Membrane organization in the preimplantation mouse embryo

HESTER P. M. PRATT

Department of Anatomy, Downing Street, Cambridge, CB2 3DY, U.K.

SUMMARY

The preimplantation mouse blastocyst consists of two differentiated tissues, the trophectoderm (a structurally and functionally polarized epithelium) and the inner cell mass. The divergence of these two cell types can be traced back to a contact dependent polarization of the surface and cytoplasm at the 8-cell stage. Membrane/cytocortical organization during this preimplantation period has been studied using freeze fracture in conjunction with the sterol-binding antibiotic filipin in an attempt to discern the molecular basis and origin of these surface asymmetries.

The distribution of filipin reactivity within the different membrane domains showed that the surface polarity exhibited by trophectoderm and by blastomeres of the 8-cell stage is underlain by a heterogeneity in molecular organization of the membrane/cytocortex which may originate prior to the appearance of any overt surface polarity.

The results are discussed in terms of the likely basis of this membrane/cytocortical asymmetry, its probable origins and the use of the preimplantation mouse embryo as a model system for studying the assembly of a polarized epithelium.

INTRODUCTION

The integrated structure and coordinated function of many animal tissues depends upon an intracellular polarity of their constituent cells. This polarity is evident in the structure of both the cytoskeleton and cytoplasm as well as the organization of the surface membrane into biochemically distinct domains that are specialized for fluid or ion transport, endocytosis, adhesion etc. (Evans, 1980). The existence of contiguous but stable domains in a membrane where many components are free to diffuse laterally implies the presence of specific constraints to prevent intermixing. Such barriers to diffusion could be due to special lipid or protein interactions within the membrane, between the membrane and the cytoskeleton and/or between individual cells linked by specialized intercellular junctions or the extracellular matrix. The relative importance of these interactions during the assembly and maintenance of membrane asymmetry is currently being studied in intact and reassembled epithelia as well as epithelia infected with envelope viruses which bud from specific domains (Nitsch & Wollman, 1980;

Dragsten, Blumenthal & Handler, 1981; Reggio Coudrier & Louvard, 1982; Van Meer & Simons, 1982; Sabatini *et al.* 1983).

Cellular asymmetries are not only important in fully differentiated tissues but may also play a significant role in early development when differential inheritance of asymmetrically localized components can initiate or reinforce cell divergence (Johnson & Pratt, 1983). One particularly accessible example of this is the preimplantation mouse embryo where a contact-induced asymmetry of membrane and cytoplasm at the 8-cell stage is distributed between two daughter 16-cell-stage blastomeres to generate two phenotypically distinct subpopulations. There is good evidence to suggest that in the undisturbed embryo these two subpopulations form the foundation of the two primary tissues of the blastocyst, the ICM (inner cell mass) and the trophectoderm, a structurally and functionally polarized epithelium (Johnson, 1985*a*).

The preimplantation mouse embryo therefore provides a system in which the development of two cell types (one polarized and the other apolar) can be traced in their entirety through six cell cycles *in vitro* and the organization of the intervening cell types studied. I chose to study this process at the level of the surface membrane since the cell surface is known to play a crucial role in early development (Johnson, 1985*b*) and the main pathways for membrane biogenesis and recycling in epithelia have been outlined (Reggio *et al.* 1982; Van Meer & Simons, 1982).

The application of freeze fracture replication of membranes to embryos provides approximately ten-fold higher resolution than scanning electron microscopy and I have used this technique in conjunction with the cholesterol-binding polyene antibiotic filipin (Severs & Robenek, 1983) to infer changes in the molecular organization of the membrane and cytocortex during cleavage. The main questions I have posed are the following: are the polarized membrane morphologies present at the 8-cell stage and in trophectoderm underlain by heterogeneity at the molecular level and if so does this heterogeneity increase in complexity as trophectoderm develops? Does molecular reorganization precede the overt morphological surface changes at any stage? The results of the freeze fracture cytochemical analysis are discussed in terms of these embryological questions as well as in the wider context of assembly and maintenance of asymmetry in other cell types.

MATERIALS AND METHODS

1. *Recovery of eggs and embryos*

Female HC-CFLP mice (Hacking & Churchill Ltd) aged 3–5 weeks were superovulated by intraperitoneal injections of 5 i.u. PMS (Folligon) followed 48 h later with 5 i.u. hCG (Chorulon) and caged overnight with HC-CFLP males if fertilized eggs or embryos were to be obtained. The presence of a vaginal plug the following morning was taken as an indication of successful mating.

Eggs (or oocytes from non-mated females) were removed from oviducts at approximately 20 h post hCG and freed of cumulus cells by brief exposure to 0·1 M-hyaluronidase (Sigma) followed by a rinse in medium 2 containing 4 mg ml^{-1} BSA (M2+BSA) (Fulton & Whittingham, 1978).

Fertilized eggs with two pronuclei and oocytes without pronuclei (i.e. that had not been activated) were selected for further analysis. Cleavage-stage embryos and blastocysts were flushed from oviducts or uteri using M2+BSA at the times (hours post hCG) indicated. ICMs were isolated from expanding blastocysts using immunosurgery as described by Johnson & Ziomek (1982). Mural trophectoderm was obtained by removing ICM and polar trophectoderm from 'giant' blastocysts which were generated by aggregating several 8- and 16-cell embryos together. The zonae were removed from the 8- to 16-cell embryos by brief exposure to acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975), followed by a rinse in M2+BSA. They were then aggregated together in groups of ten to twelve using a 1:20 dilution of stock phytohaemagglutin (PHA, Gibco Laboratories, Grand Island Biological Co., Grand Island, N.Y.) and cultured to the expanded blastocyst stage in medium 16 plus $4\,mg\,ml^{-1}$ BSA (M16+BSA, Whittingham, 1971) at 37°C in 5 % CO_2 in air. Microsurgery was conducted under a binocular dissecting microscope using a scalpel blade.

The role of the cytoskeleton was examined by incubating embryos in cytochalasin D ($0\cdot5\,\mu g\,ml^{-1}$), colcemid ($5\,\mu g\,ml^{-1}$) or low calcium medium (Pratt, Ziomek, Reeve & Johnson, 1982) for 4 to 6 h prior to fixation.

Embryos (with intact zonae) were fixed for freeze fracture in duplicate groups of 30–40 embryos in either the presence or absence of filipin. Early experiments also included a control containing the appropriate concentration of dimethylsulphoxide (DMSO). A 30 mM solution of filipin ($19\cdot6\,mg\,ml^{-1}$ U-5956 (complex) Reference 8393-DEG-11-8, a gift from Dr Joseph E. Grady, The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.) was made up in DMSO and immediately diluted 1:100 to 300 μM into a fresh solution of 3 % glutaraldehyde in 0·1 M-sodium cacodylate pH 7·3. Control samples were fixed in buffered glutaraldehyde alone or 1 % DMSO in glutaraldehyde. Samples were fixed overnight at room temperature in the dark. Tomatin (Serva Heidelberg, Germany) was used in an identical manner to filipin at a final concentration of $150\,\mu g\,ml^{-1}$. Following fixation embryos were washed in 0·1 M-sodium cacodylate (pH 7·3) and stored in the same buffer at 4°C.

