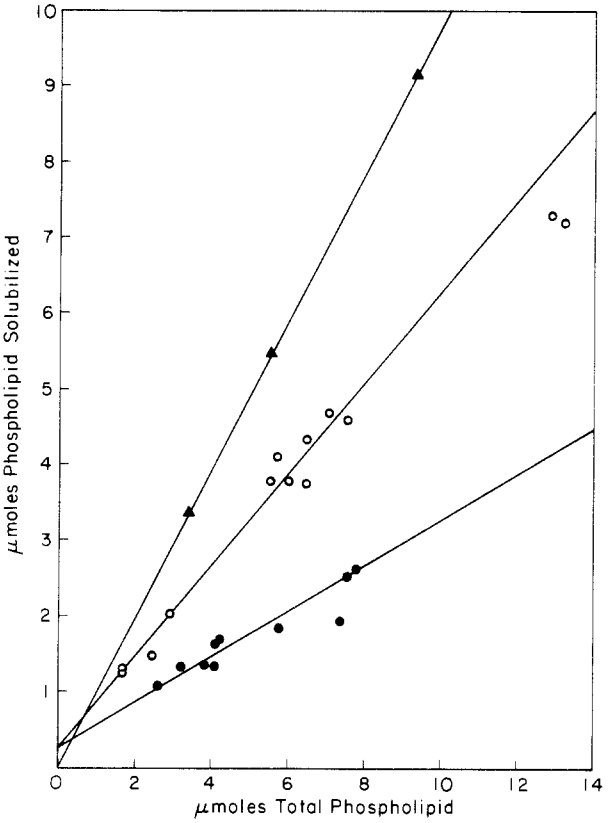


FIGURE 1: Solubilization of increasing amounts of phospholipid from red cell ghosts (open circles), red cell lipid vesicles (filled circles), and cholesterol-depleted red cell lipid vesicles (filled triangles) at 0.5% Triton X-100 or Triton/lipid mole ratios of 25:1–2.9:1. Three points shown were omitted from calculations of least-squares regression lines to move the $y$ intercept closer to 0.

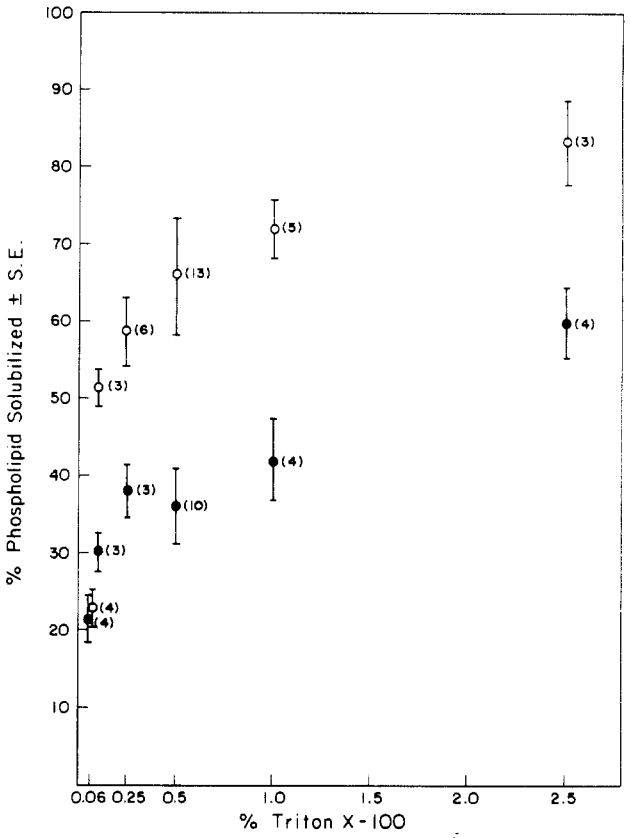


FIGURE 2: Percent phospholipid ± SE solubilized from red cell ghosts (open circles) and from red cell lipid vesicles (filled circles) by increasing concentrations of Triton X-100, which correspond with Triton/lipid mole ratios of 1:1 to 40:1. The number of samples analyzed is shown within parentheses.

innermost lamellae of the liposomes had occurred during the 30 min prior to centrifugation of insoluble from soluble lipid, since addition of Triton at its final concentration to dried ghost lipid yielded the same amount of soluble phospholipid as addition of Triton to a preformed liposome suspension. In addition, membrane integrity was measured [see MacDonald et al. (1974) for details of the procedure] by loading liposomes with a low molecular weight, impermeant solute, *p*-nitroanilinesulfonate, the release of which was determined as a function of Triton concentration under conditions of the standard solubilization assay. A control sample of liposomes not treated with detergent was processed simultaneously to distinguish Triton-independent from Triton-dependent release of dye. Even at the lowest Triton concentration of 0.06%, 95% of the solute was released into the supernatant with the solubilized lipid, affirming that Triton had equilibrated with all layers of the multilamellar liposomes.

