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Membrane fractions display different lipid and enzyme content in three cell types in 16-cell stage embryos of sea urchins

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Three cell types were isolated from dissociated 16-cell sea urchin embryos. Four membrane density fractions from discontinuous gradients have different proportions of lipids, surfacer markers and enzymes for the three cell types. Assays of lipid content, CH/PLIPID and SPH/PC ratios, acyl chain length, level of unsaturation by proton NMR and assays of enzyme activity revealed variation at the same density between the three cell types and among different densities from one cell type. There were also differences between whole embryos and dissociated embryo cells. There was no typical membrane domain at a particular density common to the cell types. Cell surface characteristics and polarity of adult cells rely on which lipid domains and enzymes are present, their association with cytoskeleton and how they are localized. At the 16-cell stage these characteristics are still very dynamic as revealed by cytochemical localization of Na^+/K^+-ATPase which varied with cell type and suggests endocytosis at set times in the division cycle. Polarity has not been permanently set for Na^+/K^+-ATPase yet. Membrane enzyme and lipid distributions unique to the three cell types seen in this study suggest parcelling out or insertion of new membrane domains occurs during early sea urchin cleavage. Perturbation of membrane density distribution and lipid content occurs after treatment of embryos with animalizing and vegetalizing teratogens which alter development.

Introduction

Differentiation during embryonic development results in establishment of cellular polarity and the different cell types found in the adult. Regional membrane domains are set up in polarized epithelial cells reflecting structural differences (microvilli, zonula adherons) and functional differences (ion transport, enzyme localization, permeability) together with differences in distribution of intrinsic membrane proteins and lipids involved, as well as associated cytoskeleton. A classic demonstration is seen in comparison of the apical versus the basolateral domains (BLM) in adult cells or monolayer tissue culture cells [1–12]. These studies have shown that the apical membrane lacks Na^+/K^+-ATPase, may have microvilli with associated villin, α-actinin meshwork, Na^+ channels, secretion and virus budding; the BLM has Na^+/K^+-ATPase with associated fodrin, ankyrin, may have hormone receptors. At the border between these two domains the belt desmosomes or zonula adherons have associated α-actinin and prekeratin. The two domains vary in lipid fluidity [13–16], which reflects diffences in lipid content and relative concentration, the unsaturation of the acyl chains of the phospholipids, the cholesterol to phospholipid ratio (CH/PLIP) and the sphingomyelin to phosphatidylcholine ratio (SPH/PC) [17,10]. The lipid headgroups contribute to the charge on the membrane surfaces and help regulate which proteins are more likely to be associated with which domain [5].

There are many pieces of evidence for a non-homo-

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Abbreviations: CE, cholesterol ester; CH, cholesterol; CEREB, cerebroside; CMFSW, calcium, magnesium free sea water; Con A, concanavalin A; DOL, dolichol; DMSO, dimethylsulfoxide; DTT, dithiothreitol; ER, endoplasmic reticulum; FA, fatty acids; GANG, gangliosides; G6Pase, glucose-6-phosphatase; G6PD, glucose-6-phosphate dehydrogenase; LPC, lysophosphatidylcholine; MAC, macromeres; MES, mesomeres; MIC, micromeres; NM, nuclear membrane; NMR, nuclear magnetic resonance spectroscopy; NPP, *p*-nitrophenyl phosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PM, plasma membrane; PS, phosphatidylserine; PV, pinocytotic vesicle; SPH, sphingomyelin; SULF, sulfatides; TG, triacylglycerols; TLC, thin-layer chromatography.

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geneous pattern of surface domains even in early embryos. Regional differences in lateral mobility properties of plasma membrane lipids show an animal/vegetal polarity in *Xenopus* eggs that is enhanced after fertilization, with smaller diffusion coefficient in the animal hemisphere (where lipids are almost immobilized [15]). However, the fluorescent probe used in the study was localized in small spots in the animal hemisphere, therefore it was thought that these may be isolated immobile domains. The cell membrane also shows polarity in distribution of intramembranous particles [18]. Lipid gradients in eggs could influence the protein localization which would be lipid dependent.

The distribution of membrane domains gradually changes during development due to the insertion of new membrane or protein or activation of protein already there and segregation during cleavage. Previous work in this laboratory revealed a change in the lipid content in cortical hulls of eggs of two species of sea urchin after fertilization [19]. This was to be expected since the cortical granule fusion occurs at that time, an insertion of membrane. The embryo after first cleavage is composed of 2 cell surfaces: the original external surface complex which is thought to be permanently associated with the hyaline layer and therefore cannot be transferred to the furrows and the new internal surfaces created by the cleavage [20]. However, the possibility that some lateral diffusion of molecules occurs is definitely not ruled out, since Con A-receptor complexes and membrane-attached microfilaments become concentrated in the division furrow or other regions of cells undergoing deformation [21]. Other cytoskeletal regionalization in the cell cortex is also common, with networks below the microvilli or adhesion complexes [6] associated with the myoplasmic segregating region in Ascidian eggs [22] or as contractile rings at the cleavage furrow [23] and this could be influenced by the ionic environment, since cytoskeletal subunit polymer addition or subtraction can be regulated by ions and pH. Following formation of tight junctions and desmosomes, segregated domains are stabilized in place [16].

