down that petiole; the maximum rate observed for air was 60 ml/min (*5*). The significance of this diurnal pattern of oxygen transport is reflected in the pattern of oxygen abundance in the rhizome, often varying from less than 10 percent O_2 at night, to ambient (21 percent O_2) during daylight. Isotope experiments have shown that most of this O_2 originates in the atmosphere, not in photosynthesis (*12*).

The efflux of gas through the petioles of the older emergent leaves also has physiological and ecological significance. It carries CO_2 from the rhizome (often exceeding 3 percent during daylight) to those leaves. Experiments with $^{14}CO_2$ have shown that most of this CO_2 is fixed by photosynthesis (*12*). This flow also carries ecologically significant quantities of methane from the lake sediment to the atmosphere (*13*), and explains the diurnal pattern of CH_4 efflux previously reported (*13*).

Further studies are necessary to determine how widespread this circulation phenomenon is among plants. Earlier data on pressurization in other plant leaves (*6*) suggest that similar flow patterns may occur in them. The purely physical basis of the pump suggests that such pressure differentials may be common in other plants, although the magnitude and significance of the phenomenon is certain to be highly variable. Such a ventilation system would be most advantageous wherever plant parts are buried in anaerobic soils.

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8. When the pores are small (less than about 0.1 μm at 1 atm total pressure), the flow of gas is predominantly diffusive [L. B. Loeb, *The Kinetic Theory of Gases* (Dover, New York, 1934); E. H. Kennard, *Kinetic Theory of Gases* (McGraw-Hill, New York, 1938)]. Mass flows of the gas mixture do not occur until the pore size increases. As the pore size enlarges, any tendency for diffusive processes to generate gradients in total pressure is offset by mass flows that dissipate those gradients. The pressure gradients sustained by the young leaves of *Nuphar* denies the importance of mass flows between these leaves and the atmosphere: their exchanges must be essentially diffusive. On the other hand, the loss of resistance to mass flow in the older leaves demonstrates their increased porosity.
9. A temperature differential across such a diffusive partition leads to a pressure gradient due to differences in diffusion rates on the two sides, so that $p_1/p_2 = (T_1/T_2)^{1/2}$ [see references in (*8*)].
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11. Gas exchanges between the lacunae of *Nuphar* and the atmosphere occur through the epidermis and palisade parenchyma of the upper surfaces of the leaves [C. D. Sculthorpe, *The Biology of Aquatic Vascular Plants* (Arnold, London, 1967)]. My study of stomatal apertures in young leaves suggests that they are too large to present the limiting porosity. The flow-restricting porosity must therefore lie between the cells of the palisade tissue. A general feature of leaf growth in dicotyledons may be that during the later stages of development, the palisade cells cease dividing and enlarging before the overlying epidermal cells cease enlarging. This results in an expansion of the intercellular spaces in the palisade tissue [K. Esau, *Plant Anatomy* (Wiley, New York, 1953)]. This is the most probable explanation of the changing porosity of *Nuphar* leaves, since the declining ability of individual *Nuphar* leaves to sustain pressures was accompanied by expansion of the leaf blade area.
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14. I thank M. J. Klug for his support of this research under NSF grant DEB-78-05321; and K. Hogg Dacey for her help in all phases of this research. This is publication 380 of the W. K. Kellogg Biological Station; and publication 9065 of the Michigan Agricultural Experiment Station. The final manuscript was prepared at Woods Hole Oceanographic Institution.
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Opposite Polarity of Filipin-Induced Deformations in the Membrane of Condensing Vacuoles and Zymogen Granules

Abstract. *Filipin binding to membrane sterols induces deformations of the membrane that are detected by freeze-fracture either as 20- to 25-nanometer protuberances or as pits on the fracture faces. By using the filipin probe in pancreatic acinar cells, it was found that the polarity of filipin-induced deformations in the membrane limiting the Golgi condensing vacuoles is opposite that in the membrane limiting the mature zymogen granules. This asymmetry could be due to unequal partitioning of cholesterol between the membrane leaflets in these two compartments during the transformation of the condensing vacuole into the zymogen granule.*

