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DETERGENT EXTRACTION OF ERYTHROCYTE GHOSTS

COMPARISON OF RESIDUES AFTER CHOLATE AND TRITON X-100 TREATMENTS

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SUMMARY

1. Human erythrocyte ghosts were extracted with individual free and conjugated bile salts and, for comparison, with Triton X-100 under conditions approximating to physiological temperature, pH and tonicity.

2. Treatment with cholate, glycocholate, taurocholate, or with Triton X-100 gave lipid-depleted residues. These could still be seen as ghost-like profiles by phase contrast microscopy. Deoxycholate brought about complete membrane dissolution.

3. The cholate residue gave a trilamellar image by electron microscopy and in condensed form gave a smaller membrane repeat than untreated membranes. It had a polypeptide composition representing mainly integral proteins.

4. The Triton X-100 residue had a granular profile in the electron microscope and a polypeptide composition largely representing peripheral proteins.

INTRODUCTION

The aim of much of the previous work on detergent-solubilization of membranes has been to obtain membrane proteins in "soluble" form, preferably with retention of biological activities (see refs. 1 and 2 for reviews). However, the nature of the residual material has been largely ignored, probably one of the most notable exceptions being the effects of Triton X-100 upon erythrocyte ghosts [11, 12].

We wish here to focus attention on the interesting nature of the residues remaining after extensive treatment of erythrocyte membranes with the trihydroxy bile acid, cholic acid, and its conjugates. Our interest in these detergents is stimulated by the fact that the liver secretes large quantities of these and other bile salts without apparent gross damage to its membranes. We have therefore chosen to study their effects upon erythrocyte membranes under conditions approximating to physiological. Since solubilization studies with other detergents have usually been carried out at 0 °C or at room temperature, we have also found it necessary to study the effects of Triton X-100 and deoxycholic acid at 37 °C to provide controls (and comparisons) with cholic acid.

MATERIALS AND METHODS

Cholate and deoxycholate were obtained from Sigma Chemical Co., London, U.K., glycocholate and taurocholate from Calbiochem. Ltd., Hereford, U.K., and Triton X-100 from Rohm and Haas Ltd., Croydon, U.K. Erythrocyte ghosts were prepared in 40 imosM bicarbonate buffer pH 7.4 containing 1 mM EDTA; such ghosts are haemoglobin-free and largely unsealed [3]. They were finally suspended in isotonic NaCl containing 40 imosM bicarbonate, pH 7.4, and adjusted to 2 μmol phospholipid per ml.

2 ml of ghost suspension were added to 6 ml of appropriate detergent/saline/bicarbonate mixtures, pH 7.4, to give the final detergent concentrations shown. After incubation at 37 °C for 30 min, the mixture was sampled for phase contrast or electron microscopy and then centrifuged at 150 000 $\times g$ for 60 min at 4 °C. The supernatant was then carefully removed and the residue resuspended in isotonic saline/bicarbonate to a known volume.

Analyses of the supernatants and residues from each incubation included phospholipid phosphorus [4], silicic acid thin-layer chromatography of lipid extracts [5], protein [6], and sodium dodecyl sulphate-polyacrylamide gel electrophoresis [7], usually after removal of lipid [8].

Specimens were prepared for electron microscopy either by adding an equal volume of buffered 6.25 % glutaraldehyde to the incubation mixture and then centrifuging at 10 000 $\times g$ for 10 min, or by direct addition to the centrifuged residue. Both types of sample were then fixed with osmium tetroxide, embedded, sectioned and stained with uranyl acetate prior to examination. Some residues were also "condensed" into membrane stacks by partial dehydration [9], they were subsequently fixed with osmium tetroxide and processed for electron microscopy.

RESULTS

At 37 °C and pH 7.4 and under approximately isotonic conditions, deoxycholate solubilized the erythrocyte membranes completely (Fig. 1). Triton X-100 solubilized most of the phospholipid but only about 70 % of the protein. Cholate also solubilized most of the phospholipid but only about 40 % of the protein. The conjugated cholate derivatives, taurocholate and glycocholate, solubilized approximately the same amount of protein as cholate but were less effective in phospholipid solubilization.

