Published in final edited form as:

Histochem J. 1981 July ; 13(4): 535–546.

β -Hydroxysterol distribution as determined by freeze-fracture cytochemistry

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Summary

Filipin, a polyene antibiotic, fluoresces and forms 15–25 nm aggregates when combined with β -hydroxysterols, rendering sterols detectable by fluorescence microscopy and by electron microscopy of thin sections and freeze-fracture replicas. We applied filipin in a glutaraldehyde fixative to tissue-cultured cells of *Drosophila melanogaster* larvae, in which sterol concentration can be regulated. Since the number of filipin–sterol aggregates observed in membranes was found to be proportional to the amount of sterol experimentally inserted, utilizing filipin is a valid method for quantifying, as well as for mapping, sterol distribution in biological membranes. Other antibiotics may be similarly used for localizing some species of negatively charged phospholipids.

In addition to cytochemical identification of specific lipids, rapid freezing and deep etching of unfixed, non-cryoprotected cells may permit us to examine membrane lipids in different physical states: liquid–crystalline and gel. Combining these several techniques has resulted in new data concerning the disposition of lipids during the intimate juxtaposition of membranes preceding fusion. For example, in guinea-pig sperm, foci of closely apposed membranes are bereft of β -hydroxysterols and intramembranous particles. Such regions of membrane sometimes exist in a crystalline state and may be rimmed by negatively charged phospholipids. As previously noted in other areas of cytochemistry, the *in situ* localization of specific substances provides information unobtainable by morphological or biochemical techniques alone.

Introduction

Overview

Though there are many cytochemical methods for localizing enzymes, membrane components, and tracer molecules by the electron microscopy of thin sections, few can be applied for identifying specific substances in freeze-fracture replicas. Several of the agents used to this end (for example, detergents such as digitonin and tomatin) have disclosed the sites of sterols with which they interact by disrupting areas of the membrane rich in these compounds (Elias *et al.*, 1978). Relatively crude, but nonetheless useful biological information has been obtained with detergent application, including the discovery of the virtual absence of sterols in coated pits involved in receptor-mediated endocytosis and the lack of sterols in regions of tightly packed transmembrane proteins – that is, in the sperm tail zipper and in the gap and tight junctions of epithelial cells (Elias *et al.*, 1978). Very recently, even among agents such as polymyxin B and melitin employed with eukaryotic cells to localize acidic phospholipids, reflection of local lipid composition depends upon indirect alterations in membrane contour (Bearer & Friend, 1980). While important and practicable

data will likewise be garnered from the use of these compounds, the polyene antibiotic filipin, which complexes with membrane sterols, comes forth as a uniquely convenient agent for the discrete localization of a specific class of membrane lipids, the β -hydroxysterols (Kinsky *et al.*, 1966). In addition to its well-defined chemical interaction, filipin's small distinctive geometry contributes to its cytochemical efficacy (Verkleij *et al.*, 1973; DeKruijff & Demel, 1974; Elias *et al.*, 1978; Robinson & Karnovsky, 1980).

Kinsky pioneered the search of filipin's properties in the early 1970s and discovered its stoichiometric binding to membrane sterols. This finding was put to good use by Tillack and Kinsky, and separately, by Verkleij and his colleagues in the mid-1970s for detecting membrane sterols by the electron microscopy of freeze-fracture replicas (Tillack & Kinsky, 1973; De Kruijff & Demel, 1974; Kitajima *et al.*, 1976; Andrews & Cohen, 1979; Seikiya *et al.*, 1979). During the last two years, more easily interpreted biological applications of the filipin-sterol complexing in membranes have emerged, based on the knowledge that glutaraldehyde fixation is a necessary preparative adjunct for assessing the distribution of 3- β -hydroxysterols in the cell membrane. Elias and I, as well as Robinson and Karnovsky, have stressed this point and it has been effectively used by Montesano & Orci (Elias *et al.*, 1979; Montesano, 1979a, b; Montesano *et al.*, 1979; Friend, 1980; Robinson & Karnovsky, 1980).

