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DIFFERENTIAL SOLUBILIZATION OF PROTEINS, PHOSPHOLIPIDS, AND CHOLESTEROL OF ERYTHROCYTE MEMBRANES BY DETERGENTS

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SUMMARY

Red cell membranes were treated with increasing concentrations of Triton X-100, sodium dodecylsulfate, or sodium deoxycholate at pH 7.5, and the solubilization of total protein, total phospholipid, cholesterol, and of individual proteins and phospholipids was determined as a function of detergent concentration.

The results suggest that each detergent solubilized membrane components by a different mechanism. Sodium dodecylsulfate extracted individual protein and lipids separately, each membrane component having a sigmoid extraction curve with a different dodecylsulfate concentration at midpoint. Sodium deoxycholate solubilized all proteins and phospholipids in parallel, with little fractionation, but cholesterol was not solubilized significantly until 60% of the protein and phospholipids were extracted. The data suggest that deoxycholate initially solubilizes membrane lipoproteins by displacement of cholesterol into the residual membrane matrix, followed at higher deoxycholate concentration by removal of phospholipids from both soluble and insoluble proteins into deoxycholate–phospholipid micelles which in turn solubilize cholesterol. Triton X-100 initially solubilizes most proteins and lipids in parallel, but proteins are released faster while sphingomyelin is preferentially retained in the pellet. At higher Triton X-100 concentration, net protein solubilization ceases while residual lipids are completely solubilized.

INTRODUCTION

Detergents are widely used in the study of membranes, both to isolate particular proteins and to probe the structure of the membrane itself. Despite the extensive body of literature describing treatment of membranes and derived fractions with detergents, there is little information on the mechanisms by which detergents interact with and eventually solubilize membrane components.

In the case of sodium dodecylsulfate, it is believed that in its interaction with protein it first binds to positive hydrophilic groups [1] or to specific hydrophobic

Abbreviation: CMC, critical micelle concentration.

receptors [2, 3], followed by less specific hydrophobic binding and eventual formation of a complex with a high ratio of dodecylsulfate to protein (1.5–2.3 g dodecylsulfate per g protein) [4, 5]. This complex is believed to have a rodlike shape [6] and other unusual hydrodynamic characteristics [7] which account for the utility of dodecylsulfate in polyacrylamide gel electrophoresis [6]. The interaction of dodecylsulfate with phospholipids and cholesterol is less defined, but several studies have shown [8–11] that at high detergent concentration, dodecylsulfate removes lipid from proteins.

Deoxycholate is reported to solubilize phospholipids by incorporation into mixed micelles [12]. This detergent action is probably the major biological function of bile salts [13]. The ratio of deoxycholate to lecithin in micelles is apparently variable [12] although a fixed ratio has been reported [14]. Cholesterol is poorly soluble in deoxycholate alone, but is incorporated into deoxycholate–lecithin micelles [12, 13, 15]. Deoxycholate binds to protein both at specific sites, e.g. with serum albumin [16, 17], and in larger quantities to "hydrophobic" but not to "hydrophilic" proteins [17, 18], and induces conformation changes in erythrocyte membrane proteins [7].

The interaction of Triton X-100 with lipids is not well characterized. Triton X-100, like dodecylsulfate and deoxycholate, binds in small amounts to definite sites on serum albumin [17], and binds in larger amounts to delipidated membrane proteins [18]. Triton X-100 is known to gradually deplete membrane lipoproteins of their lipids, as a function of detergent concentration and ionic strength [19, 20] probably by displacement of lipids since readdition of phospholipid is necessary to restore enzymatic activity [19, 20]. Triton X-100 can solubilize the red cell membrane with little loss of enzymatic activity [21]. Triton causes minimal or no changes in protein conformation when used to solubilize mitochondrial proteins [22] or red cell membranes [7]. It has been suggested [17] that Triton (and deoxycholate) are less denaturing than dodecylsulfate because Triton X-100 and deoxycholate cannot form the "saturated complex" found in protein–dodecylsulfate interactions [6]. This attractive hypothesis unfortunately does not account for the large conformation changes produced by dodecylsulfate below the concentration producing the saturated complex [7], nor does it explain the differences between deoxycholate and Triton X-100.