Phase contrast examination of the solubilization process revealed that the ghost morphology was lost with deoxycholate, which caused fragmentation of the ghosts prior to complete solubilization. On the other hand, the other detergents, cholate, glycocholate, taurocholate and Triton X-100, brought about a progressive shrinkage of the ghosts as the concentration of the detergent was increased. They did not, however, cause fragmentation of the ghosts which remained as full fields of ghost-like residues, slightly smaller than the controls and of lower contrast.

Though these extracted "ghosts" looked somewhat similar by phase microscopy, irrespective of the detergent used, fundamental differences were observed by electron microscopy. When fixed in suspension with glutaraldehyde the Triton-extracted "ghosts" were shown to be composed of lines of granules (Fig. 2a) which had lost the

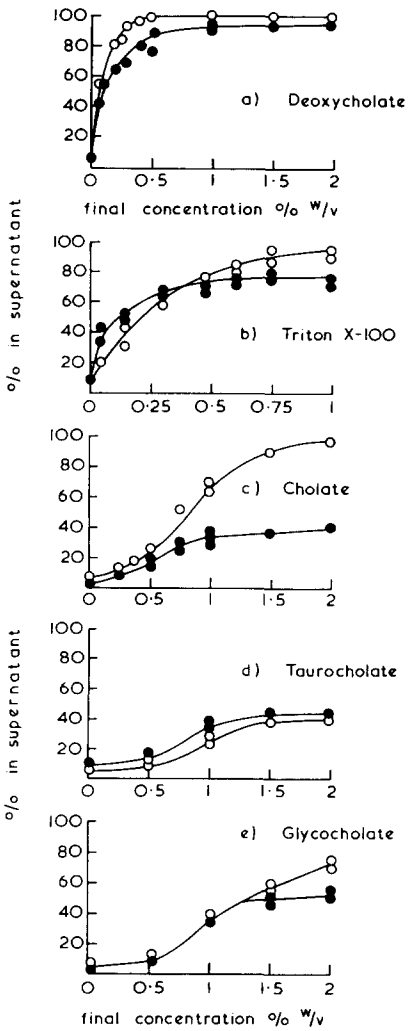

Fig. 1. Solubilization of components from erythrocyte ghosts. The data are derived from 2–5 experiments per detergent studied; ○, phospholipid; ●, protein.

trilamellar image of the original membrane. The Triton-extracted "ghosts" were rather fragile and, if ultracentrifuged prior to glutaraldehyde fixation, fragmented. This caused the granules to pack to a less structured mass.

The ghosts resulting from cholate-extraction preserved a membrane-like form whether centrifuged before or after glutaraldehyde fixation. When fixed in suspension the material showed, at low magnification, full fields of internally vesiculated "ghosts" (Fig. 2b). When centrifuged prior to fixation the membrane-like form was still preserved and the material sedimented as sheets of tightly-packed "membranes" and flattened vesicles. The apparently trilamellar image given by the material at high levels of detergent-extraction was however different from that of the original ghosts in that it appeared more sharply defined and narrower. Comparisons of "condensed"