2. *Freeze fracture replication of embryos*

In order to ensure that the maximum surface area was exposed to the freeze cleaving knife the embryos were embedded in gelatine on the flat, square bases of Beem capsules. This was achieved under a dissecting microscope by pipetting the fixed embryos (30 to 40) into a group on the base of the Beem capsule withdrawing the excess buffer and replacing it carefully with warmed 20 % gelatine in 0·1 M-sodium cacodylate pH 7·3. When the gelatine had set, the blocks were removed from the capsules and cryoprotected by infiltration overnight at 4°C with 25 % glycerol in 0·1 M-sodium cacodylate pH 7·3. Samples were then trimmed to fit the specimen mounts of a Balzers BAF 300 freeze fracture machine. The specimen table was at −100°C and the sample cut by a knife maintained at −180°C in a vacuum of 5×10^{-7} mbar. Samples were etched for 3 min, shadowed at 45° with 2 nm platinum/carbon followed by deposition of a strengthening layer of carbon, 20 nm thick, evaporated perpendicular to the sample. Specimens were removed from the chamber, a drop of 0·6 % Formvar in chloroform added and then equilibrated to room temperature by thawing in frozen methanol. Replicas were released in 50 % sodium hypochlorite, cleaned in 100 % sodium hypochlorite, washed in three changes of double distilled water, mounted on 200 HS grids (Gilder) and the Formvar removed by exposure to chloroform vapour. Replicas were viewed on a Phillips 300 Electron Microscope fitted with a goniometer stage. The density of filipin-cholesterol complexes was quantified from micrographs taken at ×50 000 to 70 000 magnification. Measurements from freeze fracture replicas can be problematic. One potential source of error is distortion which may occur during fixation of the sample and preparation of the replica. Any obviously distorted samples were therefore eliminated from analysis. A second problem not so easily circumvented is the fact that the area of interest on the replica is unlikely to be orientated at right angles to the electron beam when loaded at zero calibration of the goniometer stage. This can lead to errors of ±20 % in point-to-point measurements and hence the estimated densities of filipin–cholesterol complexes (Table 1) are likely to be subject to similar errors. (For a more detailed discussion of freeze fracture methodology and quantitation of replicas see discussion by Navaratnam, Thurley & Skepper (1982).)
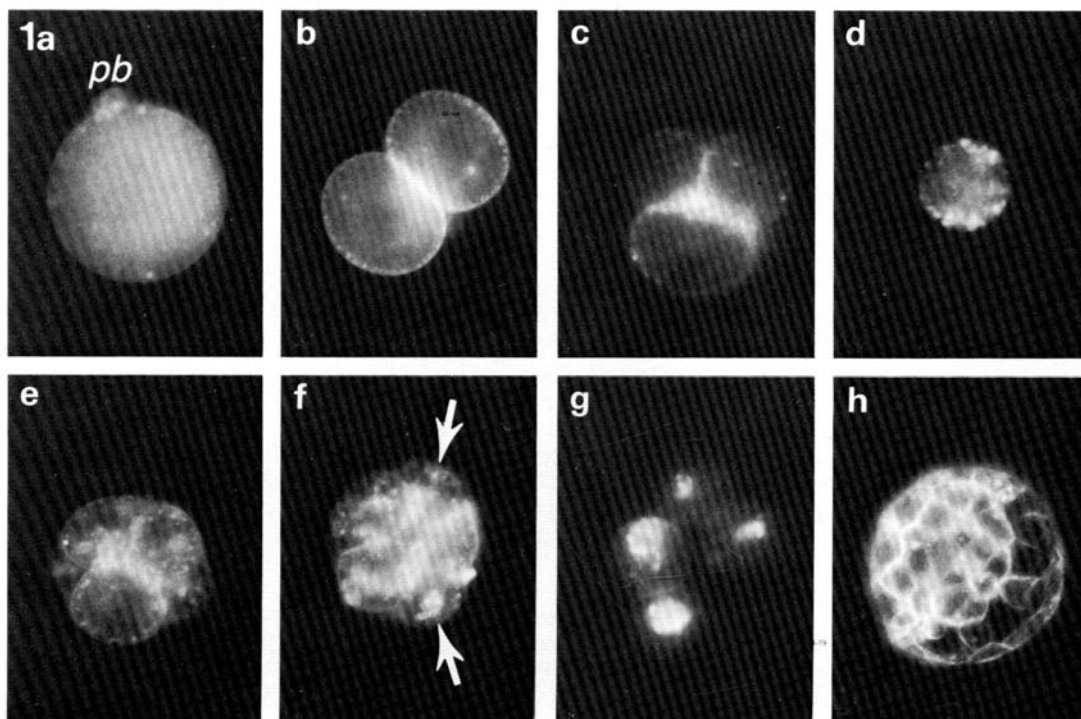
Fig. 1. Filipin fluorescence in fixed preimplantation mouse embryos (experimental details in Materials and Methods). (a) Unfertilized egg, *pb* = polar body. (b) 2-cell embryo. (c) 3-cell embryo. (d) Single blastomere from 4-cell embryo. (e) Non-compact early 8-cell embryo. (Note that the multivesicular aggregates or endosomes which are responsible for most of the cytoplasmic staining (see Results) are distributed randomly or peripherally at these early stages.) (f) Compact 8-cell. (g) Two pairs of blastomeres from a compact 8-cell. (Note that the multivesicular aggregates or endosomes (arrows f) have polarized in f & g.) (h) Blastocyst. Magnification ×340 (except g ×680).

3. *Nomenclature*

The two membrane fracture faces exposed are referred to as P (or protoplasmic) and E (or exoplasmic) faces throughout.

4. *Transmission EM*

Embryos were fixed in 3 % glutaraldehyde in 0·1 M-sodium cacodylate pH 7·3 at room temperature for 30 min, stained with 1 % osmium tetroxide, dehydrated through a graded series of alcohols and embedded in Spurr. Sections (30–40 nm) were stained with uranyl acetate and lead citrate and viewed with a Phillips E.M. 300.

5. *Filipin fluorescence*

The binding of filipin to cholesterol in embryo membranes was also assessed by taking advantage of the fluorescent properties of filipin (Hatten & Burger, 1979). Embryos fixed for freeze fracture were observed on a Zeiss epifluorescent microscope, incident source HBO 200 using the Zeiss Hoechst dye filter set No. 02 and photographed on Kodak Tri-X film (Fig. 1). The stock solution of filipin retained its cholesterol-binding properties for at least 3 days as judged by fluorescence of the embryos which was stable for at least a week at 4 °C. However the

stock solution of filipin was always prepared on the day of use and embryos were fractured within a few days of treatment.

RESULTS

During the freeze fracture process membranes are cleaved at random and the resulting replicas are only of use for analysis if they include features that enable the original orientation of the membrane within the embryo to be deduced. Membrane from regions of cell apposition is easily identified by transitions in the fracture plane between P and E faces as well as multilamellate, particle-free blebs of membrane which appear in these areas (discussed later). The presence of numerous microvilli is a marker for the apical surfaces of 8-cell blastomeres and trophectoderm while focal tight junctions and zonula occludens locate a replica within the basolateral membrane of 8-cell blastomeres or trophectoderm respectively.

Several preliminary experiments on the effects of filipin were undertaken to ensure reproducibility of action. The solvent (DMSO) alone had no detectable effect on embryos and the replicas obtained were identical to untreated controls. Increased age of the stock filipin solution decreased its capacity to interact with membrane cholesterol (as judged by both fluorescence (Hatten & Burger, 1979) and freeze fracture) and consequently all filipin solutions were made up freshly on the day of the experiment. Length of exposure and strength of solution were varied to ensure reproducible penetration and interaction which were assessed by effects on the membrane and intracellular organelles. The conditions chosen for all subsequent experiments were fixation in 300 μM-filipin in 2·5 % glutaraldehyde at room temperature overnight in the dark. Filipin–sterol complexes appeared in freeze fracture as circular protuberances (P face) or depressions (E face) approximately 25 nm in diameter (Fig. 6b) and as plications of the bilayer when observed in transverse section by TEM (Fig. 2c) (Elias, Friend & Goerke, 1979; Severs & Robenek, 1983).

The extent of membrane perturbation induced by filipin is not necessarily a direct measure of the amount of cholesterol present due to the existence of constraining mechanisms within membranes which may hinder the binding of filipin to cholesterol and the subsequent aggregation of these complexes to yield visible lesions (Severs & Robenek, 1983; Miller, 1984 and see Discussion). Nevertheless filipin-induced disruption of membranes of intracellular organelles can sometimes be related to their cholesterol composition (Severs & Robenek, 1983) and this seemed to be the case in embryos. Throughout preimplantation development membranes which were likely to be deficient in cholesterol (*e.g.* those of nuclei, mitochondria and secondary lysosomes) showed no reaction with filipin whereas cholesterol-rich membranes (*e.g.* multivesicular aggregates and other endocytic vesicles) were extensively disrupted. The plasma membrane on the other hand reacted heterogeneously with filipin, the extent and localization of the lesions being characteristic of a particular developmental stage. Quantitation
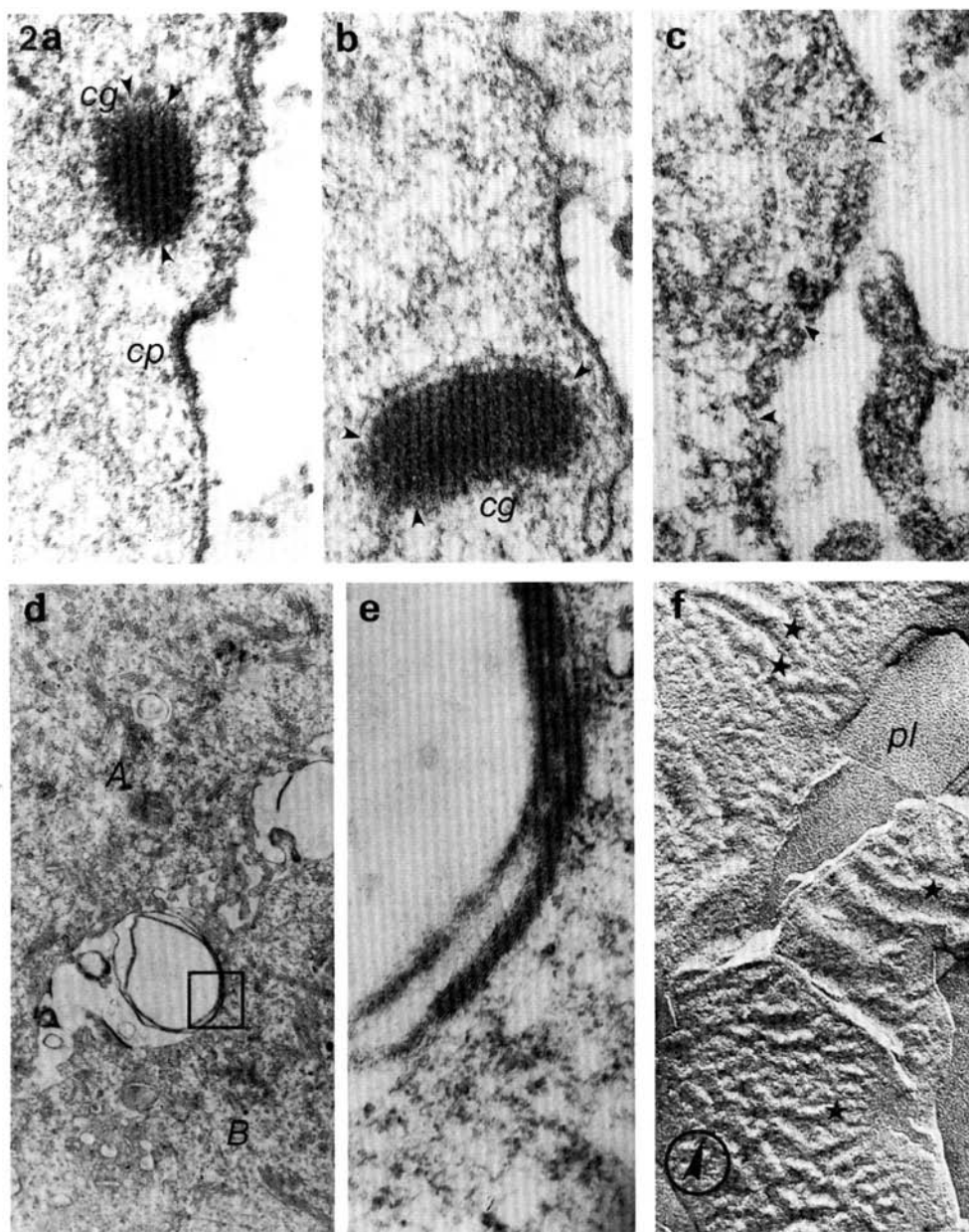
Fig. 2. (a,b) Unfertilized egg treated with filipin. Thin section showing intact plasma membrane and coated pit (*cp*). Arrows indicate some regions of cortical granule (*cg*) membrane which appear to be disrupted by filipin (×100 800). (c) Fertilized egg treated with filipin. Thin section showing regions of plasma membrane apparently disrupted by filipin. Arrows indicate areas of most prominent lesions (×100 800). (d,e) Region of cell apposition in control 2-cell embryo. Thin section of area between two blastomeres (*A* & *B*) showing membrane blebs and extrusions (×12 750). (e) High magnification of area indicated in (d) showing multilamellate nature of membrane blebs (×100 000). (f) Freeze fracture replica of basolateral region of polarized 8-cell blastomere treated with tomatin. Tomatin induces characteristic ridges (indicated by asterisks) in the regions of membrane that do not react with filipin. Plaques (*pl*) are negative (×100 800). Arrowhead indicates direction of shadowing.