(2) The second concern was prompted by the observation by phase contrast microscopy of vesicles in Triton-insoluble fractions, which seemed smaller than those characteristic of a handshaken, multilamellar vesicle preparation. It was necessary to rule out the possibility that Triton treatment could generate unilamellar vesicles of the size obtained by sonication, because the procedure for separation of insoluble and presumably bilayer lipid from soluble or presumably micellar lipid consisted of centrifugation at 210000$g$ for 30 min, conditions unlikely to pellet very small vesicles (Barenholz et al., 1977). Identical pairs of aliquots of red cell lipid vesicles were treated either with 8-mol parts Triton or with 40-mol parts Triton and centrifuged at 210000$g$, one for 30 min and the other for 3 h. The lipid contents of supernatant fractions spun for 30 min and for 3 h and of pellet fractions similarly centrifuged were within 4% of each other, contraindicating the presence of small, unilamellar liposomes in Triton-treated samples. Under the present conditions, 210000$g$ for 30 min seems adequate for separation of insoluble from soluble lipid.

*Lipid Solubilization as a Function of Triton Concentration.* Since the percent of phospholipid solubilized at a Triton/lipid ratio of 8:1 from red cell ghosts was almost twice that solubilized at the same ratio from liposomes of red cell lipid, compositional differences between the two groups of lipid extracts might reflect different stages of detergent action. The solubilization of both types of membrane was determined as a function of the Triton/lipid ratio to find a Triton/lipid ratio which would yield the same percent of lipid from red cell lipid vesicles as that removed from red cell ghosts at a Triton/lipid ratio of 8:1. Amounts of treated lipid varied between 4 and 8 μmol, and amounts of Triton ranged from 4.7 to 194.7 μmol. Cholesterol, as well as phospholipid, release was assayed in view of the apparently "antidetergent" effect of cholesterol previously mentioned.

The curves obtained by plotting the percent phospholipid (Figure 2) and cholesterol (Figure 3) released against the appropriate Triton concentrations, which correspond with increasing Triton/lipid mole ratios, are roughly exponential. Data not shown indicate the $y$ intercept to be less than 5% for ghost membrane solubilization. As will be clear from the results, Triton levels designated as low (1:1 Triton/lipid or 0.06% Triton), intermediate (8:1 Triton/lipid or 0.5% Triton), and high (40:1 Triton/lipid or 2.5% Triton) are correlated with low, intermediate, and high detergent activities in quantitative and qualitative terms of liposome and ghost responses. A Triton/lipid ratio of 40:1 (see 2.5% Triton, Figure 2) was

