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INTERACTIONS BETWEEN DIGITONIN AND BILAYER MEMBRANES

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The morphology of interactions between digitonin and cholesterol has been investigated. When precipitated from ethanolic solutions, digitonin-cholesterol complexes form in flat lamellar sheets. In contrast, when the complex is formed in a bilayer membrane, the membrane is deformed into corrugations of hemitubules. The polarity of the deformations formed in bilayer membranes is highly correlated with the direction of entry of digitonin into the membrane. We suggest that the morphology of digitonin/cholesterol hemitubules is dependent upon the complex being formed within a bilayer and, in addition, is not correlated with asymmetry of cholesterol concentration across the membrane.

Incubation of cholesterol containing membranes in media containing digitonin [1–13] and other cholesterol-binding chemical (see, for example, Refs. 14–19) produces characteristic structures which are visible through thin-section and freeze-fracture electron microscopy. In the case of digitonin, membranes which otherwise have smooth contours become scalloped by hemitubules and are subsequently disrupted. The disruption of the membranes (see, for example, Refs. 7 and 9) has been attributed to the budding of tubules (approx. 0.05 μm in diameter) from membranes incubated in digitonin. The ability of digitonin to produce hemitubules and tubules has led to its use as a morphological marker for cholesterol (see, for example, Refs. 1–4, 10, 12 and 13). In these studies, it has generally been assumed that the hemitubules and tubules represent the normal configuration of the cholesterol-digitonin complex (see, for example, Refs. 4, 10, 12 and 13, but also Ref. 8). We report here that the formation of the tubular structures requires that the complexes be formed in a bilayer membrane and that such tubular structures can be formed by digitonin alone. Formation of precipitable digitonin-cholesterol complexes, on the other hand, produces neither hemitubules nor tubular structures, but rather flat lamellae. In addition, we report that there is an asymmetry to the hemitubules seen in a bilayer membrane and that the asymmetry appears to be brought about by an asymmetric delivery of the digitonin.

The freeze-fracture morphology of digitonin, cholesterol and 1 : 1 complexes of the two was studied by precipitation of ethanol solutions by addition of water. In order to be assured that the 1 : 1 molar mixture of digitonin and cholesterol coprecipitated (i.e., that a complex was being formed) [^{14}C]cholesterol was precipitated with ethanol solutions in 1 : 0, 1 : 1 and 1 : 2 molar ratios of digitonin. Diagram 1 indicates that under the conditions employed considerable coprecipitation of cholesterol occurs upon the addition of 80% ethanol and is nearly complete upon the addition of 60% ethanol (final ethanol concentration 65%). On the other hand, in the absence of digitonin, [^{14}C]cholesterol is not significantly precipitated until the concentration of ethanol falls below 60%. In another experiment (Diagram 1, dotted lines)

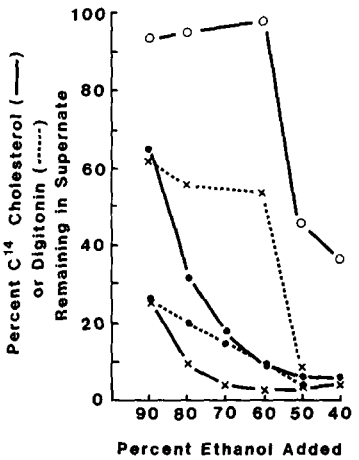

Diagram 1. Solid lines: cholesterol remaining in solution as a function of ethanol concentration with and without digitonin. 0.9 ml of different concentrations of ethanol were added to 0.12 ml of 1.33 mM cholesterol ([4-^{14}C]cholesterol from Amersham with cholesterol carrier from Sigma) with no digitonin (open circles), equimolar (closed circles), or 2.67 mM (crosses) digitonin (Sigma, 'approx. 80%'. All molar concentrations are calculated as if purity was 100%). The resulting suspensions were incubated in an ice bath for over an hour and centrifuged at 1000× g for 10 min. Aliquots of the resulting (clear) supernates were removed for determination of amount of cholesterol remaining in the supernate. Dashed lines: digitonin remaining in cholesterol-containing ethanolic solution as a function of ethanol concentration. 0.9 ml of different concentrations of ethanol were added to 0.1 ml 2 mM digitonin containing either 2 (closed circles) or 4 (crosses) mM cholesterol. The resulting suspensions were spun down as described above and aliquots of the supernates were assayed for digitonin with the anthrone reagent as described by Cook [23].