of filipin–cholesterol complexes (Table 1) was undertaken as described in Materials and Methods and is subject to the limitations described there. Detailed observations on the surface membranes of control and filipin-treated embryos during development are described below.

Fertilized and unfertilized eggs

The majority of membrane replicas obtained from eggs bore microvilli and hence were inferred to be of membrane from the body of the egg rather than the smooth area overlying the meiotic spindle ('nipple') or enclosing the polar body (Eager, Johnson & Thurley, 1976). These microvilli appeared as stumps on the P face and cavities on the E face and were distributed in small clumps (Fig. 3a,b). No replicas devoid of microvilli and hence attributable to the 'nipple' or polar body membrane were obtained. There were no clear qualitative differences between untreated membranes from unfertilized (non-activated) and fertilized eggs but their reactivity with filipin differed markedly. Membrane from unfertilized eggs always exhibited a low density of filipin–sterol complexes (although these were often distributed non-uniformly within the membrane) whereas fertilized or activated eggs had such a high density of complexes that the membrane was frequently too disrupted to permit accurate quantitation of individual lesions (Fig. 3c,d,e and Table 1). Two potential contributors to this change in membrane organization were considered viz. the cytoskeleton and fusion of cortical granules. Disruption of microfilaments with cytochalasin D did not increase the density of filipin–sterol complexes in unfertilized eggs. On the other hand areas that could

Table 1. *Density of filipin–cholesterol complexes (fc) in surface membranes of preimplantation embryos*

Stage	(h post hCG) membrane domain	Mean no. of fc μm^{-2} $\pm$ s.d.*	(n)†
Unfertilized oocytes	(18–20)	89 ± 27	(7)
Fertilized eggs	(18–20)	315 ± 91	(7)
2 to 4 cells	(47–56)	380 ± 90	(12)
		158 ± 39	(5)
Compact 8-cell	(67–70)		
	Apical	370 ± 111	(6)
	Junctions‡	148 ± 43	(4)
	Basolateral	74 ± 26	(9)
ICM	(approx. 120)	89 ± 36	(12)
Trophectoderm	(approx. 120)		
	Apical	162 ± 44	(5)
	Zonula occludens	12 ± 4	(4)
	Basolateral	76 ± 54	(6)

*Since the replica is unlikely to have been orientated at right angles to the electron beam these values are subject to an error of approximately 20 % (discussed Materials & Methods).

† Number of replicas analysed – taken from at least two different samples.

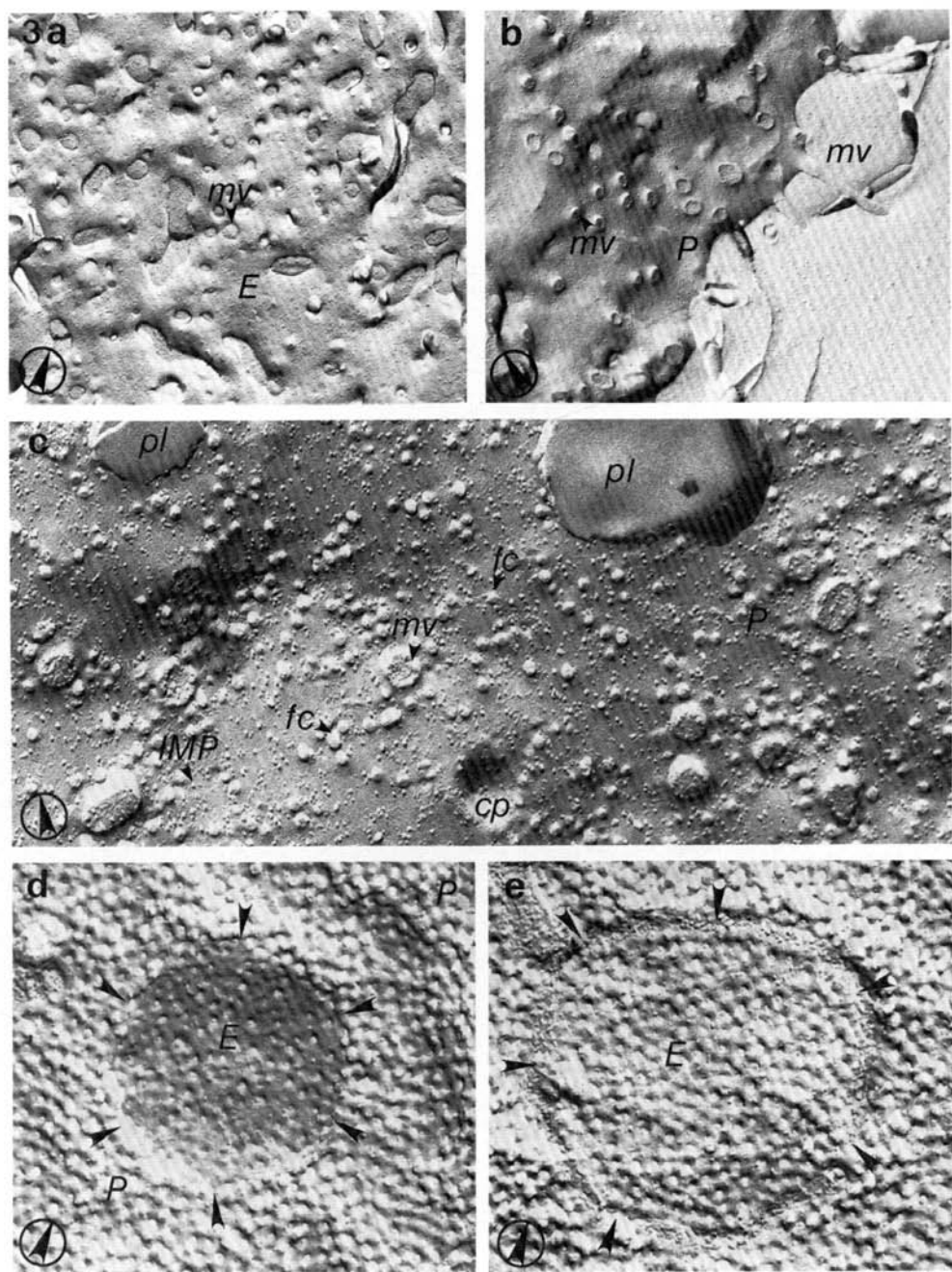‡ This refers to the region of membrane including and immediately surrounding a junction.

Fig. 3. Control and filipin-treated egg membranes. (a) Control unfertilized egg. Surface membrane E face (×2490). (b) Control fertilized egg. Surface membrane P face (×24 900). (c) Filipin-treated unfertilized egg. Surface membrane P face (×69 300). (d,e) Filipin-treated fertilized egg. Surface membrane showing possible areas of fusion (arrowheads) (×70 200). Arrowhead indicates direction of shadowing. P,E indicates P or E face. mv, microvilli; fc, filipin cholesterol complex; IMP, intramembranous particle; cp, coated pit; pl, 'plaque'.

correspond to fusion of filipin-reactive cortical granule membrane were found in fertilized eggs (Fig. 3d,e). When eggs were examined by TEM following filipin treatment areas of scalloped lesions, presumably corresponding to filipin–cholesterol complexes were observed to disrupt the membranes of fertilized (Fig. 2c) and to a lesser extent, unfertilized eggs (Fig. 2a,b). Furthermore, filipin abolished the surface integrity of cortical granules (Fig. 2a,b) consistent with the notion that their fusion with the egg membrane could contribute to the increase in filipin–cholesterol complexes observed after fertilization.

