SELECTIVE SOLUBILIZATION OF PROTEINS AND PHOSPHOLIPIDS FROM RED BLOOD CELL MEMBRANES BY NONIONIC DETERGENTS

John Yu, Donald A. Fischman and Theodore L. Steck

Departments of Biophysics, Biology, Anatomy, Medicine, and Biochemistry, University of Chicago, Chicago, Illinois 60637

(Received March 19, 1973)

Treatment of isolated human erythrocyte membranes with Triton X-100 at ionic strength $\simeq 0.04$ preferentially released all the glycerolipid and glycoprotein species. At low ionic strength, certain nonglycosylated polypeptides were also selectively solubilized. The liberated polypeptides were free of lipids, but some behaved as if associated into specific oligomeric complexes. Each detergent-insoluble ghost residue appeared by electron microscopy to be a filamentous reticulum with adherent lipoid sheets and vesicles. The residues contained most of the membrane sphingolipids and the nonglycosylated proteins. The polypeptide elution profile obtained with nonionic detergents is therefore nearly reciprocal to that previously seen with a variety of agents which perturb proteins. These data afford further evidence that the externally-oriented glycoproteins penetrate the membrane core where they are anchored hydrophobically, whereas the nonglycosylated polypeptides are, in general, bound by polar associations at the inner membrane surface. The filamentous meshwork of inner surface polypeptides may constitute a discrete, self-associated continuum which provides rather than derives structural support from the membrance.

INTRODUCTION

One approach to the elucidation of the molecular organization of membranes is to analyze the way in which selectively disruptive agents liberate various membrane constituents (1, 2). In the preceding paper (3), we showed that several different protein perturbing reagents eluted the same set of relatively polar polypeptides from the inner surface of the human erythrocyte membrane, while all of the more hydrophobic, externally-oriented glycoproteins were quantitatively bound to the lipid core. We have now attempted a complementary approach: disrupting the hydrophic continuum with mild nonionic detergents without disturbing polar protein-protein and protein-lipid associations. By this technique, the glycoproteins were preferentially solubilized along with the glycerolipids, while cytoplasmic surface polypeptides remained together in a distinctive filamentous meshwork associated with sphingolipid-rich segments.

METHODS

Materials

Triton X–100 and saponin were obtained from Sigma, Lubrol WX from General

Journal of Supramolecular Structure

Biochemicals (Chagrin Falls, Ohio), Ammonyx LO from Onyx Chemical Co. (Jersey City, N. J.). Nonidet P–40 from Shell, Brij 35 and Brij 58 from Pierce Chemical Co., and Tween 40 and Tween 60 from Nutritional Biochemical Corp. (Cleveland, Ohio). Other reagents were as listed in the accompanying report (3).

Elution Reactions

Hemoglobin-free human erythrocyte membranes (ghosts) were prepared according to Fairbanks et al., (4) from fresh or out-dated bank blood in either 5 mM Na phosphate, pH 8.0, or 56 mM Na borate, pH 8.0. One volume of packed ghosts was incubated with 5 or 7 volumes of detergent solution with time, temperature, and buffer specified. The suspensions were then centrifuged at 15,000 rpm for 30 min in a Sorvall SS–34 rotor. The supernatant fractions were recentrifuged as above while the pellets were resuspended directly for analysis.

Analytical Procedures

Electrophoresis in 5.0% polyacrylamide gels containing 0.2% sodium dodecylsulfate was executed as previously detailed (3, 4). Each gel was loaded with protein derived from 10 μl of packed ghosts (34–40 μg protein, for unextracted membranes). The following assays were performed as described in the accompanying report (3): protein, sialic acid, neutral sugars, and phospholipid phosphorus. Triton X–100 introduces two types of interference with the phosphorus determination: contamination by traces of inorganic phosphorus and spurious phosphomolybdate complex color formation. Organic solvent extraction and ashing (3) eliminate these artifacts.

Two -dimensional thin layer chromatography of extracted phospholipids was performed on 250μ thick Silica gel H plates from Analtech, Inc. (Newark, Delaware) according to Broekhuyse (5). Spots visualized with I_2 vapor were scraped into digestion tubes and incubated with 10 N H_2SO_4 at 180°C for 10 min. Four drops of 30% H_2O_2 were introduced into the bottom of each tube followed by 20 min more incubation. Phosphorus was then assayed by a modification of the Fiske-Subbarow method (6). Cholesterol assays were performed as described by Leffler and McDougald (7).

Sucrose gradient densities were estimated by the measurement of refractive index.

Electron Microscopy

Specimens for transmission electron microscopy were examined both by negative staining and thin sectioning techniques with an AEI EM6B electron microscope calibrated with a diffraction grating (54, 864 lines per inch). One volume of packed ghosts was incubated for 15 min on ice in 7 volumes of 5 mM Na phosphate (pH 8.0) with or without Triton X–100. For negative staining, aliquots were placed on carbon-coated, celloidin films on 400-mesh copper grids, and immediately stained with aqueous 1% uranyl acetate. Although not presented here, parallel specimens were fixed on the grid with 2.5% glutaraldehyde before negative staining. This fixation caused some alteration of the image, but did not affect our conclusions.

For thin section analysis, control and extracted ghosts were pelleted and fixed directly in 2.5% glutaraldehyde (in 0.1 M Na cacodylate buffer, pH 7.4). Alternatively, samples were embedded in 0.4% agarose prior to glutaraldehyde fixation in an attempt to protect the delicate structure of the Triton-extracted ghost residues. After primary fixation, the samples were washed in 0.1 M Na cacodylate (pH 7.4) and post-fixed in 1% OsO_4 in

the same buffer. Before dehydration in ethanol, the fixed material was stained en bloc with 0.5% uranyl acetate and then embedded in Araldite. Thin sections were examined on uncoated grids after staining with uranyl acetate and lead citrate (8).