The filipin-sterol complex

Filipin interacts stoichiometrically with β -hydroxysterols to form complexes, either in hydrophobic domains of the membrane in some instances (DeKruijff & Demel, 1974) or on the cytoplasmic side of the membrane in the case of guinea-pig sperm (Elias *et al.*, 1978). The reagent-substrate clusters result in 20–25 nm membrane protuberances visible in freeze-fracture (Fig. 1), each complex including at least four molecules of sterol and filipin. Tested with liposomes containing various sterol, filipin was found to react equally well with cholesterol, cholestanol, 7-dehydrocholesterol, desmosterol and lanosterol, but did not react with α -hydroxysterols (Elias *et al.*, 1979). In thin sections, affected membranes appear prominently scalloped (Fig. 2). Upon binding to sterols, filipin fluoresces at a wavelength similar to that of fluorescein.

Methodology

One practical manner of applying filipin is to solubilize the powder (available from Upjohn Co., Kalamazoo, Michigan) in dimethylsulphoxide (DMSO), then add it to a mixture of buffered glutaraldehyde, 4% sucrose, 0.05% calcium chloride, and 1% DMSO, at pH 7.4 (Elias *et al.*, 1979; Friend, 1980). Next, we fix the cells and tissues in the solution at 22° C for 1 h before further processing for freeze-fracturing or embedding for thin sections, both with the use of routine techniques.

Purification of filipin, if desired, can be readily accomplished by thin-layer chromatography in chloroform:methanol:water:acetic acid (60:35:4.5:0.5, by vol.), and the major band used (Elias *et al.*, 1979). For reaction with intracellular membranes, the fixation time is increased. Control solutions contain DMSO but no filipin.

Observations and discussion

The uses of filipin

Work in our laboratory has focused on three facets of filipin freeze-fracture cytochemistry: (1) the potential of filipin as a reagent for the quantitative measurement of plasma-membrane sterols; (2) its capacity for mapping sterol distribution in a plane of the plasma membrane; and (3) its utility in detecting changes in membrane-sterol distribution preceding a biological event such as membrane fusion. Moreover, by virtue of its diminutive size (570 daltons) and the presence of sterols in essentially all cell membranes, filipin can also be effectively used as a permeability tracer perceptible in fractures and comparable to lanthanum nitrate, for example, as an electron-dense permeability tracer in thin sections.

Filipin as a permeability tracer

The use of filipin as a permeability tracer is perhaps its simplest application, since this is a situation unconcerned with minor movements of sterol or sterol-filipin complexes in the plane of the membrane, but merely depends on the fact that the reagent has had access to a membrane in a given compartment – the typical geometry of the complex within the membrane directly indicating that exposure to filipin has transpired. For example, the normal Sertoli–Sertoli cell junction composing the mammalian blood–testis barrier is impermeable to filipin perfused together with fixative through the testicular artery, since aggregates only arise on the basal vascular side of the junction (Fig. 3). After altering the permeability of the junction (for instance, after feeding the experimental contraceptive agent gossypol to guinea pigs) filipin–sterol perturbations occur between the junctional strands and within compartments formerly protected by the junction (Fig. 4) (Pelletier & Friend, 1980).

Quantitative aspects

To test the potential of filipin as a reagent for assessing the quantity of sterol in a membrane, we combined it with *Drosophila* larval cells (Kc and Schneider lines) depleted of cholesterol and after the cell membranes had incorporated various amounts of serum lipoprotein and ethanol-dissolved sterols (Havel *et al.*, 1980). Examined in freeze-fracture images, the number of filipin–sterol aggregates was found to be proportional to the amount of sterol incorporated (the precise amount incorporated was determined by enzymatic fluorometry and thin-layer chromatography) (Havel *et al.*, 1980). Plotting the μmol of sterol thus established against the number of protrusions seen in replicas results in a sigmoid curve. The values from a representative experiment are recorded in Table 1. An increasing number of protuberances is revealed by Figs. 5–8 at progressively higher sterol concentrations. Above 7.5 mg sterol/g cell protein, no further increments were noted in the quantity of protuberances (800–1100)/ μm^2 .