At the 8-cell stage of sea urchin embryo development, the next (fourth) division pattern differs for the four cells at the animal pole and those at the vegetal pole. The four animal pole cells divide meridionally so that there is a layer of eight mesomeres at the animal pole. The four cells at the vegetal pole undergo an unequal horizontal division, so that the middle tier of cells is four large macromeres and the bottom tier at the vegetal pole is four small micromeres. The result is three kinds of cells with different fates in development [24]: (a) the eight mesomeres give rise to the external ectodermal structures; (b) the four macromeres give rise to the gut and skeleton; and (c) the four micromeres give rise to skeletal structures and coelomic pouches.

This presents an ideal test system for determining if these 16-cell stage three blastomere types have different membrane domains or components. Segregation or insertion of lipids or ATPase during cleavage could influence the cell polarity and behavior before obvious differences in gene activities. The stabilization of regional domains has not yet occurred since cell adhesion is not yet structural at this stage. To test for such domain differences, the cell types must be separated and their membrane fractions investigated, characterized and compared.

Methods have been developed for separation of the three cell types [25,26]. Research on the separated cells has shown mainly similarities of RNA [27,26], proteins [28] synthetic processes [29,30]; and differences in distribution of Con A receptors [31].

The purpose of this investigation was to analyze for different membrane lipid content in the three cell types as one of the bases of differentiation by the 16-cell stage due to resulting membrane domain fluidity and charge differences. A new method, using high-resolution proton nuclear magnetic resonance (NMR) to determine lipid content in extracts of purified cell membranes has been used and compared to thin-layer chromatography results [32,33]. A cytochemical test for ATPase was used to demonstrate the pattern of domains containing or excluding the enzyme.

Preliminary reports of this work have been published [1–3].

Methods

Fertilized eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* (supplied by Pacific Biomarine, Los Angeles, CA) were obtained by combining eggs and sperm after injection with 0.55 M KCl. Fertilization membranes (FM] removal postinsemination resulted after adding an equal volume of 0.1% DTT and 0.1% proteinase in calcium-magnesium-free sea water (CMFSW] pH 8 until the FMs dissolved as monitored microscopically (5–10 min) [34]. Immediately, the settled eggs were washed in series with CMFSW and sea water (pH 8) by settling and decanting. The embryos from four or five females were cultured separately at 16°C and at the 16-cell stage (4–5 h), settled on ice and combined. Embryos were alternately centrifuged (1000 rpm, 30 s) and washed twice for several minutes on ice with CMFSW-0.5 mM EDTA to dissociate the cells [26]. Dissociation was completed by homogenizing gently with five strokes in a large Teflon pestle homogenizer with a large volume of CMFSW-EDTA. Cells were sedimented at 1500 rpm for 1 min and resuspended in 25–50 ml of 1% Ficoll in CMFSW by gentle homogenization.

The cell separation was accomplished in a unit gravity cell separator Periput chamber (BIOSEP, Walnut

Creek, CA) [35] by loading from the bottom with the following sequence: (1) 75 ml CMFSW-EDTA, (2) 25–50 ml cell suspension (50–100 million cells total), (3) 400 ml of linear 2–6% (w/v) Ficoll-CMFSW gradient and (4) 75 ml 30% sucrose cushion. The temperature was maintained at 4–10°C. After 55 min 30% sucrose was pumped into the bottom of the chamber and the cells were collected from the top, after discarding 60–70 ml before any cells were noted. 50–60 fractions (115 drops) each were monitored by Polaroid photomicrographs to determine the cell makeup of each fraction before combining them into three cell fractions: macromeres (MAC), mesomeres (MES), micromeres (MIC) (Fig. 1).

Surface labels. Ruthenium red [36] and concanavalin A [31] were used to label cell surfaces in sea water. Membrane density gradient fractions from labeled cells were prepared.

Animalizing and vegetalizing agents. When applied at the 2-cell stage and left until the blastula stage, lithium chloride (18 mM) in sea water causes vegetalization (lack of ectodermal derivatives and accentuation of endodermal (gut) structures) [37] and zinc sulfate (0.1 mM) in sea water causes animalization (lack of gut and mostly ectodermal derivatives are prevalent) [38]. Treatments in these studies were from 2- to 16-cell stage, since the early segmentation period has the highest sensitivity to lithium and zinc [39].

Membrane fraction preparation. Membranes fractions were prepared from whole 16-cell embryos and isolated MES, MAC and MIC by modifying an existing method [40]. After homogenizing the cells in 2 M sucrose, 0.1 mM $MgCl_2$, 5 mM Tris (pH 8); and adjusting the concentration to 40% sucrose and 10 mM EDTA, 7 ml of the homogenate was layered over 3 ml 65% (w/w) sucrose cushion. Additional layers of 6 ml each of 30%, 22.5% (w/w) and homogenizing medium were added to the tube, thereby making a stepwise gradient. Centrifugation for 20 h or more at 25000 rpm in a Beckman SW25 rotor, at 4°C yielded membrane fractions separated by flotation at each sucrose interface. Membrane fractions were recovered by pumping from the bottom of the tube and collected in fractions by a drop counter, which in some runs were combined into four fractions surrounding interfaces.