It is apparent from the above that while certain aspects of the interactions of these detergents with isolated lipids and proteins are fairly well characterized, the simultaneous interaction of detergents with lipids and proteins in complex lipoproteins is not well understood. In particular, the extent to which the native associations among membrane components are disturbed by detergents, and in what sequence, is unknown. The present experiments have been designed to provide some information on this subject.

METHODS AND MATERIALS

Red cell membranes were prepared by the technique of Hogeveen et al. [22] from fresh blood unsuitable for transfusion (provided by the Rochester Regional Red Cross Blood Bank). Membranes were resuspended to about 4 mg/ml protein in 40 mM NaCl–10 mM phosphate buffer, pH 7.5, (except as noted), and mixed with equal volumes of detergent solution of various concentrations, adjusted to pH 7.5.

After 30 min incubation at room temperature, the mixture was centrifuged at 140 000 $\times g$ for 60 min. Material not sedimenting under these conditions was defined as soluble. The supernatant was carefully removed from the pellet, the pellet was resuspended in distilled water to a defined volume, and aliquots of each were taken for analysis.

Total protein was measured by a biuret method [24], modified to include 1% final concentration of deoxycholate to solubilize membranes [25].

Total lipids of the various membrane fractions were obtained by extraction with 10-fold excess chloroform–methanol (1: 1, v/v). Aliquots of the lipid extract were digested with perchloric acid for analysis of total lipid phosphorus using the method of Harris and Popat [26]. Other aliquots were spotted on silica gel SG-81 paper (Reeve-Angel) for chromatographic analysis. The phospholipids were separated in a solvent consisting of chloroform–methanol–acetic acid–water (90 : 40 : 8 : 3, by vol.). The phospholipid bands were detected by staining with Rhodamine 6 G [27]. The various phospholipids were eluted with 1% HCl in methanol and then digested as mentioned above in order to determine the lipid P in each band.

For total chlolesterol, aliquots of 0.1–0.2 ml of the supernatants or pellets were treated with 6.0 ml of a mixture of concentrated H_2SO_4–acetic anhydride–glacial acetic acid (25 : 86 : 82, by vol.). The samples were incubated for 15 min at 37 °C, cooled, and the absorbances determined at 620 nm. Standard cholesterol solutions (50–200 mg/100 ml) were used to calculate total cholesterol in the membrane fractions. Control studies showed no interference by any of the three detergents even at concentrations 5–10-fold higher than found in the membrane suspensions.

Following dodecylsulfate–polyacrylamide gel electrophoresis and staining with Coomassie blue [28], individual proteins were determined by manual integration of gel scans. Although Coomassie blue staining is reported to be non-linear [29], summation of areas of equivalent protein peaks in gels of supernatants and of pellets gave constant (approx. 5%) values. This also indicates that errors in measurement of peak areas were small.

The composition of the final membrane suspension was 2 mg/ml protein, of which 40% was band 1 and 25% was band 3; total lipid was about 1.5 mg/ml, of which 0.4 mg/ml was cholesterol (approx. 1 mM) and 1.1 mg was phospholipid (approx. 1.3 mM in lipid phosphorous). The phospholipid was about 32% lecithin, 27% sphingomyelin, 20% phosphatidylethanolamine, and 12% phosphatidylserine, plus traces (less than 5%) of phosphatidylinositol, lysolecithin, and unidentified.