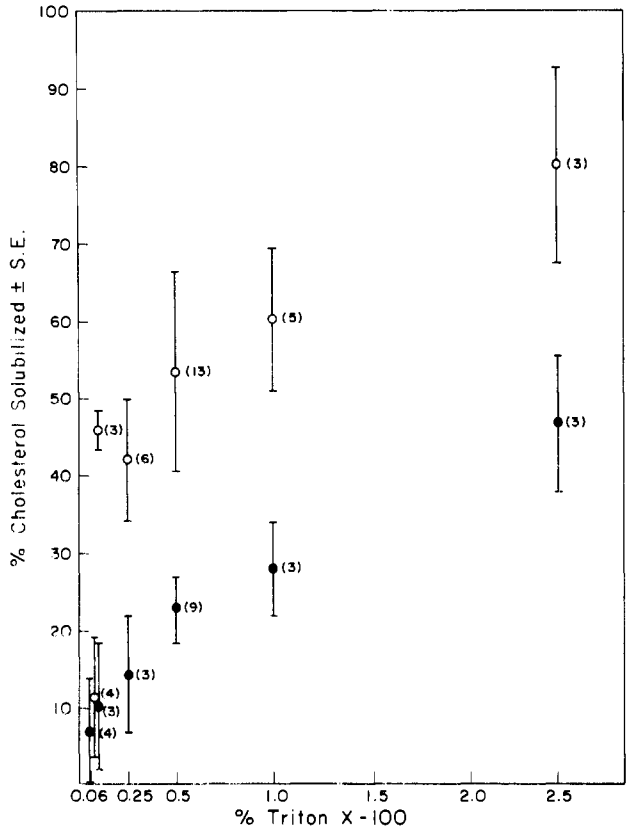


FIGURE 3: Percent cholesterol ± SE solubilized from red cell ghosts (open circles) and from red cell lipid vesicles (filled circles) by increasing concentrations of Triton X-100, which correspond with Triton/lipid mole ratios of 1:1 to 40:1. The number of samples analyzed is shown within parentheses.

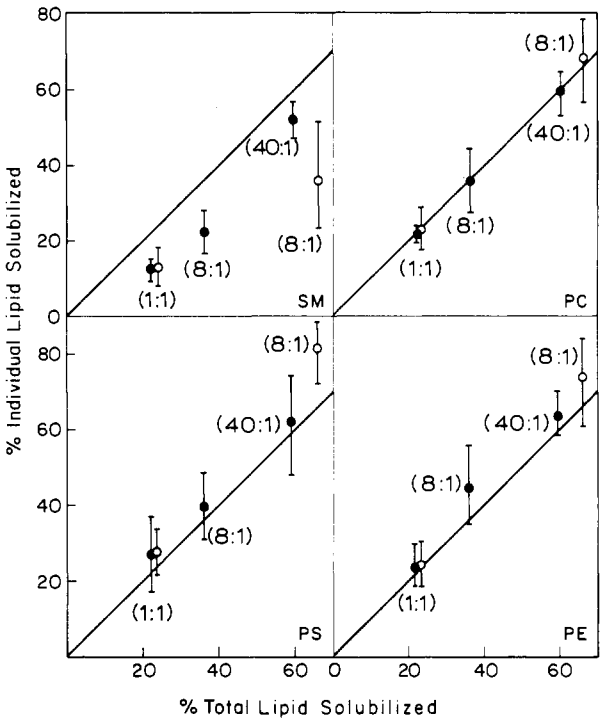


FIGURE 4: Percent solubilization ± SE of individual phospholipid species from red cell ghosts (open circles) and from red cell lipid vesicles (filled circles) plotted against the percent total phospholipid solubilized by Triton X-100. Percents of total phospholipid solubilized corresponding with Triton/lipid mole ratios (indicated within parentheses) are 21 to 22% at 1:1, 36% at 8:1 for liposomes, 59% at 40:1 for liposomes, and 65% at 8:1 for ghosts. To facilitate comparison of the data, a diagonal line indicates the percent of each species solubilized, had no selective solubilization occurred. Phospholipids indicated are sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE).

necessary to release 59% of the phospholipid from red cell vesicles, whereas a Triton/lipid ratio of 8:1 (see 0.5% Triton, Figure 2) was sufficient to release a comparable amount, i.e., 65%, of the phospholipid from red cell ghosts. Treatment of red cell lipid vesicles with the 8:1 Triton/lipid level of detergent removed only 36% of their phospholipid. At 1:1 Triton/lipid (see 0.06% Triton, Figure 2), a ratio ineffective in removing significant amounts of protein from red cell ghosts, similar amounts of phospholipid, 21.4 and 22.8% from liposomes and ghosts, respectively, were released from the two types of membranes. Characterization of supernatant and pellet proteins extracted at high (Triton/lipid 40:1 or 2.5% Triton), intermediate (Triton/lipid 8:1 or 0.5% Triton), and low (Triton/lipid 1:1 or 0.06% Triton) detergent levels confirmed a substantial and selective solubilization of protein (not shown) at high and intermediate, but not low, Triton levels, as previously reported by Yu et al. (1973).