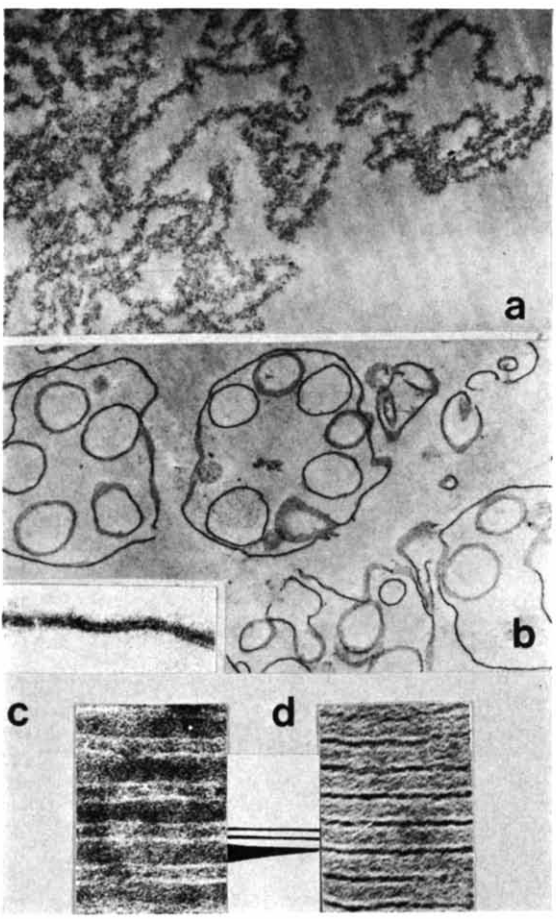

Fig. 2. Electron micrographs of detergent-treated erythrocyte ghosts. Ghosts were extracted with (a) Triton X-100, 0.75 % or (b) cholate (1.5 %), fixed in suspension with glutaraldehyde, centrifuged and post-fixed with OsO_4. Magnification × 12000, inset × 160000. (c) and (d) Control and cholate-treated (1.5 %) ghosts condensed by partial dehydration [9] and fixed with OsO_4. Magnification × 200 000.

materials indicated a reduced membrane thickness; this could be identified with a loss or pronounced condensation of the densely-staining material at the inner surface of the membrane (Figs. 2c, d).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Fig. 3) showed that the cholate-residue was largely composed of the polypeptides of bands 3, 4.5 and 4.1/4.2. (The designations of the bands are those of Steck, ref. 10). The cholate-residue also contained most of the periodate-Schiff reagent-staining material of the original membrane. The cholate-extract was largely composed of bands 1, 2, 5, 6 and 7 with a contribution from some of the components of the 4.1/4.2 band. The periodate-Schiff reagent-staining of gels of the cholate extract was very faint and no specific bands could be discerned. Glycocholate and taurocholate extractions produced polypeptide profiles similar to those of cholate.

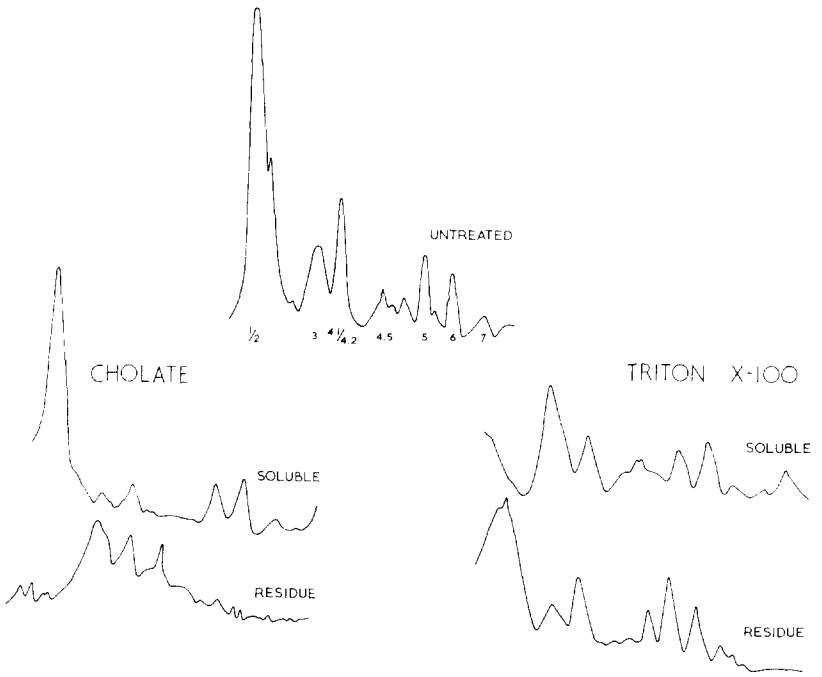

Fig. 3. Densitometer traces at 570 mμ of sodium dodecyl sulphate-polyacrylamide gel electrophoresis of detergent-treated erythrocyte ghosts. Samples were lipid-extracted [8] prior to electrophoresis and the completed gels were finally stained in Coomassie Blue R. Triton 0.75 %, Cholate 1.5 %.