RESULTS

Detergents and conditions were sought which would partially disassemble the membrane. We failed to satisfy these criteria using sodium dodecylsulfate. Brij 35, Brij 58, saponin, Tween 40, and Tween 60; that is, little or no differential release of components was observed. Selective solubilization was achieved, however, with another group of surfactants, best represented by Triton X–100.

Ghosts suspended in cold 0.1–1.0% (v/v) Triton X–100 in 56 mM Na borate, pH 8, $\mu \simeq 0.008$, immediately clarified. Centrifugation at 15,000 rpm for 30 min brought down a translucent, gelatinous pellet, barely visible until the supernatant was removed. The diminished turbidity was not caused by an increased refractive index in the surfactant solution, since washing the pellet in plain buffer did not alter its lucency.

In the phase contrast microscope, the Triton-treated membranes appeared as profiles somewhat smaller than ghosts, but so indistinct as to be nearly invisible. This phenomenon was previously observed by Heller and Hanahan (9). Performing the Triton extraction at higher ionic strength (e.g., 0.04) increased somewhat the turbidity of the suspensions and the microscopic visibility of the residues. Dark field microscopy did not resolve these bodies, in sharp contrast to the bright profiles of unextracted ghosts.

Chemical Composition of the Triton X–100 Extract and Residue

Extraction of ghosts with 0.5% Triton X–100 in ice-cold borate buffer led to the release of more than half of the membrane protein and phospholipid and almost all of the sialic acid (Table I). Significant neutral sugar was found in both the supernatant and pelleted fractions, but precise chemical determination was frustrated by the interference of the surfactant. The distribution of cholesterol roughly paralleled that of the lipid phosphorus.

Table I. Chemical Composition of the Triton X–100 Extract and Residue

Packed ghosts were incubated in five volumes of 0.5% Triton X–100 in 56 mM Na borate (pH 8.0) for 20 min on ice before centrifugation and analysis. Data (expressed per milliliter of packed ghost input) are averages of triplicate determinations from a representative experiment.

Component	Ghosts	Extract	Residue
Protein (mg)	3.43	1.88	1.53
(%)	(100)	(54.8)	(44.6)
Lipid P (μg)	64.8	42.4	27.8
(%)	(100)	(65.4)	(42.9)
μg lipid P/mg protein	18.9	22.6	18.2
Sialic acid (nmoles)	473	446	50.5
(%)	(100)	(94.4)	(10.7)
nmoles sialic acid/ (mg protein)	138	237	33.0

The phospholipid classes were not equally distributed between the Triton extract and residue (Table II). The supernatant fraction contained all the recovered lysolecithin and phosphatidic acid, 66% of the phosphatidylcholine, 73% of the phosphatidylethanolamine, and 91% of the phosphatidylserine. The residue contained 83% of the recovered sphingomyelin. A preliminary analysis of the glycosphingolipids (generously performed by Dr. Glyn Dawson) indicated that at least the major component, globoside, was also predominately retained in the detergent residue.

Table II. Phospholipid Composition of the Triton X–100 Extract and Residue

Conditions are as described in Table I. Recovery of lipid P following thin layer chromatography was 89–98%. Values are in micrograms lipid P.

Component	Ghosts	Extract	Residue
Lipid P, input	68.0	35.6	28.1
(%)	(100)	(52.4)	(41.3)
Phosphatidic acid	0.50	0.50	0
Lysophosphatidylcholine	0.53	0.64	0
Phosphatidylcholine	16.0	11.3	5.93
Phosphatidylethanolamine	13.2	10.7	4.04
Phosphatidylserine	10.0	8.44	0.84
Sphingomyelin	16.4	2.61	12.8

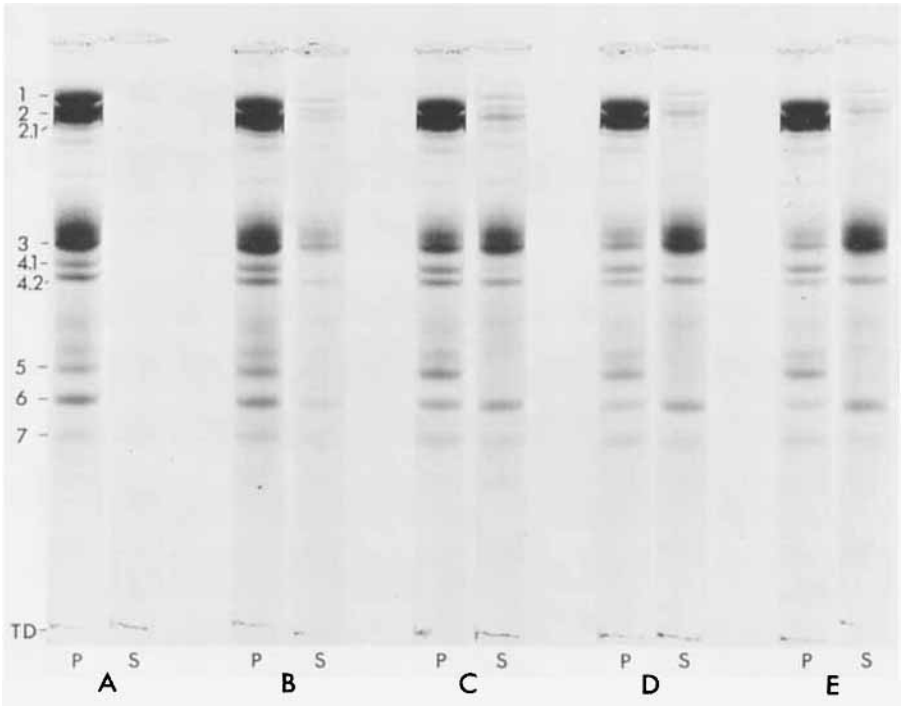


Fig. 1. The release of membrane polypeptides by Triton X–100 at low ionic strength. One volume of packed ghosts was mixed with 5 volumes of detergent in 56 mM Na borate, pH 8.0 (ionic strength $\simeq$ 0.008) and incubated on ice for 20 min. After centrifugation, aliquots of pellet (P) and supernatant (S) fractions derived from 10 μl of ghosts were electrophoresed and the gels stained with Coomassie blue. Polypeptide bands are enumerated as in (3); TD = tracking dye. Triton X–100 concentrations (%, v/v): (A) 0.01; (B) 0.05; (C) 0.10; (D) 0.50; (E) 1.0.