*Phospholipid Compositions of Supernatant and Pellet Fractions Generated by Triton Treatment.* Given Triton/lipid ratios which would solubilize equivalent percentages of lipid from red cell ghosts and red cell lipid vesicles, the phospholipid compositions of the appropriate Triton-soluble and Triton-insoluble fractions could be analyzed by thin-layer chromatography and compared. In addition to experiments with high levels of detergent which solubilize substantial amounts of intrinsic membrane proteins from red cell ghosts, experiments with a low level of detergent were performed. The comparison of lipid released from ghosts with lipid released from liposomes at a Triton/lipid ratio of 1:1, which solubilizes almost no protein from red cell ghosts, constituted a second test of the protein-dependent nature of preferential phospholipid solubility or insolubility. If the extracts from ghosts and liposomes obtained at a low Triton level differed in composition in spite of the negligible release of membrane protein from ghosts, some factor or component other than the protein, e.g., asymmetric distribution of certain phospholipids between the two halves of the ghost bilayer or larger size of ghosts than liposomes, could be construed to be responsible for that compositional difference. Results with high (40:1–8:1 Triton/lipid) and low (1:1 Triton/lipid) detergent levels will be considered separately.

*Phospholipid Composition of Extracts Obtained at High Triton Levels.* The results of phosphate analyses of thin-layer chromatographic zones of the soluble and insoluble fractions obtained with 8:1 Triton/lipid from ghosts and from liposomes and with 40:1 Triton/lipid from liposomes were converted into the percent of each phospholipid species solubilized, calculated in the following way for phosphatidylethanolamine as an example: (percent solubilized lipid represented by phosphatidylethanolamine multiplied by percent total phospholipid solubilized) divided by [(percent solubilized lipid represented by phosphatidylethanolamine multiplied by percent total phospholipid solubilized) plus (percent nonsolubilized lipid represented by phosphatidylethanolamine multiplied by percent total phospholipid not solubilized)] multiplied by 100. The percent of each species solubilized is plotted against the percent of total phospholipid solubilized in Figure 4, ghost lipid represented by open circles and liposome lipid by filled circles. In all three extracts (65% of ghost lipid at 8:1 Triton/lipid, 59% of liposome lipid at 40:1 Triton/lipid, and 36% of liposome lipid at 8:1 Triton/lipid) sphingomyelin constitutes a smaller portion than it would have, had all species been represented equally. In addition to the deficit in sphingomyelin which is

Table I: Percent of Each Fatty Acyl Chain Solubilized at Different Triton Levels[a]

| fatty acyl chains | Triton/lipid: membrane: | % solubilized 1:1 ghost | 1:1 lipo-some | 8:1 ghost | 8:1 lipo-some | 40:1 lipo-some |
|---|---|---|---|---|---|---|
| 16:0 | | 18.6 | 18.0 | 55.3 | 30.5 | 56.8 |
| 18:0 | | 25.6 | 20.0 | 75.6 | 39.8 | 57.8 |
| 18:1 | | 21.7 | 23.4 | 71.1 | 35.5 | 57.3 |
| 18:2 | | 21.8 | 23.9 | 69.3 | 36.5 | 60.9 |
| 22:0 | | 19.6 | 25.1 | 42.9 | 28.3 | 54.3 |
| 22:1, 20:4 | | 30.0 | 28.4 | 78.5 | 49.0 | 63.7 |
| 24:0 | | 18.2 | 8.3 | 24.0 | 17.9 | 58.6 |
| 24:1 | | 16.7 | 16.5 | 48.8 | 34.5 | 54.1 |
| 22:6 | | 25.0 | 30.0 | 77.8 | | 65.9 |

[a] Each value is [(FAME in supernatant)/(FAME in supernatant and FAME in pellet)] × 100.

characteristic of all extracts, there is a statistically significant, preferential solubilization of phosphatidylserine from the ghost membrane but not from the lipid vesicle membrane. This excess phosphatidylserine in ghost membrane extracts constitutes ~16% of the total ghost phosphatidylserine.

*Phospholipid Composition of Extracts Obtained at Low Triton Levels.* If data similar to those obtained at high or protein-releasing levels of Triton could be obtained at low or non-protein-releasing levels of Triton, attributes of ghosts and liposomes other than their difference in protein content might be more decisive determinants of susceptibility to detergent action. As seen from Figure 4, however, there is no preferential solubilization of phosphatidylserine, phosphatidylcholine, or phosphatidylethanolamine at non-protein-releasing levels of Triton, i.e., 1:1 Triton/lipid, which solubilize 21 to 22% of the total phospholipid from ghosts and liposomes. As at protein-releasing levels of Triton, the percentages of sphingomyelin released from both ghosts and liposomes are less than the percentages of total phospholipid released from each membrane. Finally, there is no statistically significant difference between ghost and liposome extracts obtained at low Triton levels with respect to the content of any phospholipid species.