RESULTS

Data obtained from these experiments are shown in Figs. 1 and 2. It can be seen that initially all three detergents solubilize protein somewhat faster, as a function of detergent concentration, than they do phospholipid, and that total phospholipid is solubilized faster than cholesterol. At higher detergent concentrations phospholipid and cholesterol are rapidly solubilized, eventually to levels of 95–100%. Protein solubilization tends to reach its maximum value at lower detergent concentrations. The patterns for phosphatidylserine (not shown) were approximately the same as those for lecithin and phosphatidylethanolamine. Data for proteins other than bands 1, the doublet of molecular size about 220 000 daltons, and band 3, the 90 000-

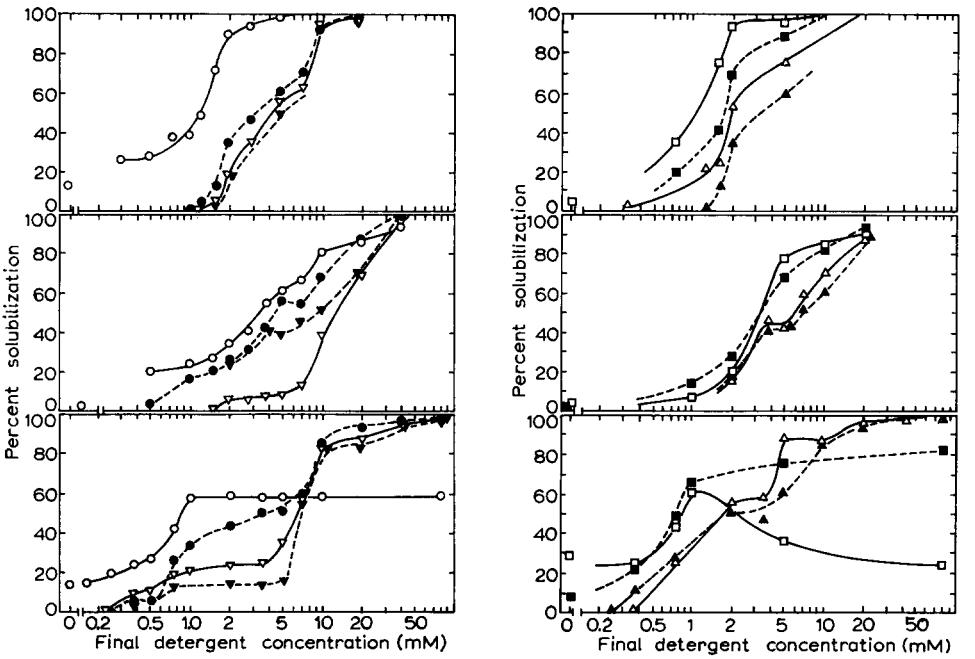

Fig. 1. Percent solubilization of membrane components at various detergent concentrations. Experimental conditions as described in text. ○–○, total protein; ●- - -●, total lipid; ▽–▽, cholesterol; ▼- - -▼, spingomyelin. Point at extreme left of graph is control (no detergent). Top, dodecylsulfate; center, deoxycholate; bottom, Triton.

Fig. 2. Percent solubilization of membrane components at various detergent concentrations. Experimental conditions as described in text. □–□, protein bands 1 ("spectrin"); ■- - -■, protein band 3 (approx. 100 000 mol.wt); △–△, phosphatidylethanolamine; ▲- - -▲, phosphatidylcholine (lecithin). Top, dodecylsulfate; center, deoxycholate; bottom, Triton.

dalton band, are also omitted. Bands are numbered according to the system of Carraway et al. [30].

In part, the faster solubilization of protein is due to the innate solubility of certain red cell membrane proteins in media of low ionic strength [31, 32]. This is especially clear with dodecylsulfate, where the water-soluble proteins, principally band 1 (and also bands 5, 7, and 10), are solubilized more rapidly, while solubilization of protein band 3 is slower (Fig. 2, top). The overall picture obtained from the dodecylsulfate data is a series of sigmoid curves for various components of the membrane. Chromatographic separation of individual phospholipids was not obtained at higher dodecylsulfate concentrations than those shown, due to interference by dodecylsulfate.

Deoxycholate solubilizes phospholipids and proteins less selectively (Figs 1 and 2, center). In particular, the two major protein bands, 1 and 3, are solubilized at essentially the same rate as total protein. Similarly, little differentiation among individual phospholipids is seen. Total protein and total phospholipid are solubilized at approximately the same rate. Cholesterol, however, is not significantly removed from the membrane pellet until 60% of total protein and total phospholipid has been solubilized (Fig. 1, center). It is of interest that cholesterol is not particularly soluble

in bile salts, but is much more soluble in deoxycholate–lecithin mixed micelles [12, 13, 15].