Membrane lipid isolation. The four fractions were diluted and centrifuged at 39000 rcf for 1 h and the pellet lipids extracted and prepared for NMR and TLC as previously described [32].

NMR method of lipid analysis. NMR samples of membrane extracts are typically about 1 mg lipid/0.6 ml 2:1 methanol-d_4/chloroform-d_1 solvent mixture. Special care is taken to ensure that the methanol-d_4 is dry prior to use. Since the membrane fraction extracts have a very low lipid concentration, high resolution NMR is required. Proton NMR spectra are recorded at room temperature or 35°C on a Bruker WM500 multinuclear NMR spectrometer, operating at a field strength of 11.74 Tesla (500.13 MHz proton frequency). Spectra are acquired in the Fourier transform (FT) mode with 32 K data points, using quadrative phase detection, a 70° pulse width, a cycling time between scans of 0.6 s and a spectral width of 6024 Hz [32]. All chemical shifts are referred to TMS as an internal standard. Integrals are taken for set PPM limits for comparison with a standard table.

These spectra of lipid extracts from the four membrane fractions were analyzed using a Fortran computer algorithm, LIPICK [33] to determine lipid content from peak areas. The resulting data were further analyzed for relative concentrations of lipids by algorithm ANALS1 (Sparling,1990). The differences in unsaturation are detected due to differences in protons next to double bonds as can be visualized in Fig. 6C at 5.4 ppm and in

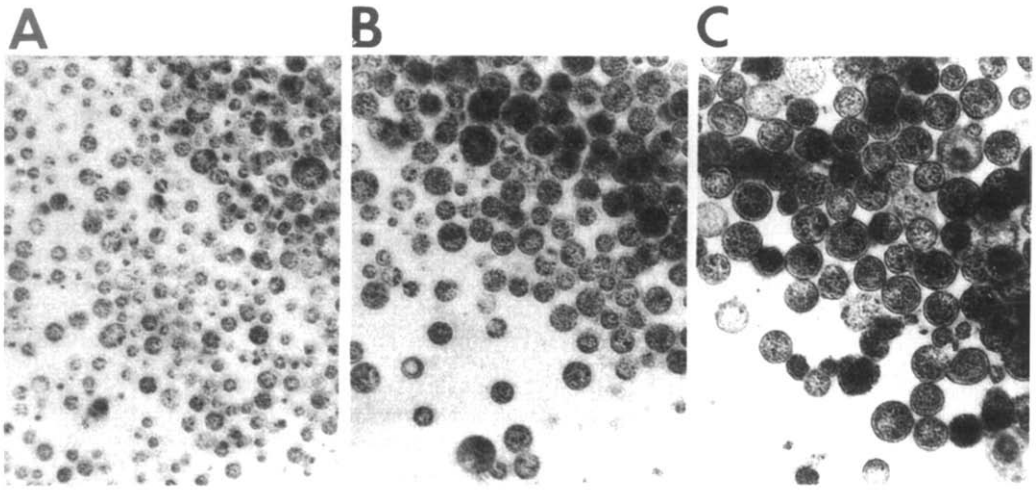


Fig. 1. Micrographs of isolated micromeres (10–15 μm) (A), mesomeres (20–25 μm) (B) and macromeres (30–35 μm) (C) from the 16-cell stage of *S. purpuratus*. Magnification: × 275.

Fig. 6A at 2.0, 2.8 ppm. The polar headgroups are shown mainly between 3 and 4.5 ppm. The chain length is calculated by adding all the acyl peaks (the peaks below 2.9 ppm plus the double bonds at above 5.3 ppm) [33].

ATPase cytochemical method. (Modified according to Ref.41 for cells living in sea water, including sodium.) All steps in the procedure involve 1 ml (unless specified) of fluid added for each treatment and then 30 s 800 rpm centrifugation followed by suctioning off. Development was stopped at the desired stage by placing embryos in tubes on ice. 16-cell embryos are fixed for 3 min on ice in 2% formaldehyde (made fresh each day) in Millipore-filtered sea water with 0.1 M cacodylate buffer (pH 7.2). Embryos are pre-incubated before the enzyme reaction in cacodylate (pH 7.6), 10% v/v dimethylsulfoxide (DMSO), in CMFSW for 30 min on ice to make the membrane permeable. Standard reaction mixture (must be made immediately before using or lead settles out) (0.5 ml for 10–20 min, 36 ° C) final concentrations: 400 mM glycine; 25–45 mM K^+; 4 mM lead; 21% (v/v) DMSO; 8.5 mM $MgCl_2$; 128 mM NaCl; 2.2 mM levamisole; 10 mM *p*-nitrophenylphosphate (NPP) or ATP; pH 8.75–9.0 (adjusted after combined). Tubes are shaken twice during incubation. Controls include substrate-free medium, medium lacking levamisole (which inhibits alkaline phosphatase,) and medium containing 10 mM ouabain (which inhibits Na^+/K^+-ATPase.) The reaction is stopped by placing tubes in ice, followed by two washs with cold CMFSW to remove all substrate. Addition of 0.5 ml 2% yellow ammonium sulfide in SW, incubation for 1 min develops the black stain which is stopped by spinning, suction, washing twice in SW. Embryos can be stored in the refrigerator in 1 ml 50% ethanol for several months without deterioration. For observation, embryos are flattened by drying on the slide, then mounted in 50% glycerol, observed with an Olympus BH-2 microscope, without phase so that only stain is seen as particles. Aliquots were embedded in paraffin and sectioned. For electron microscopy, cells are rinsed and fixed with 1% osmium tetroxide in 0.1 M cacodylate (pH 7.2), for 1 h. They are then rinsed, dehydrated and embedded in Spurr's medium. Thick sections are cut and observed using light microsopy for location of stained embryos for cutting the block and an overview of % stained. Thin sections are cut and examined without poststaining using a Zeiss Transmission EM9S2.