We tested whether the distinctive distribution of phospholipids reflected a reassociation of solubilized lipids secondary to detergent action. ^{32}P–labeled rat liver phospholipids (containing the same major classes listed in Table II) were dissolved in 0.5% Triton X–100 solution which was then used to extract the erythrocyte membranes. Less than 5% of the added radioactivity was recovered in the pellets, signifying that free phospholipids neither exchanged with the bound lipids nor deposited on the ghost residues.

Polypeptide Components of the Triton X–100 Extract and Residue

Figure 1 demonstrates that at low ionic strength polypeptide elution commences near 0.05% Triton X–100 and is optimal at approximately 0.5%. Of the Coomassie blue-stained components, band **3** is the most efficiently released, followed by bands **6, 4.2, 2.1,** and **7,** in that order. Bands **1, 2, 4.1,** and **5,** as well as several undesignated minor components, are retained in the pellet.

The very heavily glycosylated polypeptides are not stained by Coomassie blue but are made visible by periodic acid-Schiff reagent (4). Figure 2 shows that 0.05% Triton X–100 releases most of each principal glycoprotein peak and that at 0.5% detergent their elution is quantitative. Since human erythrocyte membrane sialic acid is associated

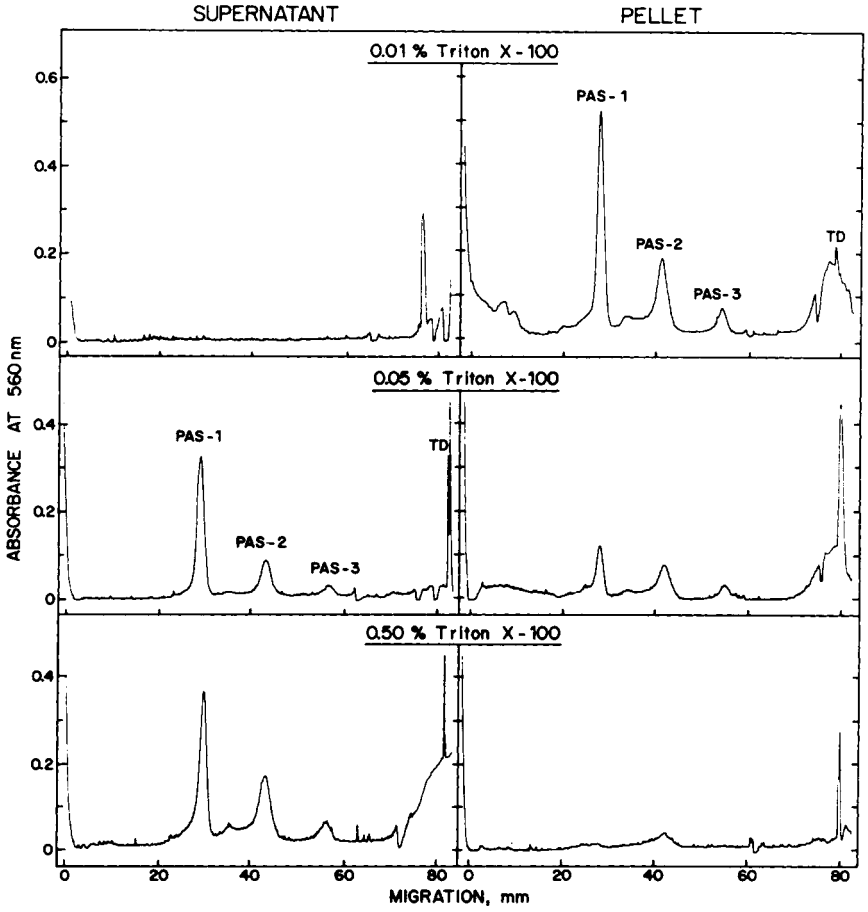


Fig. 2. The release of membrane glycoproteins by Triton X–100. Gels containing samples identical to those in Fig. 1 were stained with periodic acid-Schiff reagent and scanned at 560 nm (3). PAS 1–3 are the principal glycoprotein peaks; the unlabeled peak just behind the tracking dye (TD) corresponds to membrane lipids (4).

with the glycoproteins and not the glycosphingolipids (cf, 4, 10), these results are consistent with the preferential release of sialic acid and retention of glycolipid discussed above.

Selective elution was favored by low temperature; at 37°, the detergent tended to disperse the membranes completely. The release of proteins diminished somewhat between pH 8 and 6, and was markedly inhibited at pH 5. The selectivity of polypeptide elution could be further enhanced by increasing the ionic strength of the Triton solution (compare Figs. 1 and 3). In 25 mM NaCl – 5 mM Na phosphate, pH 8.0 (ionic strength ≃ 0.037), Triton X–100 eluted band **3** preferentially but incompletely; however, only a trace of bands **4.2** and **6** and none of bands **2.1**, **7** and the other Coomassie blue-stained membrane polypeptides were released. (The trace of hemoglobin in these ghosts, band H, was nonspecifically released, even in the absence of detergent.) The selective liberation of glycerolipids and PAS-stained glycoproteins was not diminshed at ionic strength ≃ 0.04. The presence of 10 mM $MgSO_4$ or 5 mM $CaCl_2$ had the same effect as 25–50 mM NaCl. Since band **3** is glycosylated (11, 12), Triton X–100 in the presence of these salts can be said to preferentially elute all of the known glycoprotein species and no others.