One minor feature of egg membrane replicas was the presence of small areas of membrane ('plaques') which were devoid of intramembranous particles (IMPs), were generally no more than two layers thick and did not react with filipin (Fig. 3c). 'Plaques' were located outside the cells since they were always super-imposed upon the P face but behind the E face and only visible through holes in it. These apparent membrane delaminations were more extensive at later develop-mental stages (discussed below) and were observed in material processed in the absence of either DMSO or filipin.

Cleavage-stage embryos

Membrane replicas from early cleavage-stage control embryos (2-cell and 4-cell) were microvillous and exhibited larger areas of IMP-free 'plaques'. These were found predominantly in areas of cell contact as judged by their disposition in thin sections (Fig. 2d,e) their presence in regions of transition from P to E faces and by the relative paucity of microvilli, and were more complex and multilamellate (in excess of eight layers being found frequently) than those of eggs (Figs 2e, 4a,b). Their derivation from the surface was shown by areas where there was continuity between the two forms of membrane (Figs 4a, 5c). Filipin treatment of 2-cell and 4-cell stage embryos revealed an increased complexity of surface membrane organization compared with that observed in eggs. Broadly, two types of mem-brane could be identified. One was microvillous and had an overall density of filipin–sterol complexes comparable to fertilized eggs (Fig. 4c and Table 1), the other exhibited a lower density of filipin cholesterol complexes (Table 1) and contained large areas of multilamellate 'plaques' which were free of both intra-membranous particles and filipin–sterol complexes (Fig. 4d,e). The presence of these plaques in areas where the fracture plane changed from P to E face indicated that this second domain originated from areas of cell apposition (Fig. 4b).

The surface membrane of blastomeres from untreated compacted 8-cell embryos showed clear demarcation into the domains observed by SEM (Reeve & Ziomek, 1981) namely a microvillous apical domain on the outside of the embryo which contained a high density of structures identified in other tissues as coated pits (Steer, Bisher, Blumenthal & Steven, 1984) and a basolateral inward-facing domain exhibiting few, if any, microvilli (Fig. 5). Multilamellate plaques were only found on the basolateral membrane (Fig. 5a,c,d) and were particularly extensive in regions where several blastomeres had been in contact with one another (Fig. 5a). This domain also contained small complexes of focal tight and gap
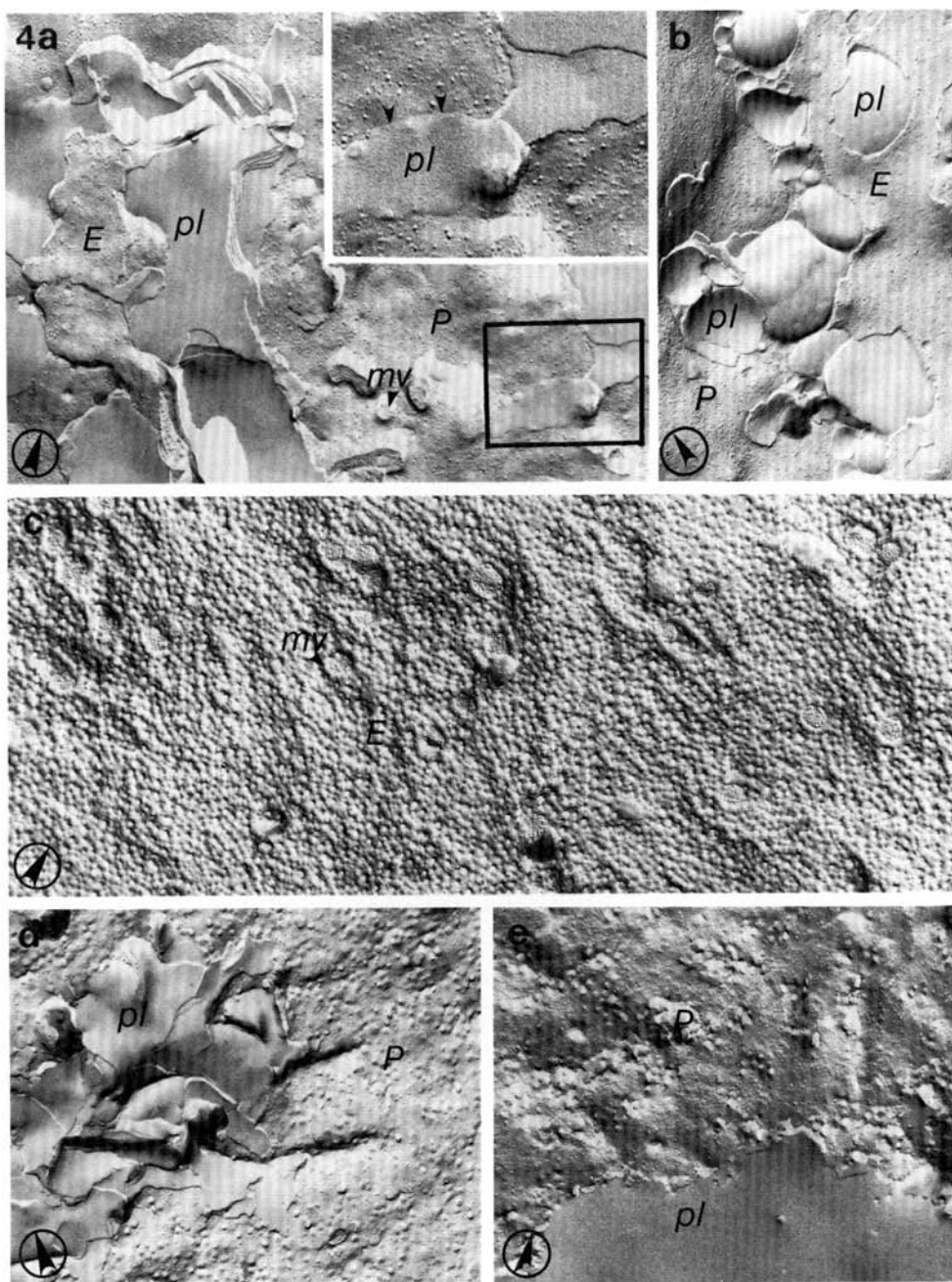
Fig. 4. Control and filipin-treated membranes of early cleavage stage embryos. (a) Control 4-cell embryo from an intercellular area showing multilamellate 'plaques' (×35 000). (Inset: higher magnification of indicated area showing continuity between membrane and 'plaque' (×70 380)). (b) Intercellular area from control 4-cell embryo showing transition between P and E faces and presence of 'plaques' between cells (×35 200). (c) Filipin-treated 4-cell embryo. Outward facing membrane showing high density of filipin complexes and microvilli (×35 200). (d,e) Intercellular region from filipin-treated 2-cell embryo showing that multilamellate plaques develop in areas of membrane reacting poorly with filipin (×35 200). Arrowhead indicates direction of shadowing. *P* & *E* indicates P or E face. *mv*, microvilli; *pl*, 'plaque'.
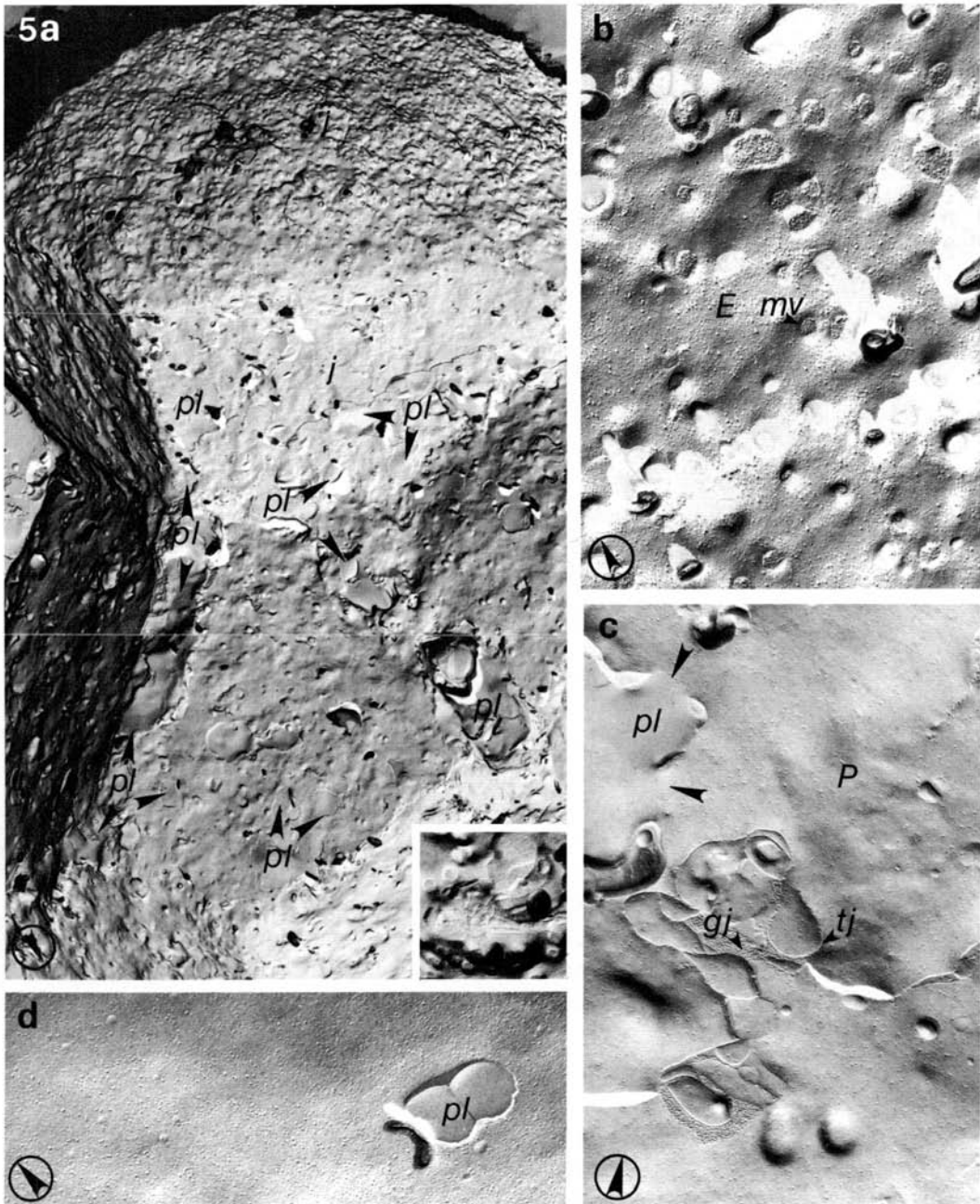
Fig. 5. Control compacted 8-cell embryo. (a) Portion of an 8-cell embryo including an intact blastomere showing apical and basolateral domains (upper portion of micrograph) and basolateral domains of other blastomeres. Note that microvilli are confined to the apical domain and 'plaques' are restricted to the basolateral regions. Focal tight and gap junction complexes (*j*) appear in both apical and basolateral regions at this stage (×4650). (Inset: higher magnification of junctional complex (*j*) from apical region (× 24 800). (b) Apical domain showing high density of microvilli (×35 000). (c) Region associated with focal tight and gap junctions (×35 200). Note continuity between 'plaque' region and the rest of the membrane (arrowheads). (d) Basolateral domain showing absence of microvilli (×34 680). Arrowhead indicates direction of shadowing. *P* & *E* indicate P & E faces. *pl*, plaques; *mv*, microvilli; *tj*, focal tight junction; *gj*, gap junction.