Enzyme assay for gradient fractions. In the ATPase assay [42], 0.1 ml membrane fraction is suspended in 4.5 ml reaction mixture: 153 mM NaCl, 33 mM KCl, 3 mM ATP, 200 mM Tris buffer (pH 8), 3.4 mM $MgCl_2$ for total $Na^+/K^+/Mg^{2+}$-ATPase. Additional 1 mM ouabain or leaving out the sodium and potassium is used for Mg^{2+}-ATPase and then Na^+/K^+-ATPase can be calculated from the difference. At 0,4 and 8 min, 1.25 ml of reaction mixture is mixed with 0.25 ml 15% TCA to stop the reaction. Then 0.7 ml filtrate is mixed with 0.56 ml 77.5 mM sodium molybdate in 2.5 M H_2SO_4 and 0.14 ml $SnCl_2$ (1/200 dilution of a 40%

TABLE I

Average distribution of ATPase in fractions of gradient runs

f, frequency of cases of activity in the fraction out of the total cases (*n*).

	Specific activity (nmol/mg per min)												Gradient density
	Na^+/K^+-ATPase						Mg^{2+}-ATPase						
	MAC			MIC			MAC			MIC			
	f	*x* (S.D.) (*n* = 5)		*f*	*x* (S.D.) (*n* = 4)		*f*	*x* (S.D.) (*n* = 6)		*f*	*x* (S.D.) (*n* = 4)		
1	1	1.4	(2.8)	1	1.25	(2.16)	4	6.67	(6.32)	3	19.5	(12.54)	1.26
2	4	3.6	(3.8)	2	3.25	(4.54)	5	16.17	(14.84)	4	29.5	(12.77)	1.26
3	1	0.8	(1.6)	1	2.5	(4.33)	6	16.67	(11.29)	4	33.25	(13.16)	1.25
4	1	0.8	(1.6)	1	3.5	(4.97)	4	21.3	(16.16)	4	26.5	(10.06)	1.24
5	2	1.2	(1.6)	2	2.75	(2.77)	6	20.3	(15.68)	4	27.75	(6.26)	1.22
6	2	11.0	(21.0)	1	2.75	(4.76)	4	16.0	(16.05)	3	27.5	(16.53)	1.21
7	1	0.4	(0.8)	1	1.75	(3.03)	6	35.5	(30.90)	3	45.0	(28.73)	1.20
8	4	6.6	(6.4)	2	15.75	(18.79)	5	32.67	(27.48)	4	62.5	(18.61)	1.19
9	1	1.6	(3.2)	2	20.5	(24.51)	6	31.67	(19.03)	3	32.5	(22.73)	1.18
10	2	4.2	(7.0)	2	8.0	(8.12)	6	58.83	(56.09)	3	27.0	(21.25)	1.17
11	4	9.8	(10.0)	1	6.75	(11.69)	6	45.0	(27.89)	3	300.5	(448.51)	1.16
12	4	20.0	(19.0)	2	26.75	(40.76)	1	13.33	(29.81)	2	15.5	(22.98)	1.13
13	0	0		0			1	0.5	(1.12)	1	4.0	(6.93)	1.12
14	0	0		0			1	2.17	(4.84)	1	30.75	(53.26)	1.10
15	2	9.6	(13.0)	0			1	31.3	(70.06)	0	0		1.08
16	2	13.0	(18.0)	0			0	0		0	0		1.06
17	0	0		0			2	45.1	(91.5)	0	0		1.06
18	0	0		0			0	0		0	0		1.06

(w/v) solution of $SnCl_2$ in concentrated HCl). The microcuvettes for the spectronic-20 are used to read at 600 nm within 5 min. Specific activity was calculated using standard phosphate curves.

The NADH-cytochrome-*c* Dehydrogenase assay was modified according to Ref.43 with 119 mM Tris-acetate buffer (pH 8.15), 0.594 mg/ml cytochrome *c*, 0.15 mg/ml KCN, 0.1 mM NADH, 0.42 ml volume with increasing absorbance measured on an ISCO double beam recording spectrophotometer using the 546 nm filter. Specific activity was calculated using a standard curve of NADH content. Glucose-6-phosphate dehydrogenase, cytochrome oxidase, glucose-6-phosphatase were all measured as described in Ref.42. DNA and RNA in the gradients were measured using the diphenylamine and Orcinol reagents [44,45]. Protein determinations were done according to Lowry et al. [46] or Bio-Rad reagent.