digitonin precipitation was monitored in solutions containing 2 : 1 and 1 : 1 molar ratios of digitonin and cholesterol. In the equimolar ratio, approx. 80% of the digitonin is precipitated with the addition of 80% ethanol. When water was added to a saturated solution of digitonin, a visible precipitate was formed as the concentration of ethanol fell below 50%. This precipitate formed slowly (over several hours) for a final ethanol concentration of 50% and more rapidly (several minutes) when the ethanol concentration was 25%. The latter preparations were used for morphological descriptions. Freeze-fracture samples were also obtained from 1 : 1 molar coprecipitates with the addition of 70% ethanol and from cholesterol precipitates with the addition of 50% ethanol. Precipitates formed from cholesterol, digitonin on 1 : 1 molar mixtures of the two were washed in water, and then in 15% aqueous glycerol. Figs. 1a, 1b and 1c illustrate the morphological characteristics of precipitates of cholesterol, digitonin and 1 : 1 mixtures of the two, respectively. Cholesterol precipitates in the form of lamellae crystals with little or no curvature. Digitonin precipitates in the form of lamellate crystals which are often serrated and usually show some curvature. In some cases, (particularly with slow formation of the precipitates) tubular forms can be observed in the digitonin precipitate. An example of this configuration is presented in Fig. 1d. The precipitate was typically composed of chips of digitonin arranged radially around a center, indicative of nucleation effects. The precipitate of 1 : 1 molar ratios of cholesterol and digitonin appeared lamellae with little or no curvature. In order to determine the morphology of complexes formed in artificial membranes, liposomes were formed by the method of Bangham et al. [20], containing 25 mol% cholesterol (Sigma) and 75 mol% phosphatidylcholine (lecithin, egg yolk, Sigma) or phosphatidylcholine alone in phosphate-buffered saline with 25% glycerol. Incubation of cholesterol free liposomes in the same buffer containing digitonin produced no hemitubules (Fig. 2a) while incubation of cholesterol containing liposomes with digitonin produced hemitubules in the plane of the membrane (Fig. 2b). In many liposomes, there were regions where the hemitubules appear to be budding off the liposome to form tubules (data not shown). When digitonin (0.02%) is included in the buffer used to make the liposomes, several structures are seen: the majority of liposomes have smooth contours and do not contain any hemitubules (Fig. 2c). Occasionally liposomes will demonstrate hemitubules, but these represent less than 5% of the images obtained. Finally, there are occasional structures which present a crystalline pattern and do not appear to be closed surfaces (Fig. 2d). It is presumed that the latter represent mixtures of digitonin, cholesterol and phospholipid which have been formed through insertion of digitonin from both sides of the 'membrane'.

In the past, the tubules that appeared to be budding from biological membranes incubated in digitonin were assumed to consist primarily or