The solubilization of membranes by Triton X-100 shows a different pattern. Protein solubilization reaches a plateau at about 1 mM, which continues beyond 50 mM. Unlike dodecylsulfate and deoxycholate, the pattern of total protein solubilization with Triton X-100 is strongly dependent on the ionic strength of the medium. The experiments shown in Fig. 1 were done with ghosts in a medium containing 4 mM NaCl–1 mm sodium phosphate buffer, pH 7.5. Under these conditions maximal protein solubilization with Triton is only 60%. The relatively large amount of protein in the pellet made it possible to observe the effects described below. If ghosts are resuspended in 2 mM $NaHCO_3$, adjusted to pH 7.5, protein solubilization reaches 90%, which is comparable with the values reported by Miller [21]. The rate of lipid solubilization, with increasing Triton X-100 concentration, also increased at lower ionic strength.

As can be seen in Fig. 1 (bottom), Triton X-100 below 1 mM preferentially extracts protein. The faster extraction of all protein is not due to increased solubilization of the water soluble proteins (bands 1, 5, 7, 10), since at 0.4 mM Triton X-100 all proteins are solubilized to the same extent (Fig. 2, bottom), while lipid solubilization is just beginning. Above 1 mM (at this ionic strength), net protein solubilization ceases, while lipids continue to be solubilized. Sphingomyelin and cholesterol are preferentially retained in the pellet up to a concentration of about 5 mM Triton X-100. It is of interest that in the plateau region for protein solubilization, band 3 continues to be solubilized while band 1 becomes distinctly less soluble (Fig. 2). At lower ionic strength this pattern of protein solubilization is still present, even though only 10% of the protein is in the pellet, and not all of it is band 1. A reasonable explanation for this behavior is that band 1, which is the principal constituent of "spectrin" [32] or "tektin" [31], is being "salted out" by competition for water hydrogen bonds with the ether linkages of the polyoxyethylene chains of Triton, and yet is sufficiently hydrophilic not to bind Triton hydrophobically [18]. "Spectrin", "tektin" and related preparations of water soluble erythrocyte membrane protein are known to aggregate at fairly low salt concentration. Evidently, the amount of Triton X-100 added is sufficient to precipitate these proteins, at an ionic strength at which they would otherwise be soluble.

DISCUSSION

It is apparent from the above results that the three detergents differ substantially in the mechanisms by which they solubilize membrane components. The mechanism of sodium dodecylsulfate solubilization appears to be that dodecylsulfate binds to all components fairly uniformly, with the release of components from the dodecylsulfate–membrane aggregate being approximately in order of the component's water solubility. The onset of solubilization for the lipids coincides approximately with the critical micelle concentration (CMC) of dodecylsulfate (assuming that only a small amount of the total dodecylsulfate is bound to the membrane components), approx. 1–2 mM at this temperature and ionic strength [6]. This is also the level at which conformation changes in the membrane proteins begin [7]. Almost all the lipid is dissolved at 10 mM dodecylsulfate. This is also the level at which formation

of the "rodlike" dodecylsulfate–protein complexes begins [7], in agreement with Tanford's statement [3] that such complexes are not formed until all lipid is incorporated in dodecylsulfate micelles. However, in this membrane system, the 10 mM dodecylsulfate that is present at the beginning of "rod" formation has not formed micelles containing a "high percentage of dodecylsulfate" [3]. It can be calculated from literature values [33, 34] that a suspension of red cell membranes containing 2 mg/ml protein will be approximately 1 mM in cholesterol and 1.3 mM in total phospholipid. Since at 2 mM dodecylsulfate the protein is substantially separated from the phospholipid, it seems likely that lipids are solubilized primarily by incorporation into dodecylsulfate–lipid micelles in the region between 2 and 10 mM dodecylsulfate. This implies that, at least initially, dodecylsulfate–phospholipid micelles have high ratios of lipid to dodecylsulfate, and also are capable of solubilizing cholesterol. This process probably occurs by binding of dodecylsulfate to the membrane pellet, followed by removal of lipids from local regions where the dodecylsulfate concentration becomes high enough to form mixed micelles. Further studies are required to determine whether the micelles found at this dodecylsulfate concentration contain protein as well as lipid. It has been reported that at dodecylsulfate concentrations of about 50 mM, lipids and proteins have been separated [35].