We, therefore, believe that developing the appropriate curves from plotting sterol concentration versus the number of protrusions/ μm^2 will allow the application of filipin for quantitative purposes in any given system, provided that the membrane is less than fully saturated with complexes.

Filipin mapping of sterol concentration

With this caveat of quantification in mind, we turn to mapping the relative sterol concentration in a single cell membrane with distinctive morphological and functional domains – the guinea-pig spermatozoon (Friend, 1977). Here our interest centres on (1) the anterior portion of the head, where the acrosomal and plasma membranes fuse during the acrosome reaction; (2) the post-acrosomal area of the plasma membrane, where fusion between sperm and egg will eventually take place; (3) the cytoplasmic droplet region of the midpiece, which may incorporate cytoplasmic microcisternae during preparation for the two above-mentioned fusions; and (4) the stable principal piece of the tail (Fig. 9). In sperm removed from the tail of the epididymis, abundant filipin–sterol complexes can be seen in the plasma membrane between the geometric lines of the quilt on the anterior portion of the head (Fig. 10). Few complexes are present in the post-acrosomal segment (Fig. 10), while many are observed in the retained cytoplasmic droplet (Fig. 11). The zipper of the tail's principal piece is entirely spared (Fig. 12). The major differences in sterol concentration in diverse regions are also clearly demonstrable by fluorescence microscopy.

The message here is that whereas sterol and sterol–filipin aggregates can move in the plane of the membrane, even after glutaraldehyde fixation (Robinson & Karnovsky, 1980), in this cell, there are distinct regional variations in the number of complexes. Therefore, some constraints to the lateral mobility of sterols must exist in the membrane; and what these constraints *are* remains an intriguing biological puzzle (Friend, 1980).

Focal alterations in sterol concentrations preceding fusions

Nevertheless, while incubating sperm *in vitro* to prepare them for the acrosome reaction, local changes in membrane sterol–filipin protuberances do become evident (Friend, 1980). As the quilt pattern disbands, filipin–sterol complexes spread throughout the acrosomal-cap portion of the head (Fig. 13). Where fusions between the acrosomal and plasma membranes will occur, foci of membrane clear of both filipin–sterol aggregates (Kitajimae *et al.*, 1976) and intramembranous particles (Figs. 14, 15) (Friend *et al.*, 1977; Friend, 1980). The same changes in pattern happen in the retained cytoplasmic droplet, where microcisternae may become incorporated into the plasma membrane (Friend & Heuser, 1980). The stable portions of the membrane, the post-acrosomal segment (Fig. 13) and principal piece, maintain their former, precapacitation filipin–sterol distributions. Following the addition of calcium to the medium, the fusions proceed (Friend *et al.*, 1977).

Our interpretation of these observations venture that sterols and then proteins move from the hydrophobic core of the membrane during intimate membrane apposition before membrane fusion. This well fits some models of protein dispersal advanced for the early molecular events of membrane fusion – we add to that the deletion of unesterified sterols.

Further considerations

Although the foregoing observations are seductive, the fact that fixatives, membrane-perturbing agents, and glycerol were applied to the cells limits the interpretive certainty of membrane clearings and calls for the provision of ancillary evidence. We have approached the gathering of confirmatory data about the reality of cleared patches preceding fusion by

two routes: (1) by applying rapid freezing (Heuser *et al.*, 1976, 1979) to unfixed, non-cryoprotected cells, and (2) by using an anionic lipid detector, polymyxin B, which can also be effectively employed in unfixed cell (Bearer & Friend, 1980).

Pertinent points of the new data we have obtained are as follows: in capacitated guinea-pig sperm frozen rapidly at liquid helium temperatures, clear circles are still visible in the membrane, identical to those observed in fixed, glycerinated tissue (Fig. 17). Many of these circles, however, particularly in the area of plasma membrane covering the cytoplasmic droplet, display an 8.4 nm 'grid-iron' orthogonal-lattice pattern (Figs. 18, 19). The same design appears in fractures of the underlying microcisternae. Because the plane of the lattice is comparable to that of the surrounding lipids in bulk phase, we interpret the crystalline domain to be composed of lipid. We think that it may even represent an intermediate stage of fusion, in which the inner leaflet of the plasmalemma after the normally intervening leaflets have dissipated. In any event, the sharply demarcated boundaries between the crystalline patch and the fluid lipid phase of the membrane present an unstable interface, subject to perturbation upon exposure to calcium ions.