Preparation of cilia. The embryos were grown to the gastrula stage and treated with sea water containing an additional 0.55 M of NaCl for 1.5 min [47], for removal of cilia, filtered through nytex cloth three times to separate the embryos and cilia. The cilia, embryos without cilia and untreated embryos were centrifuged at

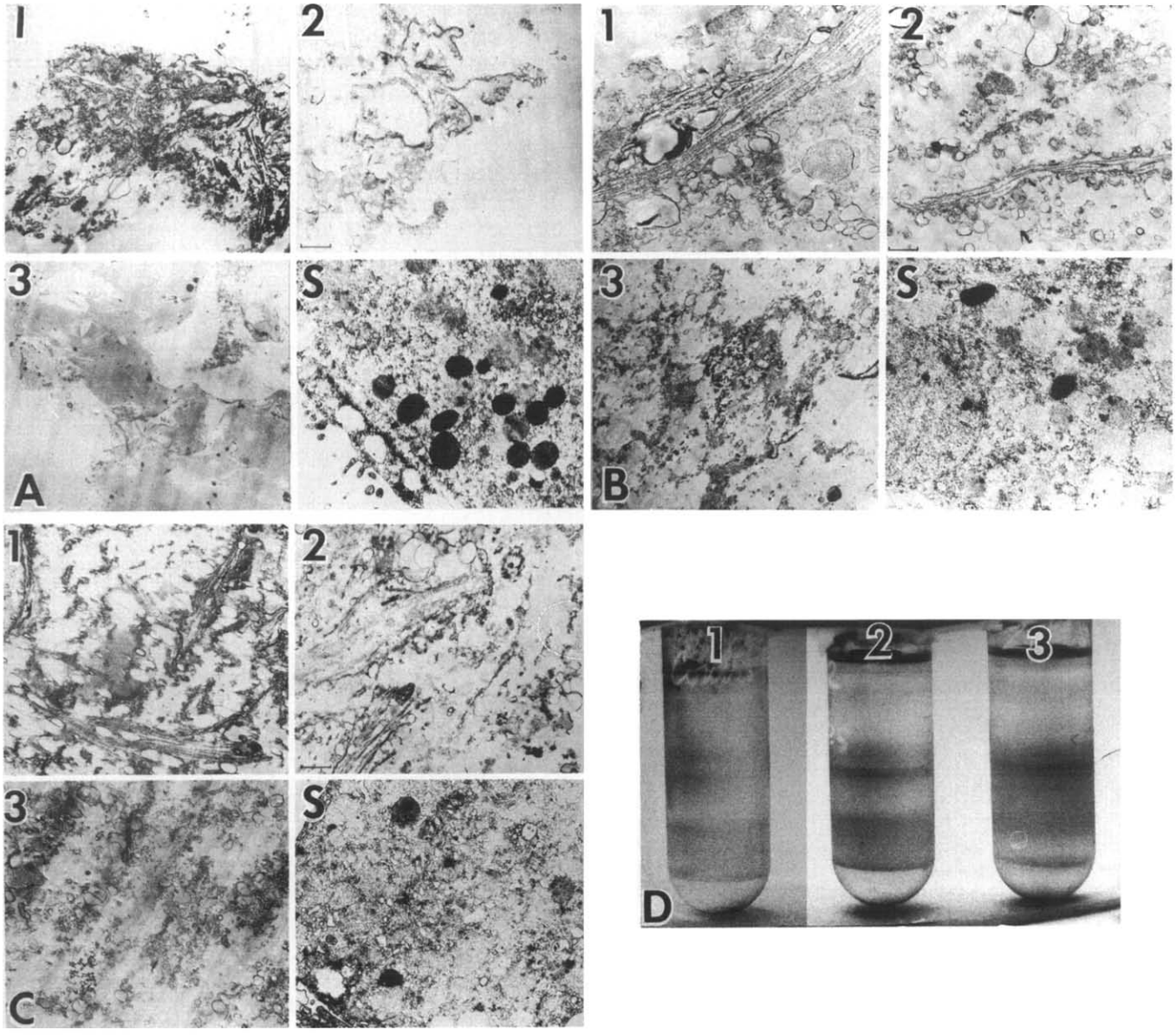


Fig. 2. Each composite of four electron micrographs represents fraction M-1, 2, 3 sectioned pellets and S sectioned whole cell of mesomeres (A), macromeres (B) and micromeres (C). The bar in each fraction 2 represents 1 μm. (D) Density gradients of homogenates of (1) untreated, (2) animalized and (3) vegetalized whole 16-cell *S. purpuratus* embryos.

27 000 rcf for 20 min and pellets frozen. When observed the next day after thawing, the cilia were still flashing. The pellets were extracted for lipid.

Materials

Deuterated methanol (99.8% purity) (obtained in 0.5 ml vials), chloroform-d_1 ($CDCl_3$, 99.8 atom%) and tetramethylsilane (TMS) were purchased from Aldrich Chemical or Stohler Isotope Chemicals.