Selective Polypeptide Extraction with Other Detergents

The action of Nonidet P-40 was the same as that found for Triton X–100, as expected from their nearly identical chemical structures. Lubrol WX, a polyoxyethylene cetyl alcohol, was less potent than these two polyoxyethylene alkylphenol surfactants, but it

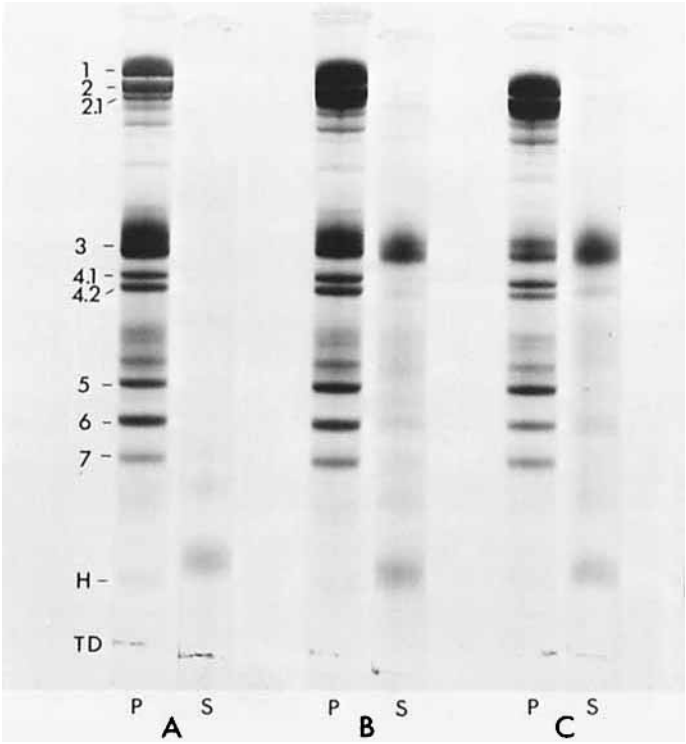


Fig. 3. The release of membrane polypeptides by Triton X–100 at moderate ionic strength. One volume of packed ghosts in 5 mM Na phosphate (pH 8) was mixed with 7 volumes of detergent in 5 mM Na phosphate (pH 8) – 25 mM NaCl (final ionic strength ≃ 0.037) and incubated on ice for 20 min. After centrifugation, aliquots of pellet (P) and supernatant (S) fractions derived from 10 μl of ghosts were electrophoresed and the gels stained with Coomassie blue. H represents the polypeptide chains of residual hemoglobin. Triton X–100 concentration (%, v/v): (A) 0; (B) 0.1; (C) 1.0.

effected the same selective release of polypeptides. At pH 8, Ammonyx LO (dimethyldodecylamine oxide) was nearly 10 times more potent than Triton X–100. For example, 0.075–0.1% Ammonyx produced the same differential elution of polypeptides shown in Fig. 1D and E, while 0.25% caused total dissolution of the membrane. The efficacy of this surfactant declined markedly with decreasing pH, so that only band **6** was eluted at pH 6. Protonation of this detergent below neutrality presumably raises its critical micelle concentration and decreases its potency (cf. 13).

Further Characterization of the Triton X–100 Residue

In order to ascertain whether the sedimentable lipids and proteins were associated with one another, we centrifuged the residues on sucrose density gradients in detergent (Fig. 4). A significant amount of protein and phospholipid was released from the residues and remained at the top of the gradient. The remaining particles formed a zone between density 1.14 and 1.20 gm/ml, the breadth of which appeared to reflect variation in the lipid/protein ratio. Table III demonstrates that the soluble (top zone) fraction contained the most readily elutable species (namely, bands **3, 4.2,** and **6,** plus glycerolipids). Conversely, the sedimentable zone was rich in the constituents preferentially bound in the original Triton residues (i.e., sphingomyelin and polypeptides **1, 2, 4.1,** and **5**). This experiment indicates that the lipid and protein species selectively retained in Triton residues are intimately associated with one another, while the remaining traces of the

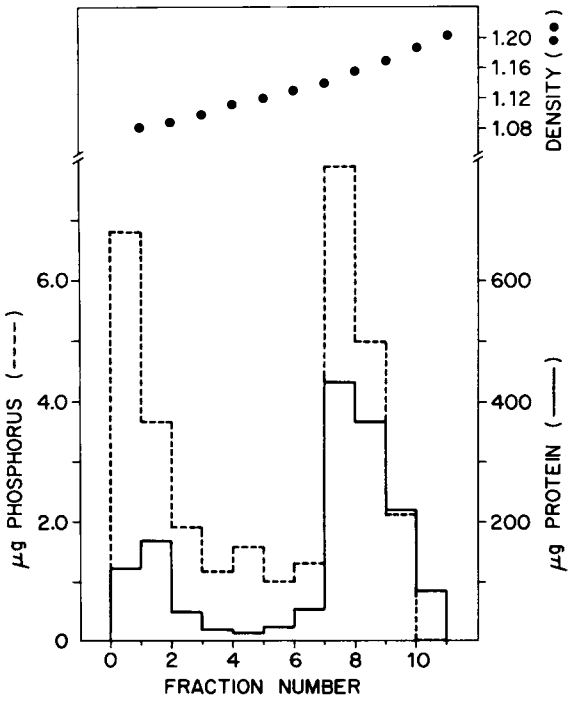


Fig. 4. Sucrose density gradient centrifugation of Triton X–100 residues. One volume of packed ghosts was mixed with five volumes of 0.5% Triton X–100 in 56 mM Na borate, pH 8, and incubated on ice for 20 min. After centrifugation, the pellet was resuspended in 0.42% Triton in 56 mM Na borate, pH 8, and layered on a 20–55% (w/v) sucrose gradient in the same buffered detergent. After centrifugation at 40,000 rpm for four hours in a Spinco SW 41 rotor, 1 ml fractions were collected and dialyzed free of sucrose before analysis.

Table III. Sucrose Density Gradient Fractionation of the Triton X–100 Residue

Light (Fractions 1–3) and dense (Fractions 6–11) zones and the starting Triton residue from the sucrose density gradient experiment shown in Fig. 4 were analyzed as described in Methods.