*Fatty Acyl Compositions of Supernatant and Pellet Fractions Obtained by Triton Treatment.* Fatty acyl compositions of fractions obtained by Triton treatment of ghosts or of red cell lipid vesicles had not been previously reported. Differences between Triton-soluble and Triton-insoluble fractions with respect to fatty acyl compositions were expected since there is some preferential attachment of specific head groups of red cell phospholipids to certain fatty acyl chains (Dodge & Phillips, 1967).

The fatty acyl chain compositions of the supernatant and pellet fractions of ghosts and liposomes treated with Triton are given in Table I as the solubilized percent of each fatty acyl chain, i.e., [(FAME in supernatant)/(FAME in supernatant + FAME in pellet)] × 100, calculated in the same way as the solubilized percent of each phospholipid species. Generally, the percentages of fatty acyl chains solubilized were slightly larger than the percent of total phospholipid solubilized with the exceptions of 16:0, 22:0, 24:0, and 24:1, the percentages of which were correspondingly smaller. At the lowest level of Triton (1:1 Triton/lipid), most fatty acyl methyl esters were solubilized to a slightly greater extent than the total phospholipid extracted, i.e., 24.8 ± 3.4% for ghosts and 25.1 ± 3.6% for liposomes. Exceptions are 16:0, 22:0, 24:0, and 24:1 with a mean of 18.3 ± 1.2% for ghosts and 16:0, 24:0, and 24:1 with a mean of 17.3 ± 1.1% for liposomes. Examination of data for ghosts treated at 8:1 Triton/lipid shows most fatty acid methyl esters to be clustered around a mean of 74.5 ± 4.1%, with the exception of 16:0, 22:0, and 24:1 which have a mean of 49.0 ± 6.2% and 24:0 which is 24.0%. Similarly, data for liposomes treated at 8:1 Triton/lipid show most fatty acid methyl esters to fall near a mean of 40.2 ± 6.1%, again with the exception of 16:0, 22:0, and 24:1 which have a mean of 31.1 ± 3.1% and 24:0 which is 17.9%. Data for liposomes exposed to detergent at 40:1 Triton/lipid exhibit less marked differences with most fatty acid methyl esters near a mean of 61.1 ± 3.7%, the exceptions being 16:0, 22:0, 24:0, and 24:1 which have a mean of 55.9 ± 2.1%.

## Discussion

The physical properties of Triton X-100 solutions and detergent solutions in general are sufficiently appreciated that they have been used with great effectiveness in the study of membrane proteins (Helenius & Simons, 1975; Tanford & Reynolds, 1976). The interaction of Triton with lipid bilayers of the complexity of a natural membrane on the other hand has received less attention, although effects of Triton on membranes composed of one or two phospholipid species have been examined (Dennis & Owens, 1973; Ribeiro & Dennis, 1975; Inoue & Kitagawa, 1976; Hertz & Barenholz, 1977; Schlieper & DeRobertis, 1977; Bangham & Lea, 1978). The comparison of Triton solubilization of ghost membranes with Triton solubilization of membranes, composed solely of lipids previously extracted from ghosts, was undertaken to characterize the interaction of Triton with a lipid membrane of complex composition, as well as to probe indirectly the relationship of ghost membrane proteins with their native lipid environment. A discussion of the results of this investigation is divided into two parts, the first dealing with quantitative aspects of the solubilization process and the second dealing with qualitative aspects.

*Quantitative Aspects of the Solubilization by Triton of Red Cell Lipid Vesicles and Red Cell Ghosts.* The first three figures depict the importance of three factors affecting the detergent activity of Triton X-100. (1) The presence of cholesterol in purely lipid membranes at a level characteristic of a red cell ghost reduces the extractability of phospholipid by more than 50% (Figure 1). Similarly, Inoue & Kitagawa (1976) observed that cholesterol-containing phospholipid vesicles retained more glucose following Triton treatment than phospholipid vesicles without cholesterol. Neither observation indicates whether cholesterol prevents Triton insertion into the lipid bilayer and/or directly alters the structural properties of the bilayer so as to inhibit bilayer–micelle conversion. Cholesterol does not seem to be preferentially solubilized by Triton so as to reduce the amount of detergent available for phospholipid micellization, since the percent release of cholesterol as a function of Triton concentration is less than, but parallels, that of phospholipid release (compare Figures 2 and 3). (2) Extraction of phospholipid by Triton is affected significantly by the presence of protein, as release of phospholipid from red cell ghosts is greater than that from red cell lipid vesicles over a wide range of Triton/lipid ratios (Figures 1 and 2). The protein effect inferred from the data in Figures 1 and 2 is probably a net result of opposing tendencies fostered by different membrane proteins. In preliminary experiments (not shown) ghosts from which extrinsic membrane proteins had been selectively removed with 0.1 M NaOH could be completely solubilized by Triton treatment insufficient for complete solubilization of intact ghosts. Similar observations have been made by Yu et al. (1973), and it is tentatively concluded that intrinsic membrane proteins promote whereas extrinsic membrane proteins and cholesterol inhibit the solubilization of