In contrast, however, Triton X-100 extracted most of bands 3 and 4.5 together with some of the material of bands 4.1/4.2, 6 and 7. Periodate-Schiff reagent-staining polypeptides were extracted. The residue, which was apparently devoid of periodate-Schiff reagent-staining material, was largely composed of bands 1, 2 and 5, with some of the material of bands 4.1/4.2, 6 and 7.

Neither cholate and its conjugates nor Triton X-100 showed the same high degree of specificity with respect to lipid extraction as they did for proteins. Phospholipid thin-layer chromatography failed to show any major differences in differential extraction at either high or low extraction values except that at the highest levels of Triton X-100 the pellet became selectively enriched in sphingomyelin relative to the other phospholipids. Cholesterol was extracted by both detergents but at a slower rate than the phospholipids. Thus the pellets at high extraction values were enriched with cholesterol relative to phospholipid, e.g. at 1.5 % cholate the pellet contained approx. 12 % of the original phospholipid and 20 % of the original cholesterol of the ghost membrane.

DISCUSSION

The complementary polypeptide patterns of residue and supernatant obtained by incubation of erythrocyte ghosts with Triton X-100 are essentially similar to

those obtained, under different conditions of temperature, pH and tonicity, by Yu et al. [11] and Kirkpatrick et al. [12]. The lipid-depleted residue, which possessed a granular profile, was selectively enriched in those polypeptides representing peripheral proteins (for designation of bands see ref. 10) and the supernatant was selectively enriched in integral proteins.

These patterns provided a striking contrast to the patterns of the cholate-extracted material. The residue after cholate-extraction was selectively enriched in those polypeptides which represent many of the integral proteins originally intimately associated with, and in many cases spanning, the lipid layer of the original membrane.

Along with other less well characterized polypeptides, cholate removed principally the spectrin group of polypeptides (bands 1, 2 and 5), peripheral proteins which are usually considered to be associated with the inner surface of the erythrocyte membrane. The loss of these proteins probably correlates with the reduction in the broad band of dense material that was apparent in comparisons of electron micrographs of condensed samples of extracted and control membranes.

The material depicted in Fig. 2b represents a membrane preparation which has been depleted of about 88 % of its phospholipid and 80 % of its cholesterol. The relative homogeneity of the material by phase contrast and low-power electron microscopy suggested that the "membranous" material was typical of the sample rather than a selected sample of non-extracted material. Moreover, the altered thickness and improved definition of the trilamellar image suggest that this is a modified, rather than a native, membrane. Retention of a trilamellar image despite the extraction of most of the phospholipid would suggest that these integral proteins may contribute significantly to this trilamellar image. Such retention of a trilamellar image is reminiscent of other lipid-depleted membrane residues: acetone-extracted mitochondrial membranes [13] and chloroform/methanol-extracted sciatic nerve myelin [14].

The pronounced differences in extraction characteristics between the dihydroxy bile salt, deoxycholate, and the trihydroxy bile salt, cholate and its conjugates, were very striking. Deoxycholate was far more damaging to erythrocyte membranes than cholate under conditions chosen to approximate to physiological. It is therefore interesting to note in this context that cholate and, more particularly, its conjugated derivatives are less damaging to tissues when present in abnormal amounts (see ref. 15). It is suggested that some of these observations on the different intensities of attack by the various detergents on the membrane of the erythrocyte may be of significance also in relation to the problems associated with the secretion, by the liver, of large amounts of potentially membrane-damaging detergents, the bile salts.

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