In this guinea-pig model, rapid freezing supports the contention of circular lipid domains developing before fusion. Observations such as these have not been reported in other secretory cells (Heuser *et al.*, 1979).

The second approach, that is, using polymyxin B membrane perturbation to indicate the presence of cardiolipin and phosphatidylethanolamine in the guinea-pig sperm membrane, discloses that the clear (and sometimes crystalline) patches are rimmed by these particular acidic phospholipids but do not contain them (Fig. 16). Interestingly, the lipids bind calcium, and calcium competes with polymyxin B for these same binding sites.

Conclusions

Used in conjunction with glutaraldehyde fixation and freeze-fracture replication, filipin is an effective reagent for localizing β -hydroxysterols in the plane of the membrane, where it can be utilized for quantitative purposes and it may be employed as a permeability tracer. Preceding membrane fusion, circular patches emerge in closely apposed membranes. Seemingly, they are devoid of sterols and proteins. These patches may contain lipids in a crystalline state and may be surrounded by highly concentrated acidic phospholipids. The results are sufficiently encouraging to stimulate the search for other agents which may be used as cytochemical probes in conjunction with freeze-fracture replication.

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Fig. 1.
Freeze-fracture preparation of filipin/sterol complexes in a plasma membrane.

Fig. 2.
Scalloped plasma membrane of a filipin-treated cell.

Fig. 3.
The normal Sertoli-Sertoli cell junction impedes filipin permeation.

Fig. 4.
Filipin has access to intrajunctional compartments in a permeable Sertoli-Sertoli cell junction.

Fig. 5-8.
Plasma membrane of *Drosophila* larval cells containing 2×10^{-4} , 1.5×10^{-3} , 5.9×10^{-3} , and $18.4 \times 10^{-3} \mu\text{mol sterol/g protein}$.

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Fig. 9.
Diagram of a guinea-pig spermatozoon.

Fig. 10.
Filipin/sterol complexes are abundant in the isles of the quilt pattern in the acrosomal cap and sparse in the post-acrosomal segment (PA).

Fig. 11.
The cytoplasmic droplet.

Fig. 12.
The zipper of the principal piece lies in a filipin-sterol complex furrow.

Fig. 13. After capacitation, filipin-sterol protuberances dominate the acrosomal cap portion of the plasma membrane. Complexes remain sparse in the post-acrosomal segment (PA).

Fig. 14.
Circles (arrows) cleared of filipin–sterol complexes develop in areas of close apposition between the plasma acrosomal membranes (not shown).

Fig. 15.
These areas are also bereft of intramembranous particles.

Fig. 16.
Polymyxin B perturbations flank the band of membrane in which the cleared circles develop.

Fig. 17.
Rapidly frozen, capacitated sperm display cleared areas (arrows) on the cytoplasmic droplet.

Fig. 18.
Some of the cleared circles are crystalline.

Fig. 19.

A computer-assisted reconstructed image of the crystalline patch in Fig. 18 reveals a 8.4 nm lattice pattern.

Table 1

Representative values of sterol incorporated into *Drosophila* larval cells and the number of protrusions observed in replicas.

<i>Growth conditions</i>	<i>Sterol/cell protein (mg/mg)</i>	<i>Sterol/protein ($\mu\text{mol/g}$)</i>	<i>Protrusions (μm^2)</i>
Without cholesterol	0.05–0.11	2×10^{-4}	0.14
Cab-0-Sil-treated serum	0.38–0.78	1.5×10^{-3}	16
Lipoprotein-poor serum	1.21–3.4	5.9×10^{-3}	127
Complete foetal calf serum	6.7–7.5	18.4×10^{-3}	812