phospholipids from bilayer membranes. (3) As expected, phospholipid extraction by Triton from ghosts and liposomes is determined more by the Triton/lipid ratio than by the Triton concentration (Figures 1 and 2). It should be noted that the lowest concentration of detergent used in this study was well above the critical micellar concentration of Triton determined by Robinson & Tanford (1975) and by Hertz & Barenholz (1977). With a Triton/lipid mole ratio of 25:1, for example, a comparable amount of phospholipid was obtained with 0.5% Triton (Figure 1, 47 and 73% from 1.7 $\mu$M liposome and ghost phospholipid, respectively) and with 1.5% Triton (Figure 2, 48 and 76% from liposomes and ghosts, respectively). The curves describing percent phospholipid (Figure 2) and percent cholesterol (Figure 3) solubilized vs. Triton concentration from red cell ghosts resemble those of Coleman et al. (1976) and those of Kirkpatrick et al. (1974). The former are displaced with respect to the latter, wherein a lower range of Triton/lipid ratios is represented by the same Triton concentrations as used here.

*Qualitative Aspects of the Solubilization by Triton of Red Cell Lipid Vesicles and Red Cell Ghosts.* The results of phospholipid head-group and fatty acyl chain analyses indicate two types of selective extraction of phospholipids by Triton X-100, one which is characteristic of both vesicles and ghosts and is considered protein independent and another which is characteristic of ghosts only and is considered protein dependent. The protein-independent effect consists of an inhibition of sphingomyelin extraction demonstrable with ghosts and liposomes at low Triton/lipid ratios, which cause little protein release from ghost membranes, and at intermediate Triton/lipid ratios, which completely remove intrinsic membrane proteins from red cell ghosts (Yu et al., 1973). At a high Triton/lipid ratio of 40:1, sphingomyelin is more readily solubilized by Triton so that no selective solubilization of phospholipid would be anticipated at sufficiently higher Triton/lipid ratios. The preferential, decreased extractability of 22:0, 24:0, and 24:1 fatty acyl chains from ghosts and liposomes at Triton/lipid ratios of 1:1–8:1 clearly corresponds with the decreased extractability of sphingomyelin, which is associated with 100% of the 24:0, 24:1, and 20:0 fatty acyl chains (Dodge & Phillips, 1967). For liposomes at 40:1 Triton/lipid, the not as decreased extractability of sphingomyelin is matched by a similarly not as decreased extractability of 22:0, 24:0, and 24:1, relative to data obtained at lower Triton levels. The decreased extractability of 16:0 could reflect a decreased extractability of sphingomyelin also, but other interpretations are plausible since 66.4% of the 16:0 resides in phospholipids other than sphingomyelin (Dodge & Phillips, 1967).

The resistance of sphingomyelin to solubilization by Triton reported here seems contrary to the demonstration that egg lecithin liposomes scatter less light and lose more solute at lower Triton/lipid ratios, as their content of sphingomyelin increases (Hertz & Barenholz, 1977). This apparent discrepancy may be resolved by the finding that cholesterol had the greatest affinity for sphingomyelin of the five different phospholipids subjected to differential scanning calorimetry (van Dijck, 1979). Cholesterol was present in the membranes in this study but not in that of Hertz & Barenholz (1977). The simultaneously decreased extractability of sphingomyelin and cholesterol, relative to that of other phospholipids (Figures 3 and 4), may reflect an interaction of sphingomyelin and cholesterol, in which form both lipids tend to avoid Triton micelles.