Component	Input	Light Zone	Dense Zone
Principal polypeptides detected by gel electrophoresis	Major: **1, 2, 4.1, 5** Trace: **3, 4.2, 6**	**3, 4.2, 6**	**1, 2, 4.1, 5**
μg lipid P/mg protein	18.9	38.4	13.6
Relative % of lipid P in			
Phosphatidylcholine	24.5	51.6	15.1
Phosphatidylethanolamine	16.8	20.9	8.2
Phosphatidylserine	3.7	14.0	0
Sphingomyelin	54.8	12.1	75.0

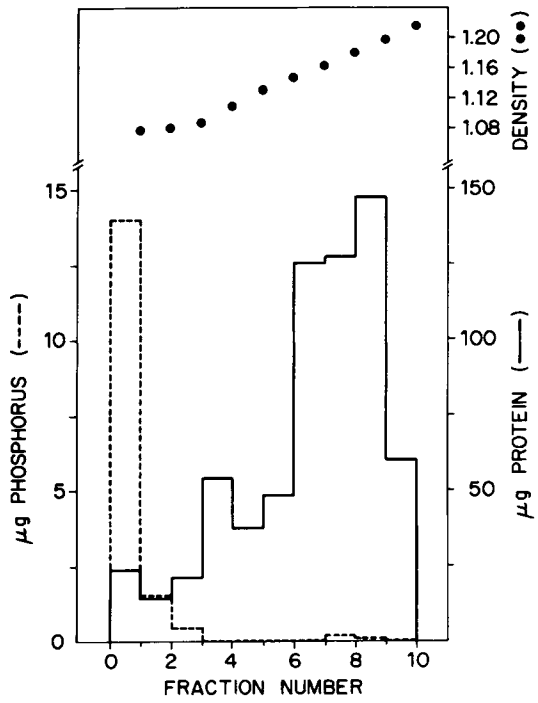


Fig. 5. Sucrose density gradient centrifugation of the Triton X–100 extract. One volume of packed ghosts was mixed with 5 volumes of 0.5% Triton X–100 in 56 mM Na borate, pH 8, and incubated on ice for 20 min. After centrifugation, the supernatant extract was concentrated approximately 10-fold by ultrafiltration (Diaflo PM–10, Amicon Corp.) and loaded on a 20–55% (w/v) sucrose gradient containing 0.42% Triton X–100, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 56 mM Na borate, pH 8. After centrifugation for 72 hours at 50,000 rpm in a Spinco SW 50.1 rotor, 0.5 ml fractions were collected and dialyzed free of sucrose before analysis.

readily elutable components continue to be released, causing further intensification of the original partition pattern.

Further Characterization of the Triton X–100 Extract

The proteins and phospholipids in Triton extracts were well separated by sucrose density gradient centrifugation (Fig. 5) and ion-exchange chromatography (not shown). While these experiments indicate that the detergent dissociates the solubilized lipids and proteins as it elutes them, small amounts of lipid-protein binding cannot be excluded. A similar action of Triton X–100 on sarcoplasmic reticulum vesicles has been reported (14).

The individual polypeptides exhibited characteristic sedimentation velocities. Rate zonal centrifugation of detergent extracts on 5–20% sucrose gradients in 0.42% Triton X–100 gave an apparent sedimentation coefficient of approximately 3.5 for PAS-1, indicating that this 50–55,000 dalton sialoglycoprotein (15) was not highly aggregated in Triton X–100, as it is in aqueous buffers (16). In agreement with McFarland and Inesi (14), we found that Triton X–100 does not markedly inactivate, dissociate, or even alter the sedimentation velocity of reference oligomeric enzymes (such as beef liver catalase and yeast alcohol dehydrogenase) nor does it greatly reduce the activity of the solubilized membrane-bound enzymes, acetylcholinesterase, glyceraldehyde 3-phosphate dehydrogenase, and NADH: ferricyanide diaphorase. It is therefore unlikely that PAS-1 is a larger multimer in the membrane which is dissociated by detergent solubilization (cf. also ref. 17).

Bands **3, 4.2,** and **6** migrated as broad overlapping zones of 7–12 S. These sedimentation coefficients are greater than expected for monomeric polypeptide chains of 88,000, 72,000, and 36,000 daltons, respectively. They are consistent with cross-linking data which indicates that band **3** is a dimer and **4.2** is a tetramer both in the membrane and in Triton solution (17) and with the identification of band **6** as tetrameric glyceraldehyde 3-phosphate dehydrogenase (18).

We find no evidence of large nonspecific aggregates in fresh Triton X–100 extracts; even the traces of residue components **1, 2, 4.1, 7,** etc., in the eluates migrate as discrete, soluble species. However, gross aggregation can be provoked by detergent removal, slow (i.e., concentrative) freezing to -20°, prolonged standing at 5°, and a reduction in pH below 5.5 (see also ref. 14).