junctions (Fig. 5c). These two regions of membrane reacted differently with filipin.

The apical membrane reacted homogeneously and intensely (Fig. 6a) whereas the basolateral membrane showed a more heterogeneous distribution of filipin-sterol complexes ranging from a moderate concentration to virtually no reaction at all (Fig. 6c, Table 1). Within areas of filipin reactivity there was clear micro-heterogeneity mostly due to coated pits and fully formed or developing tight and gap junction complexes which were all devoid of filipin–sterol complexes (Fig. 6b). Multilamellate 'plaques' did not exhibit any filipin–sterol complexes, consistent with their behaviour at earlier cleavage stages. Although relatively unresponsive to filipin the basolateral region reacted extensively with another sterol-binding reagent, tomatin, (Fig. 2f) as reported for some other filipin-negative membranes (Severs & Simons, 1983). These observations confirm the existence of three major membrane domains in blastomeres of the compacted 8-cell embryo, an apical domain, a basolateral domain and a junctional domain. The influence of the cytoskeleton on the organization of apical and basolateral domains was investigated by assessing their reactivity to filipin following 8 to 12 h incubation in either cytochalasin D plus colcemid or a low calcium medium. Neither treatment abolished the difference in filipin reactivity between the two regions (data not shown).

The organization of outer membrane in the blastocyst was analysed by examining isolated inner cell masses and trophectoderm. As anticipated from previous TEM and SEM studies, replicas of untreated ICM membrane were almost devoid of microvilli and virtually featureless apart from coated pits, gap junctions (Fig. 7a,b,c) and multilamellate 'plaques' which again showed con-tinuity with the surface membrane. Trophectoderm membrane in contrast, was more complex and clearly demarcated into the three domains anticipated for this functional epithelium (Fig. 7d,e). The apical domain (Fig. 7d) was microvillous (though the density of microvilli was lower than on the apical region of 8-cell blastomeres) and separated from the non-microvillous basolateral region by the zonula occludens (Fig. 7e). Each type of membrane was characterized by a different form of reaction with filipin. Inner cell mass generally exhibited few filipin complexes (Table 1) and areas of gap junctions and coated pits were entirely devoid of them (Fig. 8a,b). This membrane also exhibited apparent 'phase separation' effects with small IMP-free patches surrounded by filipin–cholesterol complexes (Sekiya, Kitajima & Nozawa, 1979). Apical trophectoderm on the other hand reacted intensely with filipin (Fig. 8d,f) though the basolateral region gave a range of response with some areas negative and others exhibiting a density of filipin–sterol complexes equivalent to the apical region (Fig. 8d,e). The band of zonula occludens was entirely negative (Fig. 8d, Table 1).
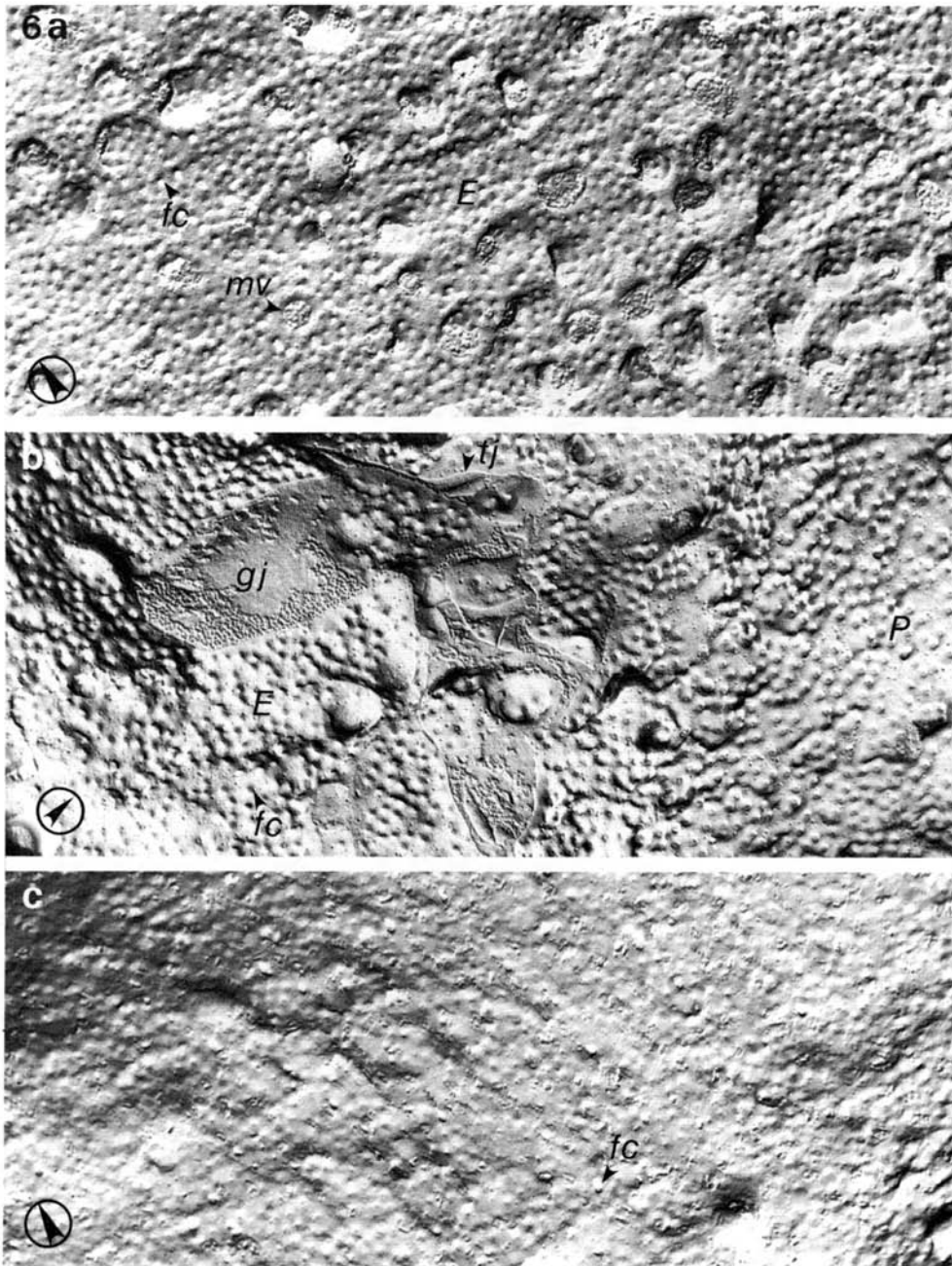