Results

Cell separation

A satisfactory cell separation of MIC, MES and MAC is achieved from the 16-cell stage, with the MIC fraction of cells 10–15 μm in diameter the least contaminated, with five other cells out of 150 and some cell fragments smaller than 8 μm (Fig. 1A). Fig. 1B shows MES (20–25 μm in diameter) but also contains 12 MIC and 7 MAC. MAC fractions (30–35 μm in diameter) contain 8 MES, 6 MIC out of about 100 cells (Fig. 1C). None of the fractions are pure, but they are considerably enriched in one cell type.

Cell homogenate membrane components

Visible particle bands from MES, MAC and MIC type homogenates were located mainly at four different densities (1.27, 1.21, 1.18 and 1.13) at the three lower interfaces and above the highest one in the discontinuous gradient. An example is shown in Fig. 2D which shows separations of homogenates from whole 16-cell embryo with and without prior treatment with animalization and vegetalization agents. Differences in the banding patterns are apparent, though there are still four major fractions in each.

Attempts to focus on one density fraction as cell membrane failed repeatedly in 16-cell density gradient separations using surface markers and enzyme distributions as criteria. Enzyme distributions were different between MACs and MICs (as shown in Table I), where enzyme assays were run on all fractions (either 14 or 18 fractions, according to the number of drops per fraction collected.) Therefore, we decided to simplify the analyses by combining fractions around the interfaces into four density gradient membrane fractions, M1–M4, each of which contained some surface membrane markers in some cell type. It was necessary to study all four of these gradient fractions for the enzyme and lipid studies. Fraction M1 extended from 60 to 50%, fraction M2 from 49 to 40%, fraction M3 from 39 to 30%, fraction M4 from 29 to 15% sucrose. Fig. 1A–C shows electron micrographs of sections of ruthenium red-treated embryos, fraction M1–3 pellets (labeled 1–3) and sections of intact cells (labeled S) for MES (Fig. 2A), MAC (Fig. 2B), MIC (Fig. 2C). Fraction M4 pellets were too small to handle for sections. The presence of membrane fragments are clearly seen in fractions M1–3. Fraction M1 stained with Ruthenium red in all three cell types and the electron dense particles can be seen in the M1 electron micrographs (Fig. 2A-C1). Fraction M2 pellet stained orange only in MES, fraction M3 stained only in MIC and MAC and particles can be seen in the micrographs of sections of those fractions. Con A

TABLE II

Enzyme distribution in gradient fraction pellets

Density	n	Specific activity (nmol/mg per min) Fraction 1 >1.237		Fraction 2 1.231–1.183		Fraction 3 1.178–1.132		Fraction 4 1.127–1.063	
		x	(S.D.)	x	(S.D.)	x	(S.D.)	x	(S.D.)
S.P.MAC									
Na^+/K^+-ATPase	6	10.17	(15.89)	11.67	(12.55)	22.83	(30.79)	31.66	(42.23)
Mg^{2+}-ATPase	5	38.00	(29.49)	60.21	(48.19)	16.00	(19.28)	24.80	(41.80)
S.P.MIC									
Na^+/K^+-ATPase	5	14.06	(18.08)	31.80	(38.02)	4.00	(1.60)	3.00	(6.00)
Mg^{2+}-ATPase	4	9.5	(10.45)	51.50	(27.50)	40.75	(38.11)	106.5	(149.9)
G6PD	1	0.033		0.011		0.003		0.0001	
L.P.MAC									
Na^+/K^+-ATPase	2	3.00	(3.00)	35.5	(35.5)	0		19.0	(19.0)
Mg^{2+}-ATPase	2	75.5	(36.5)	141.5	(3.5)	11.0	(11.0)	19.0	(19.0)
L.P.MAC AN									
Na^+/K^+-ATPase	2	34.0	(14.0)	31.0	(26.0)	0		0	
Mg^{2+}-ATPase	2	77.5	(17.5)	93.4	(19.5)	16.0	(16.0)	0	
L.P.MAC VEG									
Na^+/K^+-ATPase	2	9.0	(9.0)	86.0	(50.0)	22.0	(22.0)	22.0	(22.0)
Mg^{2+}-ATPase	2	106.0	(32.0)	102.0	(5.0)	25.0	(25.0)	2.5	(2.5)

TABLE III

Average enzyme specific activity distribution in gradient fractions (nmol/mg per min)

n = number of gradients, f = frequency of enzyme activity in the fractions, out of n.