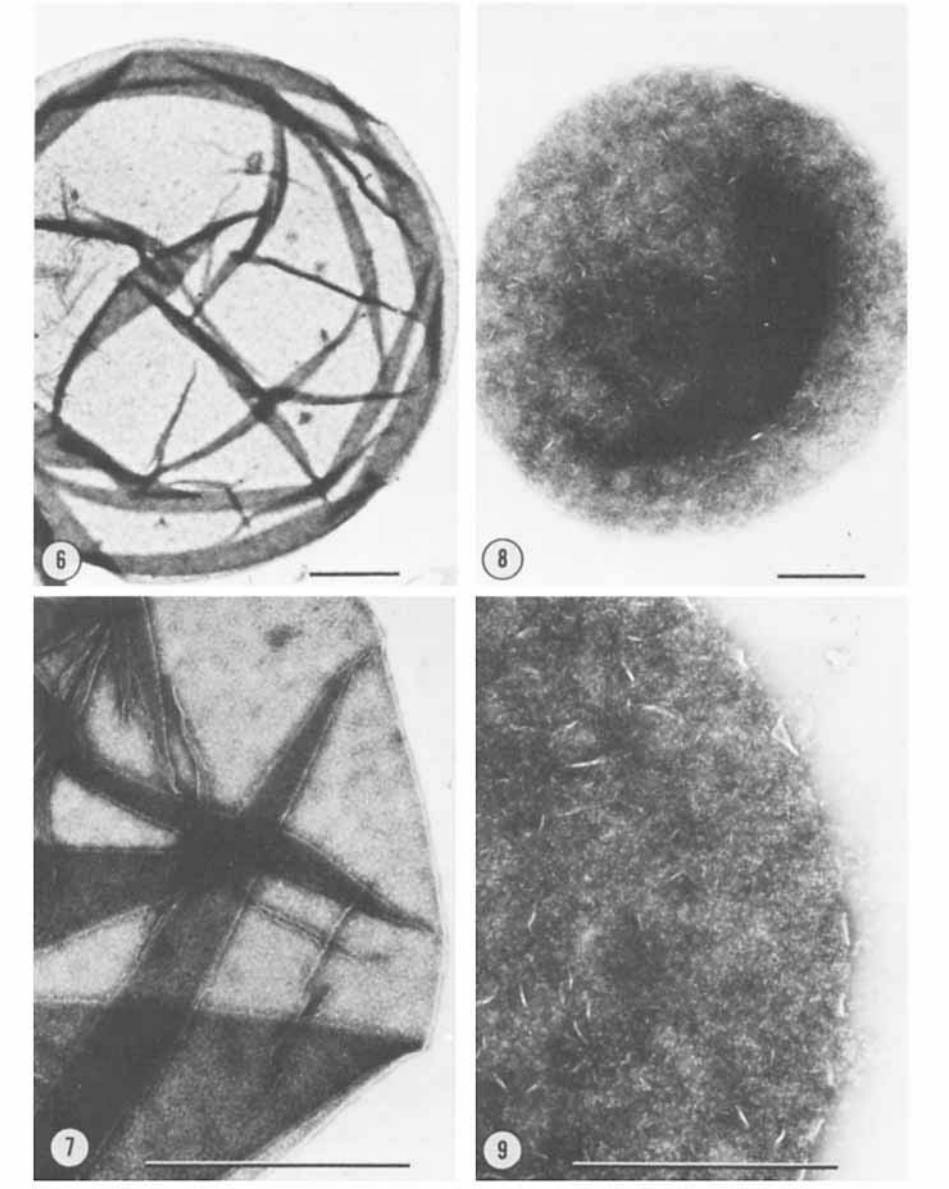
Electron Microscopy of Triton X–100 Residues

Triton-extracted membranes are fragile, being disrupted by shearing and aggregated by pelleting. However, by applying negative stain to fresh, uncentrifuged control and Triton-treated membranes, it can be demonstrated that the sedimentable structures are direct derivatives of ghosts and not merely aggregates of dispersed lipid and protein (Figs. 6–9). At high magnification, and particularly in glutaraldehyde-fixed preparations (not shown), we see that the smooth surface and sharp edge of the normal ghost membrane (Fig. 7) has been replaced by a dense reticulum of fine filamentous material associated with large lucent segments reminiscent of lipid figures (Fig. 9).

Thin section electron micrographs of unextracted membrane pellets showed characteristic, compacted ghost profiles (Fig. 10) with diffuse "fuzzy" material adherent to their inner surfaces (Fig. 11). Triton-extracted pellets prepared in the same fashion gave the appearance of aggregated masses of filaments surrounding more densely staining (presumably lipid) vesicles and fragments (Fig. 12). We attributed this loss of ghost-like morphology to the rigors of conventional fixation and imbedding techniques; we there-

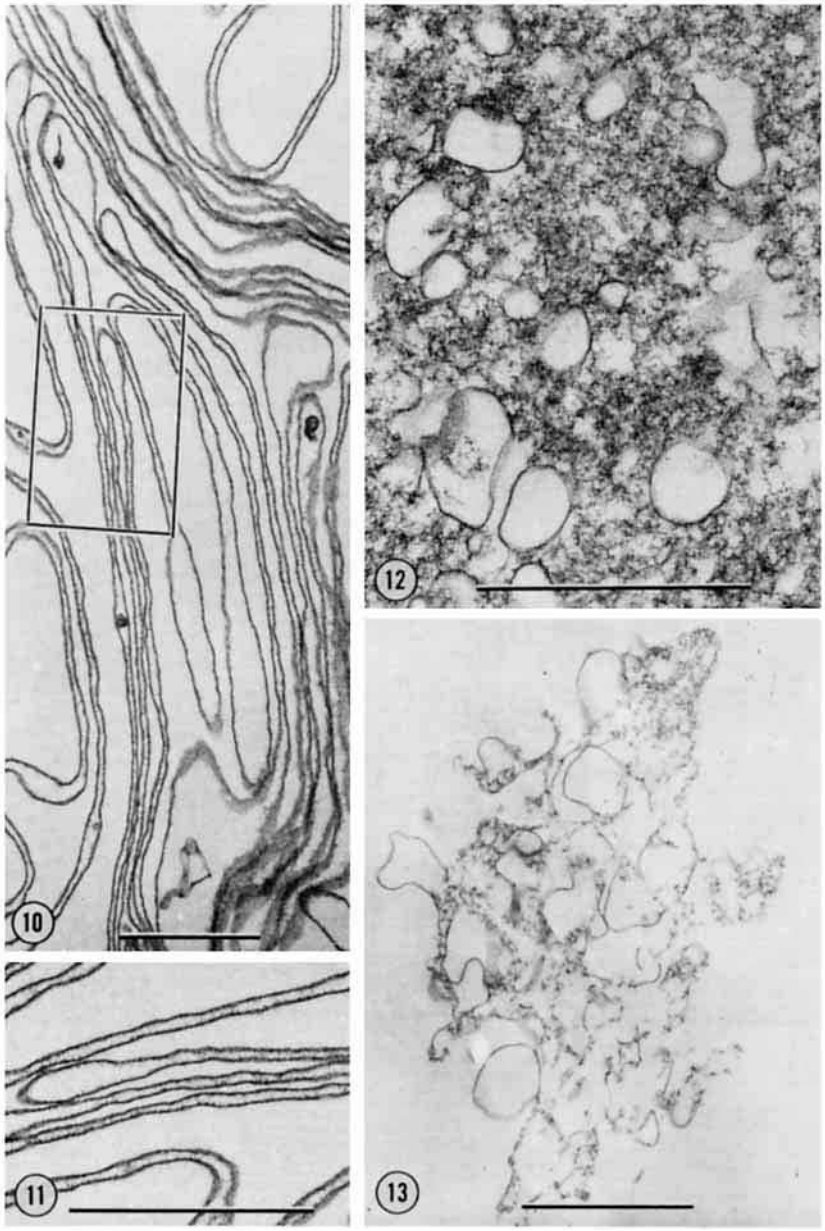
fore attempted to stabilize the residues by imbedding the Triton-extracted pellets in agarose prior to fixation. Thin sections of such agarose blocks (Fig. 13) indicated that the residues, though distorted, retained the over-all size and shape of discrete ghosts.