Lipid asymmetries have been predicted in biological membranes (*1*) and have been found in a number of systems [(*2*); for reviews, see (*3*)]. Such asymmetries have been detected by biochemical and biophysical techniques; nonpenetrating reagents were used that label the outside leaflet of the membrane when the membrane is sealed, and both leaflets when the membrane is unsealed. Biochemical and biophysical approaches average information about the inside and outside leaflets of all the membrane within a preparation. If a membrane preparation is heterogeneous, differences between the components go undetected. To obtain information about the heterogeneity of a membrane preparation, morphological approaches must be used. Some attempts along these lines have been made with freeze-fracture followed by autoradiography, but this technique is laborious and fraught with technical problems (*4*). Morphological labels are needed that are capable of marking different lipid components between the leaflets. Such markers exist for charges (for example, cationized ferritin), antigenic components (the peroxidase-antiperoxidase and protein A–gold techniques), and carbohydrates (lectins). To date, however, no such marker has been claimed to label

Table 1. Quantitative evaluation (*8*) of the polarity of filipin-induced deformations (protuberances or pits) in the fracture faces (P and E leaflets) of zymogen granule and condensing vacuole membranes. Values are numbers of protuberances or pits ± standard errors of the mean per 1.0 μm^2 of membrane face; N is the number of exposed faces studied.

	P face			E face		
	Protuberances	Pits	N	Protuberances	Pits	N
Zymogen granules	0.24 ± 0.17	195.6 ± 14.6	25	182.8 ± 14.8	2.0 ± 1.1	43
Condensing vacuoles	153.4 ± 19.3	14.5 ± 3.2	29	11.8 ± 5.2	144.9 ± 19.3	16

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any lipid or class of lipids with respect to the leaflet of the membrane in which it resides.

Binding of filipin to membrane cholesterol and related 3β-hydroxysterols (*5*) results in the formation of filipin-sterol complexes, which produce characteristic deformations of the membrane that are asymmetric with respect to the plane of the membrane (Fig. 1A). Filipin thus may be useful for labeling cholesterol asymmetries in membranes. We report here that the polarity of filipin-induced deformations changes between the condensing vacuole and zymogen granule membrane in the exocrine pancreas (*6*).

Figure 1, B and C, shows the appearance in freeze-fracture replicas of the condensing vacuole and zymogen granule membrane after filipin treatment (*7*). Figure 1B shows the characteristic irregular shape of two condensing vacuoles and the polarity of the filipin-induced deformations in the P (concave) and E (convex) leaflets of the membrane. Filipin-induced deformations appear as 20- to 25-nm protuberances or pits on the fracture faces; the E leaflet has more pits and the P leaflet has more protuberances. Protuberances are easily distinguished from the smaller intramembrane particles (probably representing proteins) that are numerous on the vacuole P face. Figure 1C shows the pattern of filipin-induced deformations in the zymogen granule membrane; the pattern is the reverse of that in the condensing vacuole, since the E leaflet has a high incidence of protuberances while the P leaflet has a similarly high incidence of pits. The P membrane leaflet of the zymogen granule is poor in particles. Among the various intracellular membranes observed in filipin-treated acinar cells, the zymogen granule membrane has the highest content of filipin-induced deformations and the most asymmetric distribution of these deformations between the two leaflets: there are virtually no pits on the E face and no protuberances on the P face of zymogen granules. Fractures of the condensing vacuoles show a broader range of polarities; the majority of condensing vacuoles contain mostly pits on the E face (and protuberances on the P face) but others have a mixture of protuberances and pits on both faces. A quantitative evaluation (*8*) of the polarity of filipin-induced deformations in these two organelles is shown in Table 1.