Enzyme		NADH–cyt-*c* dehydrogenase						G6Pase						G6PD					
Fraction		MAC ($n = 3$)			MIC ($n = 4$)			MAC ($n = 4$)			MIC ($n = 2$)			MAC ($n = 5$)			MIC ($n = 6$)		
No.	Dens	f	x	(S.D.)	f	x	(S.D.)	f	x	(S.D.)	f	x	(S.D.)	f	x	(S.D.)	f	x	(S.D.)
1	1.26	2	8.0	(10.61)	4	90.5	(89.2)	2	3.75	(5.93)	0	0		4	38.4	(33.04)	3	13.0	(18.76)
2	1.25	2	3.0	(2.45)	3	20.25	(20.4)	1	2.25	(3.9)	0	0		5	83.2	(67.07)	6	30.17	(18.3)
3	1.24	3	7.33	(1.89)	2	6.75	(6.5)	1	1.25	(2.17)	1	1.0	(1.0)	5	139.4	(142.77)	6	31.5	(17.23)
4	1.24	3	7.67	(3.86)	3	7.0	(6.96)	2	2.75	(4.21)	0	0		5	99.2	(48.6)	6	28.3	(11.2)
5	1.21	3	8.67	(3.86)	3	5.5	(3.84)	1	5.5	(9.53)	0	0		5	94.8	(79.72)	6	26.16	(13.2)
6	1.19	3	16.67	(3.3)	3	16.0	(19.7)	3	10.25	(14.91)	1	4.0	(4.0)	5	73.8	(39.91)	6	23.0	(9.36)
7	1.18	3	13.0	(4.32)	3	19.5	(17.9)	2	8.0	(9.06)	1	6.0	(6.0)	5	51.0	(35.68)	6	37.66	(24.5)
8	1.16	3	29.33	(23.16)	3	20.5	(19.5)	4	13.75	(5.93)	2	24.5	(12.5)	5	39.8	(27.76)	6	23.83	(12.8)
9	1.13	3	4.33	(2.49)	2	35.5	(36.85)	4	18.5	(10.74)	2	41.5	(4.5)	5	37.0	(26.6)	6	27.0	(8.34)
10	1.11	2	9.33	(10.5)	2	141.25	(222.7)	2	12.25	(10.78)	1	23.0	(23.0)	5	39.2	(37.75)	4	48.0	(79.74)
11	1.06	0	0		2	41.25	(52.75)	2	2.75	(3.7)	0	0		3	9.4	(8.42)	2	11.33	(17.9)
12	1.06	0	0		1	6.5	(11.26)	0	0		0	0		4	11.6	(8.45)	2	8.17	(14.9)
13	1.06	0	0		2	39.0	(54.58)	1	7.0	(12.12)	0	0		1	3.8	(7.6)	0	0	
14	1.06	1	1.67	(2.36)	1	3.0	(5.2)												

stained the dense fraction of MAC and the two middle fractions of MIC and MES.

Enzyme distribution (Enzyme work not done on MES)

$Na^+/K^+/Mg^{2+}$-ATPase activity (calculated as the difference between total ATPase minus Mg^{2+}-ATPase enzyme activity in the presence of ouabain or absence of Na^+ and K^+) was distributed throughout the gradients (Table I). There are two densities where Na^+/K^+-ATPase is found in some gradients for both MIC and MAC (1.18–1.19 and 1.13) with some activity in all fractions with density greater than 1.13, with considerable variation in different batches of eggs. Only two out of four cases of MIC gradients had activity in each of those fractions, the others having activities at different densities. This shows a higher degree of variation of density of ATPase domains in the micromeres. Some of the MAC gradients had activity at density 1.06, not found in any MIC runs and higher activity at density 1.21. As the standard deviations clearly show, the gradient runs were not identical. In 2/16 MAC gradients there was no Na^+/K^+-ATPase, only Mg^{2+}-ATPase. This may be related to the fluctuation of the intensity and location of the histochemical enzyme stain as discussed later.

Distribution of specific activity for Na^+/K^+-ATPase in all four MAC pelleted fractions (Table II) shows its highest average in fractions M3 and 4 (densities 1.06–1.17) while that of the MIC was in fractions M1 and M2 (densities > 1.18). The values of specific activity for fraction M4 may be inflated by error increases due to the exceedingly low protein concentrations and absorbance increases in enzyme assays. The most dense MAC fractions pelleted were sometimes stimulated by lack of Na^+ and K^+, suggesting a different Mg^{2+}-ATPase there. Ouabain or lack of Na^+ and K^+ (both inhibit Na^+/K^+-ATPase) also stimulated the total ATPase (in this case Mg^{2+}-ATPase) in the less dense MIC fractions.