A similar problem in interpretation occurs with deoxycholate. Philippot and Authier [36] have shown that the critical micelle concentration of dodecylsulfate is not affected by the presence of protein, and obtained a value of 3–4 mM under conditions comparable to ours. It thus appears that protein–phospholipid complexes are detached from the membranes by non-micellar dodecylsulfate, probably by displacement of cholesterol into the insoluble membrane residue. Above the CMC, phospholipids begin to be removed from protein by deoxycholate micelles, which permit solubilization of cholesterol. Then, as the "excess" cholesterol is removed from the pellet, the residual lipids and protein are also solubilized. The necessity of obtaining micelles containing a proper ratio of dodecylsulfate to phospholipid to solubilize cholesterol could account for the slight plateau in solubilization of various components seen around 5 mM deoxycholate. It is also possible that the "excess" cholesterol is present as a separate phase, as observed in the lecithin–cholesterol–bile salt–water system [16].

The pattern of solubilization by Triton X-100 is more difficult to interpret. The solution is of sufficiently low ionic strength that even without addition of Triton X-100, 25% of the water-soluble protein is already in solution. Small amounts of hydrophobic proteins are also found in the supernatant, possibly reflecting the presence of small membrane vesicles that did not sediment. Addition of more Triton X-100 increases the amount of hydrophobic protein in the supernatant and begins "solubilization" of lipid. It is likely that the lipids and hydrophobic proteins are present in the supernatant as small vesicles or membranes, while the water soluble protein has been dissociated. The supernatant material, however, is depleted in lipid relative to the whole membrane. At 1 mM Triton X-100 net solubilization of protein, sphingomyelin and cholesterol ceases. The protein plateau is the net effect of an increase in the solubilization of band 3 (and other hydrophobic proteins), little change in solubilization of small water soluble proteins, and a net decrease in solubilization of band(s) 1 ("spectrin"). A possible explanation for the latter effect has been noted above. The flatness of the plateau is an especially interesting effect, since this protein

solubilization plateau is seen at all ionic strengths. When the level of maximal solubilization varies from 30 to 90% as a function of ionic strength, the Triton X-100 concentration at onset of the plateau slowly increases from about 1 to about 2 mM. Study of the protein solubilization pattern at 90% solubilization also shows a pattern of band 1 solubilization decreasing at high Triton concentration. An apparent balance of band 1 precipitation and band 3 solubilization is needed to produce the plateau.

Between 1 and 5 mM Triton X-100, phospholipids (except sphingomyelin) are "solubilized" roughly in parallel with band 3, while sphingomyelin and chlolesterol are retained in the pellet. It is possible that acidic or amino phospholipids are preferentially associated with the "solubilized" hydrophobic protein, which could account for this phenomenon. Between 5 and 10 mM, rapid solubilization of sphingomyelin and cholesterol, and additional solubilization of other lipid, occurs. Although the CMC of Triton X-100 is less than 1 mM, it is possible that, below 5 mM, all added Triton X-100 is bound to membrane components, and thus 5 mM reflects the appearance of micelles of free Triton. This may correspond to the situation with dodecylsulfate described by Tanford [3]. The existence of the differential solubilization effects of Triton in the total protein "plateau" makes it likely that Triton disturbs native lipid–protein arrangements as much as dodecylsulfate and deoxycholate do, although by a different mechanism in each case. Hence the retention of sphingomyelin and cholesterol by the Triton pellet below 5 mM is probably a property of Triton rather than of the proteins.

NOTE ADDED IN PROOF (Received February 11th, 1974)

While this paper was under review, we became aware of the work of Yu et al [37], who treated erythrocyte membranes with Triton X-100 and other non ionic detergents and obtained results compatible with those presented here.

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