The protein-dependent effect on the solubilization of phospholipids by Triton is manifest as a preferential solubilization of phosphatidylserine, which is not obtained with ghosts at a non-protein-releasing ratio of Triton/lipid or with liposomes under any condition employed. That the preferential extractability of phosphatidylserine cannot be correlated with a preferential extractability of a particular fatty acyl chain(s) is not unexpected, since phosphatidylserine comprises 15% of the total red cell phospholipid and the more readily extractable phosphatidylserine was ~16% of that species. These findings are compatible with, if not supportive of, the copurification of band III, extracted by dodecyltrimethylammonium bromide, with phosphatidylcholine and neutral lipids (Ross & McConnell, 1978) and with the copurification of glycophorin, extracted by lithium diiodosalicylate, with phosphatidylinositol (Armitage et al., 1977; Buckley, 1978) and phosphatidylserine (van Zoelen et al., 1977; Buckley, 1978). The protein-dependent selectivity of phosphatidylserine solubilization, which was suggested by the reports of van Zoelen et al. (1977) and of Buckley (1978) and which has been definitely established here, implies that a fraction of this species is associated with intrinsic membrane protein(s) in ghost membranes not exposed to detergent. In contrast, bacteriorhodopsin (Wildenauer & Khorana, 1977), the $Ca^{2+}$-ATPase from sarcoplasmic reticulum (Moore et al., 1978), and acetylcholine receptor (Chang & Bock, 1979) are solubilized by detergents in association with lipids not differing in composition from the total membrane lipid. Either some proteins do not associate preferentially with certain lipids in situ or some preferential associations between lipids and proteins are not stable in the presence of certain detergents. Whether detergents other than Triton X-100 can be used to establish specific lipid–protein interactions in ghost membranes remains an important question.

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# Energy-Dependent Changes of the Electrokinetic Properties of Chloroplasts†

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ABSTRACT: Free-flow electrophoresis of spinach chloroplasts reveals that illumination causes a marked increase of net negative surface charge. The light stimulation is most pronounced in broken chloroplasts, missing the outer envelope membranes. Broken chloroplasts, prepared in the presence of $MgCl_2$, showed a much larger light stimulation (90–120%) than if $MgCl_2$ was omitted during preparation (40–50%). The effects of mono- and divalent cations on electrophoretic mobility and its stimulation by light are not simply explainable in terms of the Gouy–Chapman theory of the diffuse double layer. In addition to their charge-screening effect, these ions influence the membrane integrity and presumably thereby the energy-linked exposure of negative surface charges. The light stimulation is dependent on photosynthetic electron transfer and sensitive to both anionic and cationic uncouplers, to valinomycin, and to treatment with glutaraldehyde or a short heat shock. The calculated chloroplast charge densities are about one electronic charge per 3500 Å$^2$ in the dark and one electronic charge per 1700 Å$^2$ in the light. The electric potentials derived from the electrostatic interaction of cationic aminoacridine probes with chloroplasts behave qualitatively similar with respect to light stimulation and chemical or physical treatments as the electrokinetic properties, reported here. It is concluded that the light-induced increase of net negative surface charge of chloroplasts is the result of an energy-dependent conformational rearrangement of thylakoid membrane components.

Most biological membranes bear a net negative surface charge in the physiological pH domain (Gitler, 1971, 1972), including chloroplast membranes (Mercer et al., 1955; Dilley & Rothstein, 1967; Gross & Hess, 1974; Berg et al., 1974). The corresponding negative electric surface potential may be considered as an entity that controls the ionic composition in the diffuse double layer adjacent to the membrane surface and thereby intrinsic metabolic and structural membrane properties, including the translocation of ions (Gross & Prasher, 1974; Barber et al., 1977; Theuvenet & Borst-Pauwels, 1976a,b). There is clearly a mutual influence of membrane surface phenomena on the one hand and transmembrane phenomena on the other [see also Rumberg (1977)]. A number of surface-related phenomena, such as the electrostatic adsorption of dye molecules (Montal & Gitler, 1973; Kraayenhof & Arents, 1977; Searle et al., 1977) and uncouplers (Bakker et al., 1975), seem to be in good harmony with the theory of the diffuse double layer developed by Gouy & Chapman [see Davies & Rideal (1963); Aveyard & Haydon (1973)]. McLaughlin (1977) has lucidly reviewed those aspects of this theory and of electrostatic phenomena at mem-

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