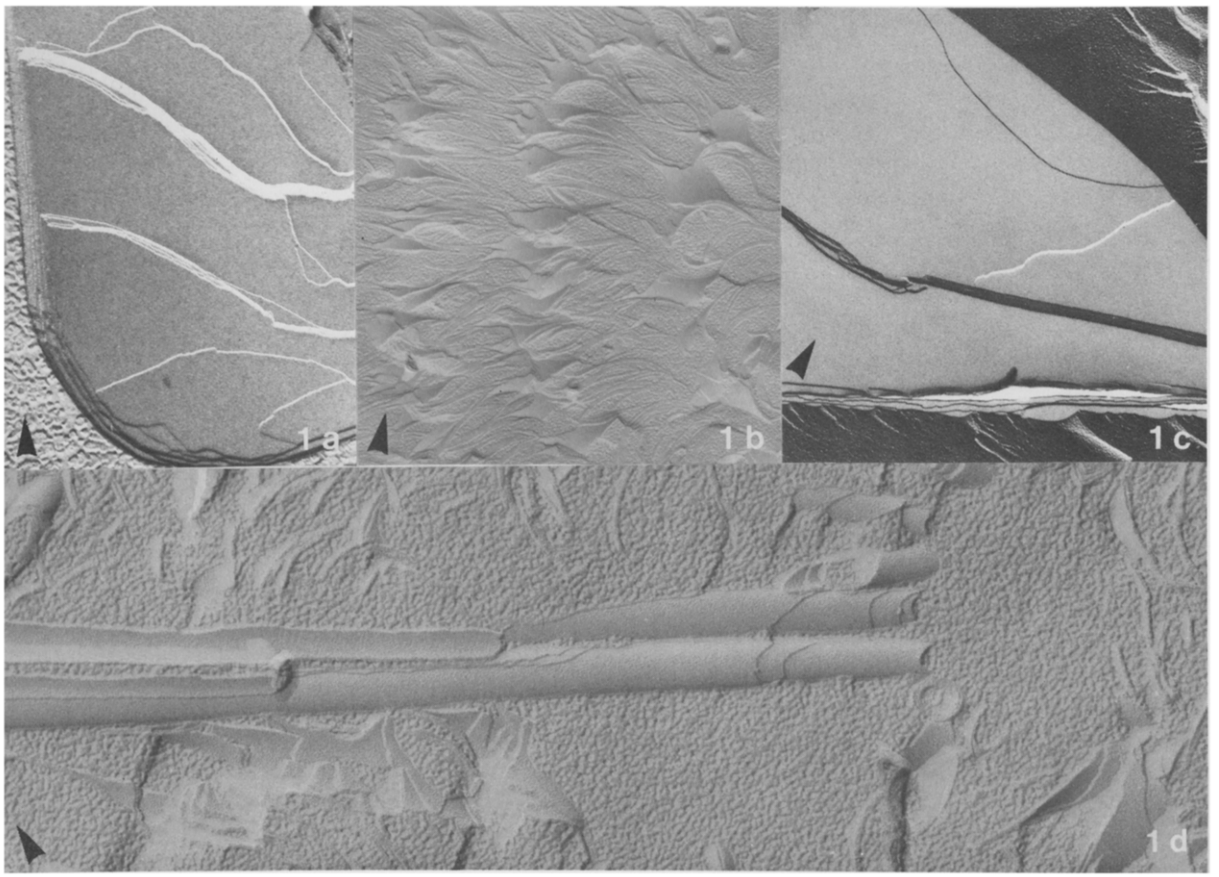

Fig. 1. (a) Characteristic morphology of cholesterol precipitated from an ethanol solution by addition of water (× 50000). (b) Characteristic morphology of digitonin precipitate resulting from the addition of three volumes of water (× 35000). (c) 1:1 molar ratio of digitonin and cholesterol precipitated from ethanol solution (× 22000). (d) Morphology of digitonin tubules formed by precipitation of digitonin with three volumes of water (same sample as shown in Fig. 1b.) (× 76000).

exclusively of digitonin-cholesterol complexes which preferentially adopted a tubular structure (see, for example, Refs, 4, 10, 12 and 13). The present results indicate that cholesterol-digitonin complexes do not, per se, arrange into hemitubules and tubules and, furthermore, that digitonin by itself is capable of forming tubules. On the other hand, formation of complexes by incorporation of digitonin into the plane of a bilayer cholesterol-containing membrane, has produced hemitubules and/or tubules in all cases studied (see below and, for example, Refs, 4, 7, 9, 10, 12 and 13). These findings suggest that it is the interaction of the complex with the bilayer membrane which is responsible for the formation of hemitubular structures and that tubular structures are possible in the absence of cholesterol.

The hemitubular structures which appeared on liposomal membranes after incubation with digitonin were highly asymmetric with respect to the plane of the membrane (see also Ref. 8). In all cases, the convex face of the fractured liposome contained only convex hemitubules while the concave face contained only concave hemitubules. In order to better define the source of this asymmetry, membranes from several biological sources (red blood cells, intestinal epithelia, endocrine and exocrine pancreas, central and peripheral myelin and fibroblasts in tissue culture) incubated in the presence of digitonin were analyzed. Cells or tis-