Fig. 6. Filipin reactivity of the three membrane domains in compacted 8-cell embryos. (a) Apical domain showing high density of microvilli and filipin-cholesterol complexes (×50 400). (b) Focal tight and gap junction complex showing that the surrounding membrane is highly filipin reactive whereas the junction itself is not disrupted by filipin–cholesterol complexes (×49 980). (c) Basolateral domain showing sparse filipin–cholesterol complexes (×50 400). Arrowhead indicates direction of shadowing. *P* & *E* indicate P & E faces. *mv*, microvilli; *fc*, filipin cholesterol complex; *tj*, tight junction; *gj*, gap junction.
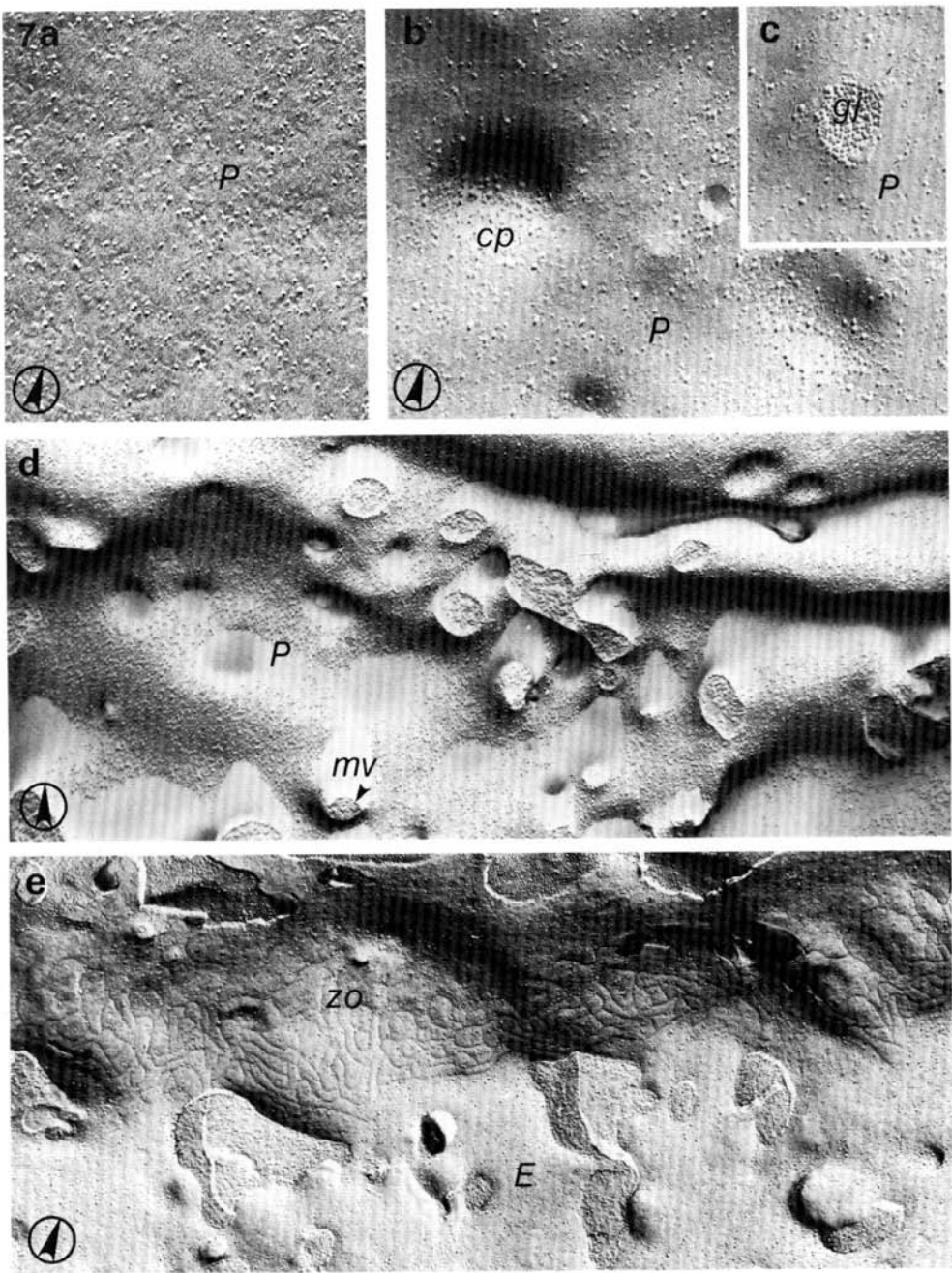
Fig. 7. Control membranes of ICM and trophectoderm. (a,b) Control ICM. Surface membrane P face (×70 400). (c) Control ICM gap junction. P face (×70 400). (d) Control trophectoderm. Apical surface membrane P face (×70 400). (e) Control trophectoderm zonula occludens E face and basolateral membrane (×34 600). Arrowhead indicates direction of shadowing. *P & E* indicates P or E face. *mv*, microvilli; *cp*, coated pit; *gj*, gap junction; *zo*, zonula occludens.
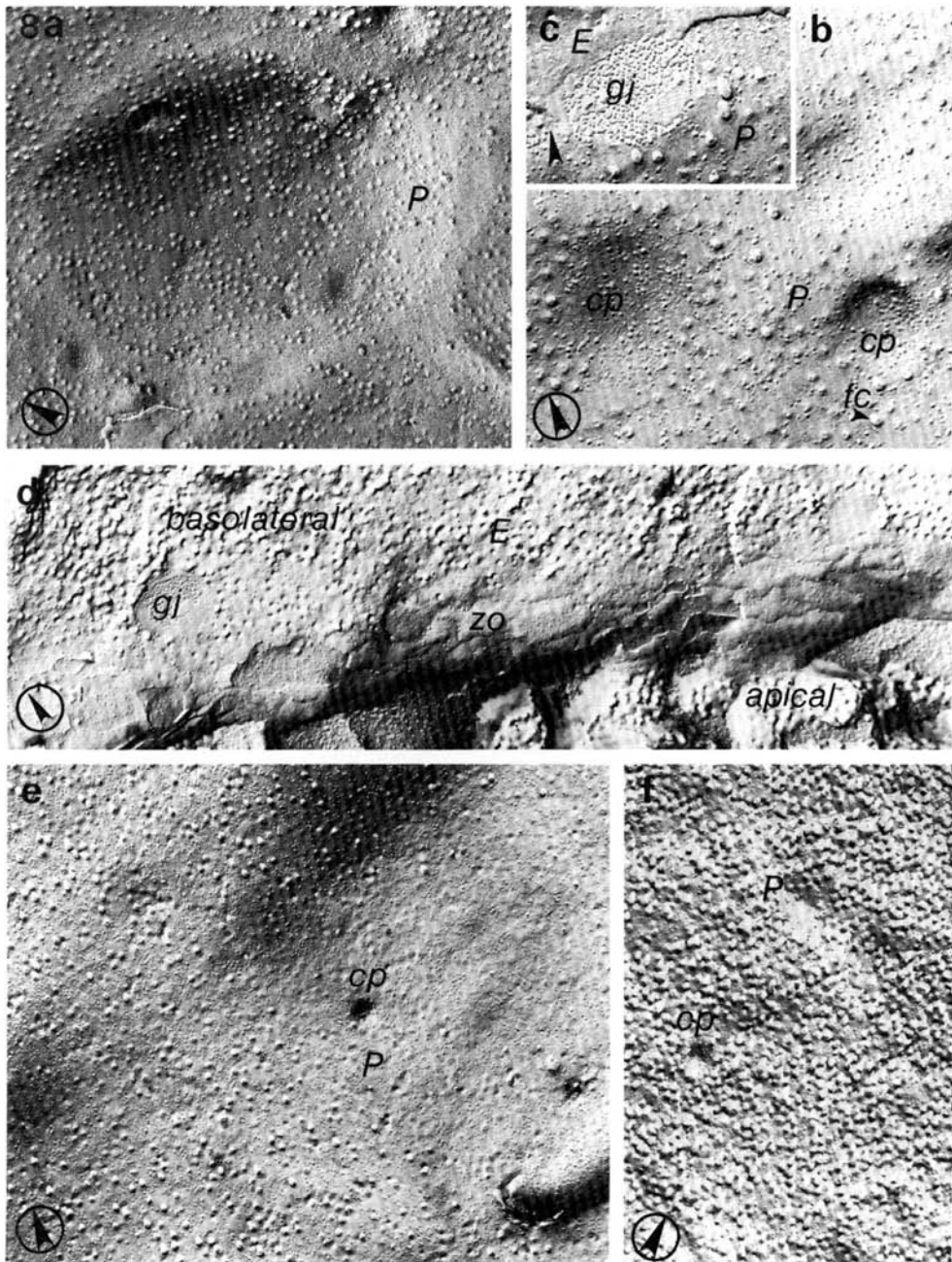
Fig. 8. Filipin-treated membranes of ICM and trophectoderm. (a) Filipin-treated ICM. Surface membrane showing impressions made by adjacent cells. P face (×35 700). (b) Filipin-treated ICM. Surface membrane and coated pits. P face (×69 300). (c) Filipin-treated ICM. Gap junction P and E faces (×69 300). (d) Filipin-treated trophectoderm. Zonula occludens E face (×35 200). (e) Filipin-treated trophectoderm. Basolateral surface membrane showing impressions of adjacent cells (×35 700). (f) Filipin-treated trophectoderm. Apical surface membrane (×35 700). Arrowhead indicates direction of shadowing. *P & E* indicates P or E face. *cp*, coated pit; *gj*, gap junction; *zo*, zonula occludens; *fc*, filipin-cholesterol complex.

DISCUSSION

The technique of freeze fracture cytochemistry using filipin has been widely criticized due to the frequent misinterpretation of the density of filipin complexes as a direct measure of local concentrations of membrane cholesterol (discussed Severs & Robenek, 1983; Miller, 1984). It is clear that the formation of visible filipin–sterol complexes is a multi-stage process involving the penetration of the bilayer by filipin, its stoichiometric interaction with 3 β-hydroxysterols, the aggregation of these filipin–sterol complexes and finally the deformation of the bilayer (De Kruijff *et al.* 1974). Intuitively it would seem that the composition of both the membrane and the cytoskeleton should influence these processes and this has been shown theoretically by consideration of the diffusion coefficient of cholesterol (Miller, 1984) and experimentally by inducing phase transitions in membrane lipids to alter membrane fluidity (Sekiya, Kitajima & Nozawa, 1979) and by modifying protein associations within both the membrane (Feltkamp & van der Waerden, 1982; Steer *et al.* 1984) and cytoskeleton (Brown, Montesano & Orci, 1982). In view of these cautionary considerations I shall adopt a conservative approach to the observations reported here and interpret them mainly in terms of changes in the organization of the cytocortex (i.e. surface membrane and under-lying cytoskeleton) during preimplantation development. Newly synthesized sterols will contribute to this organization from early cleavage stages onwards with lanosterol predominating until the blastocyst at which time cholesterol has become the main sterol to be synthesized (Pratt, 1982).