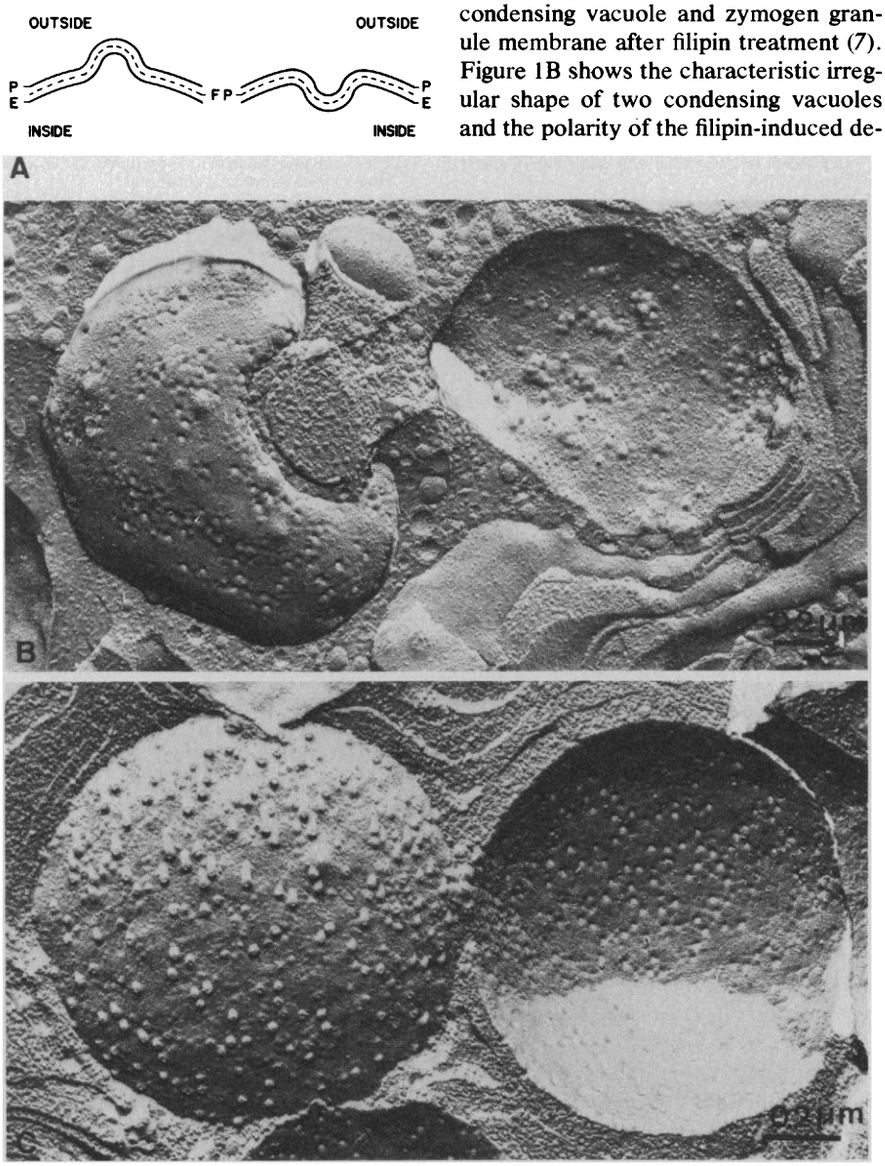

Fig. 1. (A) Schematic drawing of the membrane deformation induced by filipin binding to membrane sterols. The deformation can occur toward the outside or toward the inside of a membrane-bound vesicle such as a condensing vacuole. Outside the vacuole is the cytosol, inside the secretory proteins. (At the plasma membrane level, outward deformation occurs towards the extracellular space, inward deformation toward the cytosol.) On freeze-fracture, the deformed membrane cleaves along the dotted line (*FP*, fracture plane). The E membrane leaflet (facing the inside of the vacuole) will show a protuberance if the deformation is outward, a pit if the deformation is inward: conversely, the P membrane leaflet (facing the cytosol) will contain a pit if the deformation is outward, a protuberance if the deformation is inward. (B and C) Freeze-fracture replicas of rat acinar cells after filipin treatment. (B) Convex E membrane leaflet of a condensing vacuole and concave P membrane face of another condensing vacuole. Direction of platinum shadowing is from the bottom of the picture; with this orientation, it is evident that the E face is deformed by numerous pits and occasional protuberances, while the P face is deformed by numerous protuberances and occasional pits (× 43,000). Note that in (B) and (C) pits appear white on the bottom and black on top, protuberances black on the bottom and white on top. (C) Convex E membrane leaflet of a zymogen granule showing numerous protuberances on the fracture face, and concave P membrane leaflet of a neighboring granule dotted with numerous pits (× 54,000).

The results reported here show a change in the polarity of filipin-induced deformations associated with the transformation of condensing vacuoles into zymogen granules. One possible cause is asymmetric delivery of the marker to the membrane. The results indicate that the direction of delivery of filipin is not critical in determining the polarity of filipin-induced deformations; both the condensing vacuole and the zymogen granule are closed-membrane vesicles and filipin must enter the membrane from the P leaflet in both cases. The change in polarity of filipin-induced deformations between the condensing vacuole and the zymogen granule must therefore reflect alteration of the membrane (or something intimately associated with the membrane) during the transformation of condensing vacuoles into zymogen granules. Another possible cause is a difference in the medium surrounding the membrane. In both the condensing vacuole and the zymogen granule, the cytosol bathes the P leaflet. The difference in the interior of the two compartments (facing the E leaflet) is the degree of con-