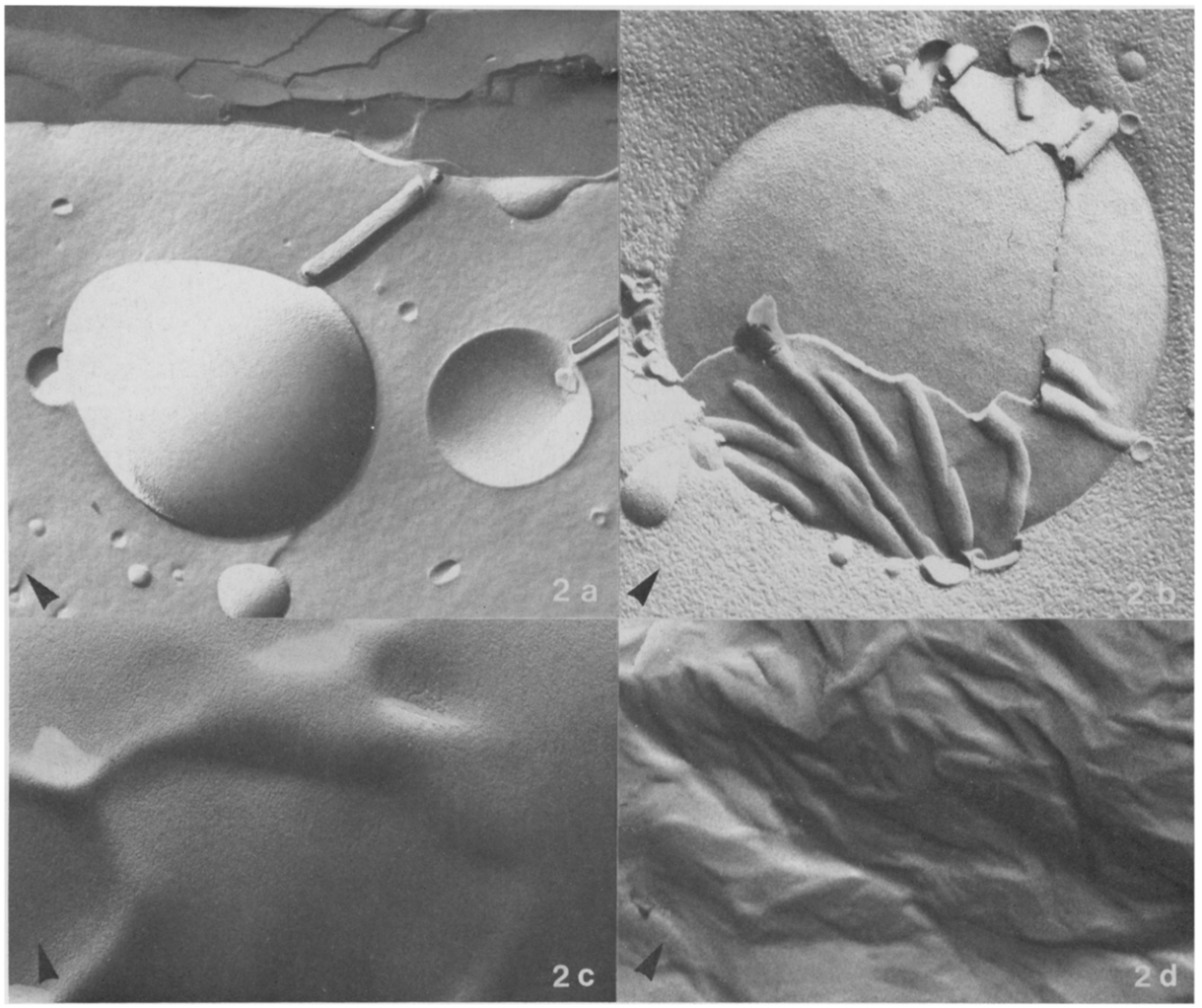

Fig. 2. (a) Incubation of cholesterol-free liposomes in 0.2% digitonin results in liposomes free of hemitubules (below) and some plates of crystallized digitonin (above): at this concentration of digitonin, some digitonin is in suspension (× 33000). (b) Hemitubules formed on cholesterol-containing liposomes (× 33000).