Fertilization (or activation) induces a dramatic reorganization of the egg surface as assessed by increased density of filipin–cholesterol complexes. The cyto-skeleton could possibly mediate this change in filipin reactivity although cyto-chalasin does not affect this transition. Alternatively the fusion of cortical granule membranes of different lipid composition (Decker & Kinsey, 1983) and attendant re-ordering of lipid domains (mouse, (Wolf, Edidin & Handyside, 1981); *Xenopus*, (Dictus *et al.* 1984)) could be responsible. The apparent disruption of cortical granule membrane by filipin is consistent with this interpretation, although the cortical reaction is not extensive in the mouse (Gulyas, 1980). A comparable study of fertilized and unfertilized eggs in the sea urchin *Arbacia punctulata* (Carron & Longo, 1983) showed a high density of filipin–sterol complexes in cortical granules undergoing fusion with the egg plasma membrane. However fertilization induced little or no change in the reactivity of the rest of the egg membrane. Since the unfertilized sea urchin egg has completed meiosis whereas the mouse oocyte is arrested at second meiotic metaphase this difference in filipin reactivity could be a consequence of progressive cytocortical reorganization during meiosis.

The outer surface of the embryo apparently remains reactive with filipin throughout cleavage and blastocyst formation. The external location of this type of membrane is unequivocal in the 8-cell blastomere and trophectoderm since it is invariably associated with an increased density of microvilli or the presence of a zonula occludens. At the 2-cell and 4-cell stages, however, its position within the embryo can only be construed by default, that is membranes reacting with filipin

never exhibit features indicating cell apposition. A different type of membrane, which shows less reaction with filipin and exhibits intercellular features, namely transitions between P and E faces and reduced density of microvilli, appears for the first time at the 2-cell stage, is clearly established as basolateral membrane at the 8-cell stage and is characteristic of the ICM in the blastocyst. A striking feature restricted to the intercellular areas of this membrane, is the presence of multilamellate 'plaques' whose disposition about P and E faces and appearance in thin sections suggest that they are derived by blebbing out of IMP-free areas of the bilayer. The clustering of IMPs and filipin complexes around these particle-free areas is highly suggestive of lateral phase separations within lipids (Sekiya *et al.* 1979) which is not unexpected in view of the fact that fixation and filipin treatment were not carried out at physiological temperature. Similar lipid domains have been observed in fusigenic areas of acrosomal and plasma membranes of sperm (Friend, 1980) and on the surface membrane of mitotic neuroblastoma cells where their presence is attributed to membrane growth (Bluemink *et al.* 1983). Although membrane vesicles can arise from areas of cell apposition during disaggregation of live cells the most likely explanation for these structures is that they are a consequence of glutaraldehyde fixation (Hasty & Hay, 1978) since they were observed in samples processed for TEM as well as freeze fracture but not in rapid frozen unfixed material (results not shown). Irrespective of whether these blebs are preserved or induced *de novo* by glutaraldehyde they presumably indicate a difference in membrane and/or cytocortical organization between apical and intercellular areas during cleavage and between apical trophectoderm and ICM in the blastocyst.

The simplest (but in view of the earlier discussion, most contentious) interpretation of heterogeneous filipin reactivity is that the membrane domains examined here have different cholesterol compositions. Such an asymmetric distribution of cholesterol would confer increased fluidity (Searls & Edidin, 1981) and adhesivity on cholesterol-depleted membranes, (e.g. intercellular and basolateral domains) and would also be consistent with the known lipid composition and properties of basolateral membrane of polarized cells isolated from intestinal epithelium or derived from canine kidney (MDCK) (Brasitus & Schachter, 1980; Van Meer & Simons, 1982). If such an asymmetry were to be established early during cleavage it would be likely to persist since the cells are so large that diffusion alone would be insufficient to overcome this polarity (Wolf, 1983).

However, the underlying cytoskeleton can influence the formation of visible filipin–cholesterol complexes (Brown, Montesano & Orci, 1982; Feltkamp & van der Waerden, 1982) and the depletion of actin (Johnson & Maro, 1984) and myosin (Sobel, 1983) in regions of cell apposition could render cholesterol unavailable for filipin binding but permit interaction with tomatin in common with some other filipin-negative membranes (Severs & Simons, 1983).

These observations and conclusions permit a simple but tentative model of cortical assembly and lineage to be constructed. Since the peripheral region of the embryo (egg membrane and apical regions of the polarized 8-cell and

trophectoderm) all react with filipin it is plausible to suggest that egg membrane persists on the exterior of the embryo and that 'new' membrane is inserted internally in a form which is less reactive with filipin (as occurs in yeast (Sekiya & Nozawa, 1983)). This scenario conforms to established lineage relationships within the preimplantation embryo and is consistent with the apparent coherent and clonal nature of blastocyst development (discussed Johnson, 1985a). 'New' membrane is evidently synthesized and/or assembled from the 2- to 4-cell stage onwards (Izquierdo, 1977; Pratt, 1980, 1982) and probably inserted internally at sites of cell apposition (Izquierdo, Lopez & Marticorena, 1980; Izquierdo & Ebensperger, 1982) as occurs during cleavage of *Xenopus* eggs (Tetteroo *et al.* 1984). Cell contact could focus its assembly and insertion although no obvious membrane precursors have been observed in these regions (unpublished observations) as they have in amphibia (Bluemink, 1970; Tetteroo *et al.* 1984). Alternatively 'new' membrane could be inserted throughout the cell surface and contact only act as a focus for its reorganization.

Although this surface mosaicism may originate from a requirement for increased surface area it could also represent the earliest signs of contact-dependent heterogeneity which develops into the clearly identifiable polarized surface morphology of the 8-cell stage (Reeve & Ziomek, 1981; Johnson, 1985b).

This mosaicism also highlights another potentially significant phenomenon, namely, the apparent persistence of the egg cytocortex during cleavage and its probable incorporation into the apical surface of outer blastomeres and trophectoderm. If verified this conclusion implies that any maternally-derived developmental information laid down in the cytocortex of the egg (discussed Johnson & Pratt, 1983) could influence the organization of the embryo up until the blastocyst and possibly even later.

These results also have important implications for studies of the biogenesis of cell polarity since they confirm that cytocortical polarity can be assembled and maintained in the absence of morphological barriers to lateral diffusion such as tight junctions, or other obvious cytoskeletal elements. Other systems where this has been shown are sperm (Friend, 1982) cleaving *Xenopus* eggs (Tetteroo *et al.* 1984) and single epithelial cells attached to a substratum (Sabatini *et al.* 1983). Trophectoderm shows all the characteristics of a conventional epithelium in terms of filipin reactivity namely a high concentration of complexes in the apical surface, fewer in intercellular regions and a complete absence of them from the zonula occludens (Robenek, Jung & Gebhardt, 1982; Gotow & Hashimoto, 1983). Since the main routes for membrane traffic within trophectoderm are now established (Fleming, in preparation) it should be possible to use the preimplantation mouse embryo to dissect out the inter- and intracellular signals which direct the assembly of membrane asymmetries.

REFERENCES

BLUEMINK, J. G. (1970). The first cleavage of the amphibian egg. An electron microscope study of the onset of cytokinesis in the egg of *Ambystoma mexicanum*. *J. Ultrastruct. Res.* **32**, 142–166.

BRASITUS, T. A. & SCHACHTER, D. (1980). Lipid dynamics and lipid protein interactions in rat enterocyte basolateral and microvillous membranes. *Biochem.* **19**, 2763–2769.

BLUEMINK, J. G., VAN MAURIK, P. A. M., TERTOOLEN, L. G. J., VAN DER SAAG, P. T. & DE LAAT, S. W. (1983). Ultrastructural aspects of rapid plasma membrane growth in mitotic neuroblastoma cells. *Eur. J. Cell Biol.* **32**, 7–16.

BROWN, D., MONTESANO, R. & ORCI, L. (1982). Patterns of filipin sterol complex distribution in membrane particle-aggregated ghost membranes. *J. Histochem. Cytochem.* **30**, 702–706.

CARRON, C. P. & LONGO, F. J. (1983). Filipin–sterol complexes in fertilised and unfertilised sea urchin egg membranes. *Devl Biol.* **99**, 482–488.

DE KRUIJFF, B., GERRITSEN, W. J., OERLEMANS, A., DEMEL, R. A. & VAN DEENEN, L. L. M. (1974). Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. *Biochem. biophys. Acta* **339**, 30–43.

DECKER, S. J. & KINSEY, W. H. (1983). Characterisation of cortical secretory vesicles from the sea urchin egg. *Devl Biol.* **96**, 37–45.

DICTUS, W. J. A. G., VAN ZOELEN, E. J. J., TETTEROO, P. A. T., TERTOOLEN, L. G. J., DE LAAT, S. W. & BLUEMINK, J. G. (1984). Lateral mobility of plasma membrane lipids in *Xenopus* eggs, regional differences related to animal/vegetal polarity become extreme upon fertilisation. *Devl Biol.* **101**, 201–211.