As seen in Fig. 13, the lipid figures in the detergent-treated ghosts are discontinuous, suggesting that the cohesive element in the residues is a continuum of protein filaments.



Figs. 6–9. Electron micrographs of negatively stained control and 0.5% Triton X–100 extracted ghosts. Figs. 6 and 7, control ghosts; figs 8 and 9, extracted ghosts. Calibration bar is 1 micron throughout. See Methods section for experimental details.

Along the periphery, large lipid segments seem to have pulled away from the underlying matrix. Closed vesicles are also seen. With this image in mind, Fig. 12 can be inferred to represent a disrupted and condensed form of ghost residues. These interpretations also apply to the negatively-stained preparations, which show discontinuous lipid figures adherent to a filamentous matrix (Fig. 9).



Figs. 10–13. Electron micrographs of thin-sectioned control and 0.5% Triton X–100 extracted ghosts. In Figs. 10–12, the preparations were pelleted and resuspended in glutaraldehyde. In Fig. 13, the Triton-extracted residue was imbedded in 0.4% agarose (final) prior to glutaraldehyde fixation. Figs. 10–11, control ghosts, with Fig. 11 being a higher magnification of the boxed area in Fig. 10. Figures 12 and 13, Triton extracted. Calibration bar is 1 micron throughout. See Methods section for experimental details.

The electron microscopic images suggest that the diminished turbidity of the ghost residues is not the result of a reduction in their over-all size. The solubilization of approximately half of the membrane mass and the reduction of the size of the "light scattering elements" to that of filaments and lipid segments might cause this striking effect.

DISCUSSION

We have defined conditions under which certain nonionic detergents can partially disassemble the isolated human red cell membrane into distinctive soluble and insoluble fractions. Previously, Miller (19) reported that Triton X–100 and Lubrol WX completely solubilized this membrane. Since his intent was total rather than selective solubilization, Miller may have favored a higher (unspecified) incubation temperature, vigorous mixing, or other variables which disrupt the fragile residues we sought to preserve. In both our experience and his, solubilization was reduced by increasing ionic strength (especially with divalent cations) and by decreasing pH. In neither study was acetylcholinesterase inactivated.

Our results are in accord with numerous studies using nonionic detergents, particularly polyoxyethylene alkylphenols like Triton X–100, to solubilize membrane-bound enzymes (9, 14, 19–25), antigens (26, 27), and receptors (28–30) in a biologically active (nondenatured) form. Like bile salts (2, 31, 32, 33), nonionic detergents often show interesting and useful selectivity in their action (34–37). As in the present case, true solubilization (rather than fine fragmentation) can occur, with proteins and lipids being well separated (14, 25). The loss of biological activity sometimes observed in such extracts may relate to delipidation, rather than to denaturation, and may be reversed by proper reconstitution of lipid-protein associations (cf. 2, 23, 24, 33).

The polypeptides preferentially released from the erythrocyte membrane by Triton X–100 at ionic strength $\simeq$ 0.04 differ from the residue species in three respects. First, the elutable class contains all the glycoproteins (principally bands **3** and PAS 1–3) and no others. Secondly, it appears that this group uniquely populates the external membrane surface, and that at least polypeptides **3** and PAS–1 extend asymmetrically across the membrane thickness (38, 39, 40). Conversely, the polypeptides in the residue (primarily bands **1, 2, 2.1, 4.1, 4.2, 5, 6,** and **7**) all appear to lack sugar and to be confined to the inner (cytoplasmic) face of the membrane (cf. 3 and 38).

Finally, the effect of Triton X–100 at $\mu \simeq 0.04$ is nearly the reciprocal of that described for several protein perturbants in our previous report (3). All of the aforementioned inner surface components (except band **7**) were quantitatively eluted from the membrane by such agents as 0.1 N NaOH, while the outer-directed glycoproteins remained bound to the lipid core.

The nearly diametrical action of the nonionic detergents and the protein perturbants can be rationalized by assuming that there are, in general, two modes of association of these major proteins with the membrane. The glycoproteins penetrate into the membrane to make apolar associations with its lipid center. This bonding is not readily disturbed by protein perturbants but would be particularly susceptible to nonionic detergents. Conversely, the nonglycosylated proteins are seen as being linked through specific "polar" (e.g., electrostatic or hydrogen) bonding to other proteins and/or lipid head groups at the cytoplasmic membrane face, hence their elution by protein perturbants and their resistance to the nondenaturing detergents.

Band **7** is an exception to these generalizations; it is not readily eluted by either class of agent. However, the preservation of lipid bilayer morphology in the detergent residues (Figs. 12 and 13) indicates that not all apolar associations have been disrupted by the Triton X–100. If band **7** were hydrophobically bound to the sphingomyelin persisting in the residue, it would survive both types of selective agents. Alternatively, if band **7** made two different types of association with the membrane, it might remain bound following disruption of either linkage.