Figs. 2(c and d). Inclusion of digitonin (0.02%) in the buffer forming cholesterol liposomes results in some liposomes containing hemitubules (similar to Fig. 2b) while the majority of liposomes (> 90%) are free of hemitubules as presented in Fig. 2c (× 20000). Other structures suggestive of crystalline patterns found on myelin after incubation in the cold (see Ref. 9) are also present and presented in Fig. 2d (× 56000).

sues were fixed (typically with glutaraldehyde 2% in phosphate-buffered saline for 2 h) and subsequently exposed to digitonin (typically 0.02%) in the fixative solution. Tissue was impregnated in 10–15% glycerol in phosphate buffered saline and processed for freeze-fracture by routine procedures. Figs. 3–5 show the appearance of hemitubules which are convex on the P-face and concave on the E-face of plasma membranes. In Fig. 3, a human red blood cell is shown with convex hemitubules on the P-face. Plasma membranes from intestinal epithelia and endocrine pancreas demonstrate convex hemitubules on the P face in Figs. 4 and 5, respectively. In all tissues with the exception of myelin, the P-face of the plasma membrane showed convex hemitubules while the E-face

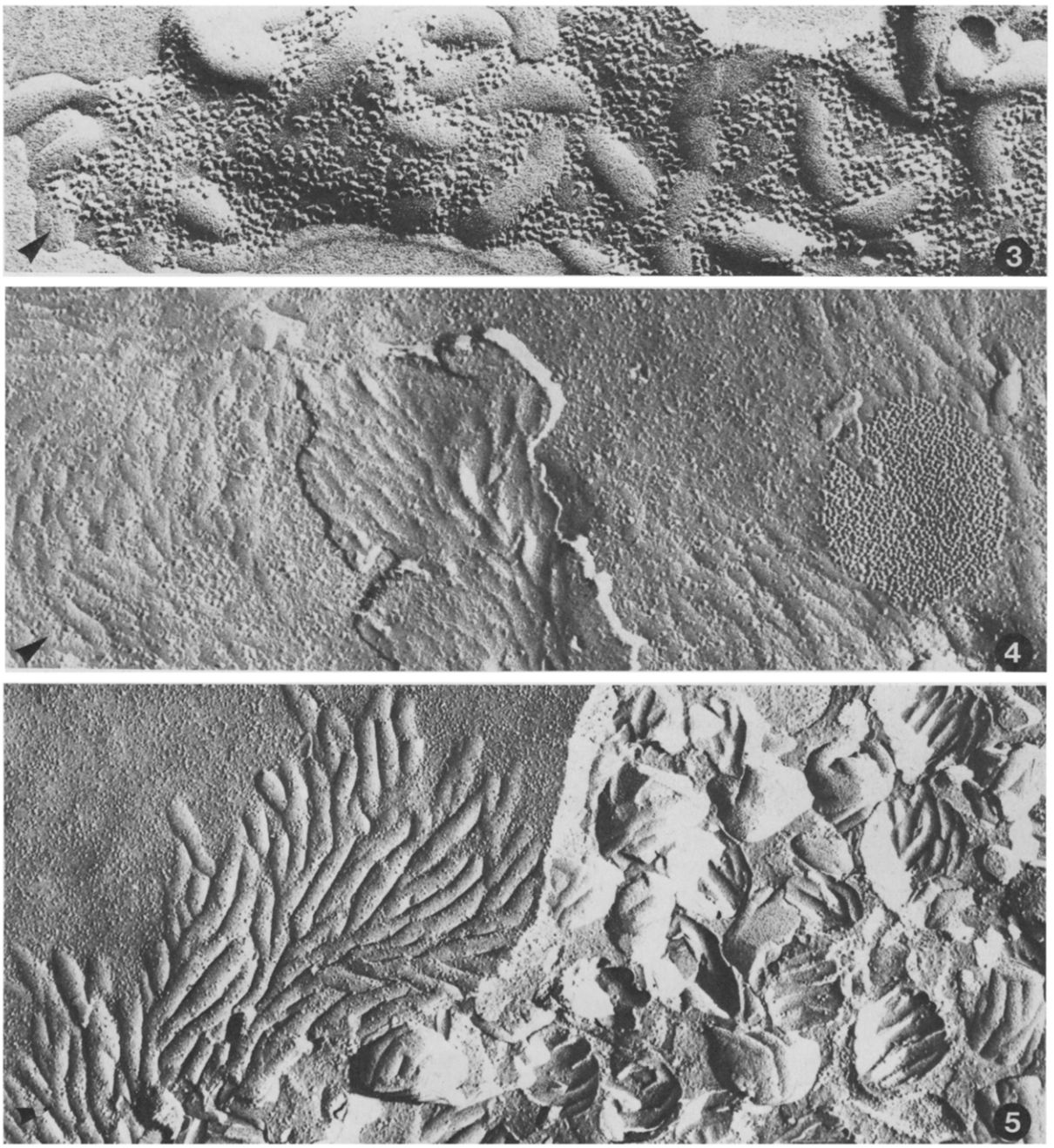

Fig. 3. Hemitubules formed on red blood cell ghosts (× 100000).