densation (concentration) of the secretory product. While this difference in concentration cannot be ruled out as the source of the observed asymmetry, it does not seem the most likely mechanism at present.

Since filipin binds to cholesterol, a possible interpretation of the change in polarity of filipin-induced deformations between the condensing vacuole and the zymogen granule membrane is that it is caused by a change in cholesterol partitioning between the two leaflets accompanying the transformation of condensing vacuoles into zymogen granules. If this is so, filipin may be the first morphological marker of membrane lipid asymmetry, and the relation between structural polarity and cholesterol partitioning polarity should be established.

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6. Condensing vacuoles represent a station at which secretory pancreatic enzymes migrating from their site of synthesis (the rough endoplasmic reticulum) to their site of release (the plasma membrane) start to be concentrated; zymogen granules, deriving from condensing vacuoles, are intracellular storage sites for enzymes until they are released by exocytosis [reviewed in G. Palade, *Science* **189**, 347 (1975)].
7. Fragments of rat pancreas were minced into very small pieces (< 1 mm), fixed in 2 percent glutaraldehyde in 0.1*M* cacodylate buffer, *p*H 7.4, for 60 minutes, and further incubated overnight at room temperature in the same buffer containing filipin (0.2 mg/ml; provided by J. E. Grady, Upjohn Co.) dissolved in dimethyl sulfoxide (final concentration of DMSO, 1 percent). After exposure to filipin, the pieces of pancreas were washed in 0.1*M* cacodylate buffer, soaked in the same buffer containing 30 percent glycerol (by volume) for 2 hours, and freeze-fractured at −110°C in a Balzers BAF 301 apparatus. Replicas were cleaned with sodium hypochlorite followed by dimethyl formamide, rinsed in distilled water, and recovered on Parlodion-coated copper grids (150 mesh). Freeze-fracture replicas were examined in a Philips EM 300 electron microscope.
8. We evaluated 36 micrographs of replicas showing fracture faces of zymogen granule and condensing vacuole membrane at a suitable magnification. The surface area and number of deformations (pits and protuberances) on regions of fractured membrane that appeared to be roughly perpendicular to the viewing axis were recorded on a graphic tablet (Tektronix, type 4953) connected to a microprocessor (IMSAI, type 8080) programmed to calculate the number of protuberances (or pits) per square micrometer of membrane.
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Stimulation of DNA and Collagen Synthesis by Autologous Growth Factor in Cultured Fetal Rat Calvaria