DRAGSTEN, P. R., BLUMENTHAL, R. & HANDLER, J. S. (1981). Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? *Nature, Lond.* **294**, 718–722.

EAGER, D., JOHNSON, M. H. & THURLEY, K. W. (1976). Ultrastructural studies on the surface of the mouse egg. *J. Cell Sci.* **22**, 345–353.

ELIAS, P. M., FRIEND, D. S. & GOERKE, J. (1979). Membrane sterol heterogenity. Freeze fracture detection with saponins and filipin. *J. Histochem. Cytochem.* **27**, 1247–1260.

EVANS, W. H. (1980). A biochemical dissection of the functional polarity of the plasma membrane of the hepatocyte. *Biochim. Biophys. Acta* **604**, 27–64.

FELTKAMP, C. A. & VAN DER WAERDEN, A. W. M. (1982). Membrane associated proteins affect the formation of filipin–cholesterol complexes in viral membranes. *Expl Cell Res.* **140**, 289–298.

FRIEND, D. S. (1980). Freeze fracture alterations in guinea pig sperm membranes preceding gamete fusion. In *Membrane–Membrane Interactions* (ed. N. B. Gilula), pp. 153–165. New York: Raven Press.

FRIEND, D. (1982). Plasma membrane diversity in a highly polarised cell. *J. Cell Biol.* **93**, 243–249.

FULTON, B. P. & WHITTINGHAM, D. G. (1978). Activation of mammalian oocytes by intracellular injection of calcium. *Nature, Lond.* **273**, 149–151.

GOTOW, T. & HASHIMOTO, P. H. (1983). Filipin resistance in intermediate junction membranes of guinea pig ependyma. Possible relationships to filamentous underlying. *J. Ultrastruct. Res.* **84**, 83–93.

GULYAS, B. (1980). Cortical granules of mammalian eggs. *Int. Rev. Cytol.* **63**, 357–392.

HASTY, D. L. & HAY, E. D. (1978). Freeze fracture studies of the developing cell surface 11. Particle free membrane blisters on glutaraldehyde fixed corneal fibroblasts are artefacts. *J. Cell Biol.* **78**, 756–768.

HATTEN, M. E. & BURGER, M. M. (1979). Effect of polyene antibiotics on the lectin-induced agglutination of transformed and untransformed cell lines. *Biochem.* **18**, 739–745.

IZQUIERDO, L. (1977). Cleavage and differentiation. In *Development in Mammals 2* (ed. M. H. Johnson), pp. 99–118. Amsterdam: North Holland.

IZQUIERDO, L. & EBENSPERGER, C. (1982). Cell membrane regionalisation as demonstrated by 5' nucleotidase activity. *J. Embryol. exp. Morph.* **69**, 115–126.

IZQUIERDO, L., LOPEZ, T. & MARTICORENA, P. (1980). Cell membrane regions in preimplantation mouse embryos. *J. Embryol. exp. Morph.* **59**, 89–102.

JOHNSON, M. H. (1985a). Manipulation of early mammalian development. What does it tell us about cell lineages? In *Manipulation of Mammalian Development* (ed. R. B. L. Gwatkin). New York: Plenum Pub. Corp. (in press).

JOHNSON, M. H. (1985b). Three types of cell interaction regulating the generation of cell diversity in the mouse blastocyst. In *The Cell in Contact. Adhesions and Junctions as Morphogenetic Determinants* (ed. G. Edelman & J. P. Thiery), pp. 27–48. New York: Neurosciences Institute Publications Series.

JOHNSON, M. H. & MARO, B. (1984). The distribution of cytoplasmic actin in mouse 8-cell blastomeres. *J. Embryol. exp. Morph.* **82**, 97–117.

JOHNSON, M. H. & PRATT, H. P. M. (1983). Cytoplasmic localisation and cell interactions in the formation of the mouse blastocyst. In *Time, Space and Pattern in Embryonic Development* (ed. W. J. Jeffrey), pp. 287–312. New York: Alan Liss Inc.

JOHNSON, M. H. & ZIOMEK, C. A. (1982). Cell subpopulations in the late morula and early blastocyst of the mouse. *Devl Biol.* **91**, 431–439.

MILLER, R. G. (1984). The use and abuse of filipin to localise cholesterol in membranes. *Cell. Biol. Int. Reports* **8**, 519–535.

NAVARATNAM, V., THURLEY, K. W. & SKEPPER, J. N. (1982). Freeze fracture replication studies of mammalian heart muscle. In *Progress in Anatomy* vol. **2** (ed. R. J. Harrison & V. Navaratnam), pp. 201–215. Cambridge Univ. Press.

NICOLSON, G. L., YANAGIMACHI, R. & YANAGIMACHI, H. (1975). Ultrastructural localisation of lectin binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. *J. Cell Biol.* **66**, 263–274.

NITSCH, L. & WOLLMAN, S. H. (1980). Ultrastructure of intermediate stages in polarity reversal of thyroid epithelium in follicles in suspension culture. *J. Cell Biol.* **86**, 875–880.

PRATT, H. P. M. (1980). Phospholipid synthesis in the preimplantation mouse embryo. *J. Reprod. Fert.* **58**, 237–248.

PRATT, H. P. M. (1982). Preimplantation mouse embryos synthesise membrane sterols. *Devl Biol.* **89**, 101–111.

PRATT, H. P. M., ZIOMEK, C. A., REEVE, W. J. D. & JOHNSON, M. H. (1982). Compaction of the mouse embryo: an analysis of its components. *J. Embryol. exp. Morph.* **70**, 113–132.

REEVE, W. J. D. & ZIOMEK, C. A. (1981). Distribution of microvilli on dissociated blastomeres from mouse embryos: evidence for surface polarisation at compaction. *J. Embryol. exp. Morph.* **62**, 339–350.

REGGIO, H., COUDRIER, E. & LOUVARD, D. (1982). Surface and cytoplasmic domains in polarised epithelial cells. In *Membranes in Growth and Development*, pp. 89–105. New York: Alan R. Liss Inc.

ROBENEK, H., JUNG, W. & GEBHARDT, R. (1982). The topography of filipin–cholesterol complexes in the plasma membrane of cultured hepatocytes and their relation to cell junction formation. *J. Ultrastruct. Res.* **78**, 95–106.

SABATINI, D., GRIEPP, E. B., RODRIGUEZ-BOULAN, E. J., DOLAN, W. J., ROBBINS, E. S., PAPADOPOULOS, S., IVANOV, I. E. & RINDLER, M. J. (1983). Biogenesis of epithelial cell polarity. In *Modern Cell Biology* **2**, pp. 419–450. New York: Alan R. Liss Inc.

SEARLS, D. B. & EDIDIN, M. (1981). Lipid composition and lateral diffusion in plasma membranes of teratocarcinoma-derived cell lines. *Cell* **24**, 511–517.

SEKIYA, T., KITAJIMA, Y. & NOZAWA, Y. (1979). Effects of lipid-phase separation on the filipin action of membranes of ergosterol-replaced *Tetrahymena* cells as studied by freeze fracture electron microscopy. *Biochim. Biophys. Acta.* **550**, 269–278.

SEKIYA, T. & NOZAWA, Y. (1983). Reorganisation of membrane ergosterol during cell fission events of *Candida albicans*. A freeze fracture study of distribution of filipin-ergosterol complexes. *J. Ultrastruct. Res.* **83**, 48–57.

SEVERS, N. J. & ROBENEK, H. (1983). Detection of microdomains in biomembranes. An appraisal of recent developments in freeze-fracture cytochemistry. *Biochim. Biophys. Acta* **737**, 373–408.

SEVERS, N. J. & SIMONS, H. L. (1983). Failure of filipin to detect cholesterol-rich domains in smooth muscle plasma membrane. *Nature, Lond.* **303**, 637–638.

SOBEL, J. S. (1983). Cell-cell contact modulation of myosin organisation in the early mouse embryo. *Devl Biol.* **100**, 207–213.

STEER, C. J., BISHER, M., BLUMENTHAL, R. & STEVEN, A. C. (1984). Detection of membrane cholesterol by filipin in isolated rat liver coated vesicles is dependent upon removal of the clathrin coat. *J. Cell Biol.* **99**, 315–319.

TETTEROO, P. A. T., BLUEMINK, J. G., DICTUS, W. J. A. G., VAN ZOELEN, E. J. J. & DE LAAT, S. W. (1984). Lateral mobility of plasma membrane lipids in dividing *Xenopus* eggs. *Devl Biol.* **104**, 210–218.

VAN MEER, G. & SIMONS, K. (1982). Viruses budding from either the apical or the basolateral plasma membrane domain of MDCK cell have unique phospholipid compositions. *EMBO J.* **1**, 847–852.

WHITTINGHAM, D. G. (1971). Culture of mouse ova. *J. Reprod. Fert. suppl.* **14**, 7–21.

WOLF, D. (1983). The plasma membrane in early embryogenesis. In *Development in Mammals* **5** (ed. M. H. Johnson), pp. 187–208. Amsterdam: Elsevier Science publishers.

WOLF, D. E., EDIDIN, M. & HANDYSIDE, A. H. (1981). Changes in the organisation of the mouse egg plasma membrane upon fertilisation and first cleavage. Indication from the lateral diffusion rates of fluorescent lipid analogs. *Devl Biol.* **85**, 195–198.