Fig. 4. Hemitubules formed on cells from the intestinal epithelia (× 80000).

Fig. 5. Hemitubules formed on the plasma membrane and secretory granule membranes of pancreatic endocrine cells (× 50000).

showed concave hemitubules. in the case of myelin, the lamellar membranes typically showed hemitubules which pushed the membrane in the direction away from the axon. A survey of available micrographs of various biological tissues from this laboratory showed that a very small proportion of the plasma membranes labeled with digitonin demonstrated both convex and concave hemitubules on a single fracture plane. In all of these exceptions, the tissue was in a block where some impediment to digitonin access can be expected.

The polarity of hemitubules in the intracellular membranes is reversed with respect to the P- and E-leaflets. Membranes from granules of the exocrine and endocrine pancreas demonstrate convex hemitubules on the convex (E) face and concave hemitubules on the concave (P) face. A survey of secretory granules from replicas obtained form digitonin treatment of endocrine pancreas indicated that less than 2% of the granules (4/280) showed hemitubules with opposite polarity (convex hemitubules on concave granule fracture faces or concave hemitubules on convex granule fracture faces).

There are three possible mechanisms by which asymmetry of the hemitubules described might be induced: (1) the asymmetry might be brought about by the asymmetric incorporation of digitonin caused by an asymmetry (presumably of cholesterol concentration) within the bilayer membrane. This is the most interesting of the possibilities for it would suggest that digitonin may be used as a marker for membrane asymmetry with respect to cholesterol. However, two observations make this interpretation unlikely: (A) Liposomes and all plasma membranes (except where access to the internal membrane is impeded) show the same polarity. It would appear unlikely that all of these membranes have the same polarity with respect to cholesterol concentration; (B) In the liposomal system, although it is possible to argue that the radius of curvature of the liposome may bring about some asymmetry, one would expect the concentration of cholesterol to be equal in all regions and in both leaflets of the membrane. Thus we believe that the polarity of digitonin-induced hemitubules cannot indicate cholesterol asymmetries in biological membranes. (2) The asymmetry could be induced by an asymmetry in the media on the two sides of the membrane. The media on the inside and the outside of the liposome is the same with the exception of digitonin. Also, the polarity of hemitubules seen in myelin membranes makes this possibility unlikely. (3) The asymmetry could be induced by the asymmetric delivery of digitonin to the membrane. This mechanism is in accordance with all of the present data; digitonin induces hemitubules which are convex in the direction from which the digitonin enters the membrane. In plasma membranes digitonin enters from the E-face and thus convex hemitubules are formed on the P-face and concave hemitubules are formed on the E-face. In secretory granules digitonin enters from the P-Leaflet, and thus convex hemitubules are formed on the E-face and concave hemitubules are formed on the E-face. The structure of digitonin, with five sugar groups on one side and a hydrophobic steroid on the other, also argues for this interpretation.

The present analysis suggests that the incorporation of digitonin into a membrane and its stabilization by complexing with cholesterol creates a membrane with more components on the outer leaflets than on the inner leaflets of membranes. Compensation for this asymmetry could be provided by an induced curvature and by flip-flop of some of the lipids in the outer leaflet to the inner leaflet. This situation is very different from that of complex formation from solution, where there is no initial asymmetry and thus the structures formed are flat plates. Determination of the degree of similarity between the molecular packing in the two (or more: see Refs. 3, 5, 6 and 8) structures of digitonin-cholesterol complexes remains to be determined.

The mechanism of incorporation of other sterol binding molecules into membranes is unknown. Work is in progress in this laboratory to determine whether any of the other cholesterol-binding moieties distribute in the plane of the membrane through the schema 1 above. The polyene antibiotic, filipin is of particular interest in that there are not bulky sugar groups on this compound and in that there is evidence [21] that direction of entry into the membrane is not the only determinant of the polarity of membrane deformations.

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