Bands **4.2** and **6** constitute another exception: they are preferentially liberated both by protein perturbants (3) and by nonionic detergents at low ionic strength (Fig. 1). Preliminary evidence favors the premise that these polypeptides are bound in situ to band **3**. They would then be released by protein perturbants which disrupt protein-protein associations, but also by nonionic detergents which elute their binding site, band **3**. Persistent association of bands **4.2** and **6** with band **3** in Triton would contribute to the large sedimentation coefficients observed for these species and to their co-migration in certain purification protocols (J. Yu and T. L. Steck, unpublished data). We presume that as ionic strength is increased these complexes of band **3** are preferentially retained in the residue, while uncomplexed **3** remains elutable (compare Figs. 1 and 3). The mole fraction of band **3** dimers which would be complexed 1:1 with band **4.2** and **6** tetramers (17) can be estimated by quantitative densitometry (4) to be approximately 30%.

Extracting red cell membranes with Triton X–100 leaves an insoluble filamentous matrix of ghost dimensions (Figs. 6–13). Sheet-like proteinaceous residues have also been observed following selective extraction by deoxycholate of *Micrococcus lysodeikticus* (31) and mitochondrial (32) membranes. The lipid figures observed here do not appear to stabilize the over-all residue structure, but rather to be imbedded therein. The residue meshwork is probably related to the fibrillar patches seen at the cytoplasmic surface of ghost membranes (11, 41, 42, and Fig. 11). This material, originally called spectrin (41), may correspond to our electrophoretic bands **1, 2,** and **5** (1, 4, 11, 43, and 44). These polypeptides are recovered exclusively in the Triton residues, to which they contribute at least half of the protein. If these three components are selectively eluted from the ghost membranes by treatment with 0.1 mM EDTA (4) prior to the addition of Triton X–100, little or no insoluble residue is obtained. Water soluble preparations of spectrin can be reaggregated to form structures resembling the inner surface fibrillar arrays (41, 42). Guidotti (11, 44) has recently suggested that bands **1** and **2** are myosin analogues, that band **5** relates to actin, and that a complex of these polypeptides might perform some actomyosin-like role in this membrane. Filamentous material has been observed at the inner surface of other plasma membranes (cf. 11, 45), and submembrane protein layers may also occur on intracellular (46) and viral (47) membranes.

Bands **1, 2,** and **5** may therefore comprise a submembrane continuum whose integrity depends on self-association rather than membrane binding. Such a configuration is compatible with a "skeletal" role for the filamentous meshwork.

The other cytoplasmic surface polypeptides, namely, bands **2.1, 4.1, 4.2, 6,** and **7,** can not be bound only to bands **1, 2,** and **5,** since the latter three can be selectively removed from the membrane without releasing any of the former components (4). In addition to the possible modes of binding of bands **4.2, 6,** and **7** entertained above, the other polypeptides might form direct polar associations with inner surface lipids retained in the residues.

The partition of lipid into Triton-soluble and insoluble fractions is distinctive: splingolipids

were retained over glycerolipids in the residues, and lipids lacking a net charge (lecithin, sphingomyelin, and glycolipids) remained bound compared to the anionic classes, phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid. Earlier studies using organic solvents to extract freeze-dried erythroctye membranes (48, 49) established that cephalins were more weakly bound than lecithin or sphingomyelin, in agreement with the present report (Tables II and III). Recently, Kramer et al. (50) observed a preferential binding of sphingomyelin to solubilized sheep red cell membrane proteins upon recombination out of 2-chloroethanol solutions. Widnell and Unkeless (22) have also reported that the 5′-nucleotidase isolated from rat liver membranes in Triton X–100 was associated with a single phospholipid, sphingomyelin.

The sheet-like and vesicular profiles in the Triton residues (Figs. 12 and 13) demonstrate that the retained lipids are not merely bound to protein but form apolar associations with one another which withstand detergent action. It is relevant that naturally occurring sphingomyelin and glycosphingolipids have considerably higher melting temperatures than the glycerolipids (51, 52). This stability could relate to the abundance of saturated and very long hydrocarbon chains in the sphingolipids of human erythrocytes compared to the shorter, more unsaturated fatty acids in the glycerolipids (53).

Recent physical studies of mixed phospholipid (54, 55) and cholesterol-phospholipid (56) systems have advanced the concept of clustering of disparate lipid species in membranes. One might speculate on whether the sphingolipid segments observed in the Triton X–100 residues reflect such clusters, and whether these aggregates arise by demixing following detergent action or exist in some form in the original ghost.

The sustained association of sphingolipid patches with the filamentous meshwork might signify sites of polar protein-lipid bonding at the inner surface of the membrane. The glycolipids retained in the sphingolipid domains should be confined to the side of the bilayer segment originally oriented toward the extracellular space (38). This point is worth testing since it would demonstrate that specific lipid interactions across the bilayer are conserved in the detergent residue. It would also be worthwhile pursuing the hypothesis that, since the glycoproteins and glycerolipids are preferentially eluted together, they might be specifically associated in situ.

We cannot now exclude the possibility that the patterns of association described in this and the companion study (3) are in part or wholly artifacts resulting from reagent action; however, the weight of the indirect evidence discussed above indicates otherwise. These selective elutions at least offer gentle and facile means of solubilization and partial purification of membrane polypeptides and a conceptual framework for further study of this and other membrane systems.

ACKNOWLEDGMENTS

The excellent technical assistance of Mrs. Benita Ramos and Mrs. Rita Gramas is gratefully acknowledged. We also thank Dr. P. Zahler for furnishing us a copy of his manuscript (ref. 50) prior to publication.

This study was aided by grant No. BC–95A from the American Cancer Society, U.S.P.H.S. Training Grant No. GM–780, U.S.P.H.S. Research Grant #HL–13505–02, U.S.P.H.S. contract #HL–4368133, and a fellowship (to T. L. S.) from the Schweppe Foundation.

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