Abstract. *Conditioned medium derived from organ or cell cultures prepared from 19- to 21-day fetal rat calvaria stimulated the incorporation of [^{3}H]proline into collagen and of [^{3}H]thymidine into DNA in organ cultures of the same tissue. Addition of cortisol enhanced the effect on collagen but not on DNA synthesis. These effects appeared to be due to a nondialyzable and heat-stable growth factor.*

The regulation of bone remodeling is incompletely understood. The importance of local factors is suggested by the close linkage between resorption and formation during remodeling and by skeletal responsivity to deforming forces. Since nonskeletal tissues and cells elaborate growth factors when maintained in culture in vitro (*1*, *2*), we have searched for such regulatory factors in bone. We report that conditioned medium obtained from fetal calvaria in organ or cell culture contains a growth factor which stimulates the incorporation of [^{3}H]proline into bone collagen in the presence of cortisol and increases the incorporation of [^{3}H]thymidine into DNA both in the presence and absence of cortisol.

The growth factor was obtained from the incubation medium of fetal rat calvaria maintained in organ or in cell culture according to techniques previously reported (*3*, *4*). In the organ culture model, half-calvaria from 21-day fetal rats were cultured in BGJ_b medium (*3*) without serum or albumin for periods of 96 hours in a shaking incubator. The medium was changed every 24 hours, pooled, and stored at 4°C. In the cell culture model, cells from 19- to 21-day-old fetal rat calvaria were dispersed by incubation in crude collagenase (Worthington Biochemical) after we removed the periosteum. Cells were washed and cultured at an initial density of 10^5 cells per square millimeter of surface area in modified BGJ_b medium without serum or albumin (*5*). The medium was replaced at 24 hours of culture and every 2 to 4 days thereafter. The medium from 6- to 12-day-old cultures was pooled and stored at 4°C.

Conditioned medium was dialyzed exhaustively against distilled water or 0.05*M* acetic acid at 4°C and lyophilized. The conditioned medium from organ culture was further purified by gel filtration chromatography. Ten milligrams of lyophilized, dialyzed, conditioned medium containing 5 mg of protein (*6*) were obtained from 100 ml of medium and chromatographed on a column of Sephadex G-75 (Pharmacia, Uppsala, Sweden) in 1*M* acetic acid at room temperature. Eluates were lyophilized before assay. Conditioned medium from cell cultures was purified by ion exchange chromatography by a modification of the method described by Dulak and Temin (*1*). Medium (500 ml) adjusted to *p*H 6.3 at room temperature was loaded onto a Dowex 50W-X8 column (4.5 by 30 cm; Bio-Rad), equilibrated with 0.15*N* NaCl, and eluted with a sequential gradient of 0.1*N* sodium carbonate and 0.15*N* NaCl (*p*H 9), 0.1*N* ammonia and 0.15*N* NaCl (*p*H 11), and 1*N* NaOH and 0.15*N* NaCl. The eluates were dialyzed and lyophilized before assay.

The effects of the growth factor on bone formation were studied in organ culture as previously described (*3*). Half-calvaria from 21-day fetal rats were cultured in modified BGJ_b medium supplemented with bovine serum albumin (1 to 4 mg/ml; Reheis Chemical), 1 m*M* proline, and 0.1 m*M* thymidine under 5 percent CO_2 in a shaking incubator at 37°C for 24 hours. Collagen and noncollagen protein synthesis were studied by adding [2,3-^{3}H]proline (5 μCi/ml; specific activity 20 to 30 Ci/mmole; New England Nuclear) for the last 2 hours of the culture period. The calvaria were extracted with trichloroacetic acid, acetone, and ether;

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