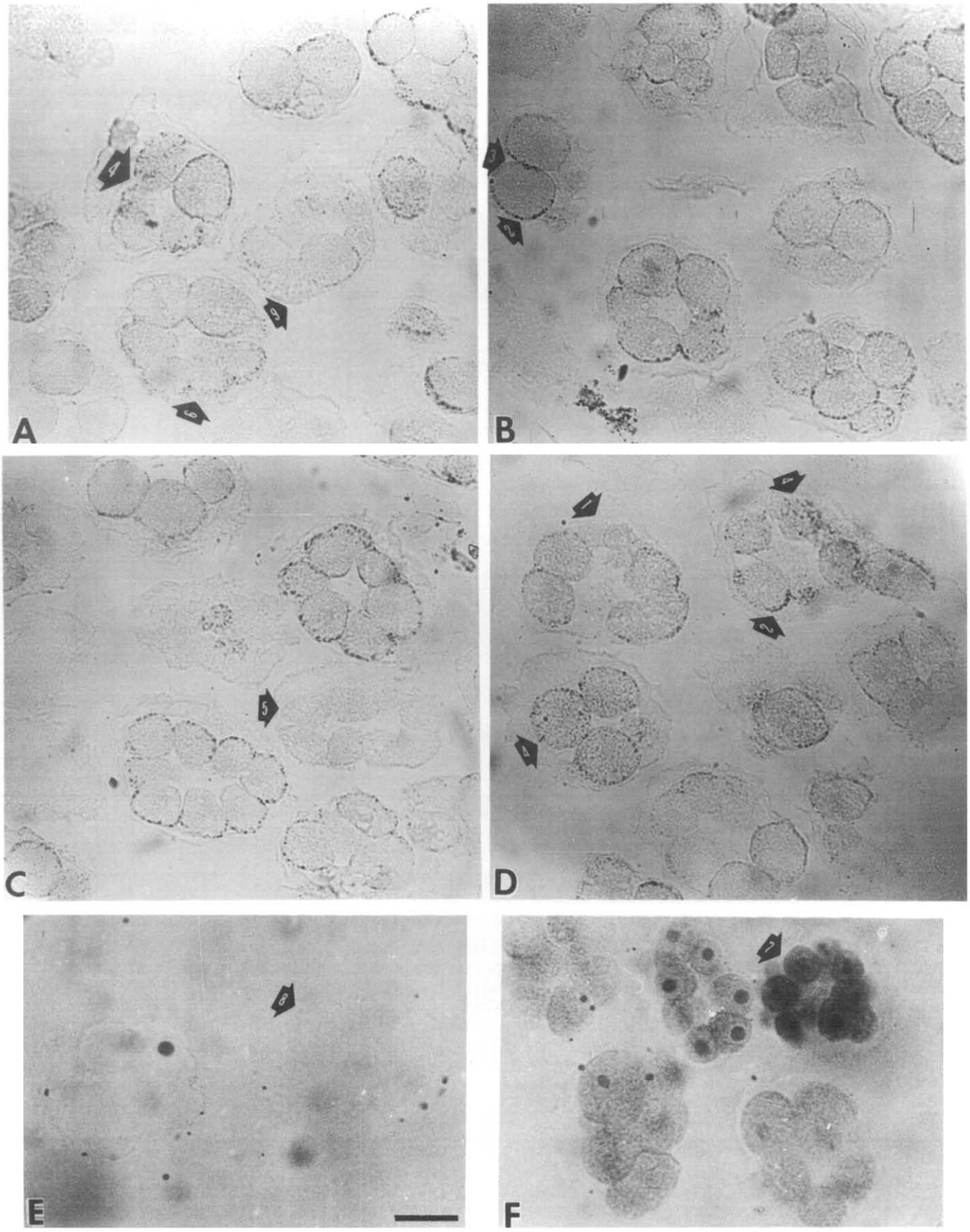
The cytoplasmic membranes as shown by marker enzyme distribution varied between MAC and MIC and were found to be most concentrated in the gradients with fraction M3. The distributions of NADH dehydrogenase (microsomes), G6Pase (microsomes) and G6PD (possibly bound on cytoplasmic matrix) (Table III) are not the same as the Na^+/K^+-ATPase though there is some overlap. G6Pase is at the same density (1.13) as one of the ATPase bands in both MAC and MIC (Table III). NADH dehydrogenase can be found in most fractions, but its highest specific activity was located at higher density (1.16, M3) in the gradient in MAC as compared to MIC (1.11, M4). Glucose-6-phosphate dehydrogenase (G6PD) was pelleted out of fractions throughout the gradient but its highest specific activity was at a higher density in MAC than MIC (Table III).

MIC gradients exhibit much less protein at the lowest densities, at the 28% interface than the MAC membranes. Most protein was found in pelleted fractions M1 and M2. This is where the homogenate was applied. DNA and RNA were distributed throughout the MAC gradients, with DNA concentrations highest at greater than 45% sucrose in both MAC and MIC and most MAC RNA was in fractions with less than 45% sucrose. MIC RNA was highest in the most dense half of the gradient. Tests for DNA and RNA in pelleted fractions showed most of it in fraction M1. This suggests that nuclear materials were in the most dense fractions and membranes with attached ribosomes were at lower densities in the gradient in MAC but higher densities in MIC gradients.

Histochemical results

Histochemical localization in the whole 16-cell embryo of Na^+/K^+-ATPase with light microscopy showed different possible distributions on the surface of cells, as seen in paraffin sections. Stain can be rather evenly spaced on only the exterior face of polarized MES or MAC cell surfaces (arrow 1, Fig. 3D) but not all cells of the same embryo stain in the same way, as shown by lack of stain in the MIC in the same section. Stain can be clumped or capped on their free edges (arrow 2, Fig. 3B, D) or between cells (arrow 3, Fig. 3B). ATPase assays stain not only the exterior, but alternatively the interior of the cell (arrow 4, Fig. 3A, D), as though there is a cycling due to endocytosis at some stages of cell division, as perhaps at arrow 6, where an embryo has the MES stained just inside the cell as if endocytosis is taking place and the MAC lack stain completely. Some clear vesicles appear which may be lysosomes where turnover of the enzyme might occur as at arrow 9. All stain is missing from the 8 cells visible (arrow 5) in one embryo. At arrow 7 is evidence that not only does

Fig. 3. Micrographs of paraffin sections of embryos stained histochemically for Na^+/K^+-ATPase as whole embryos, before sectioning. In A, arrow 4 indicates cells with internal stain granules, arrow 6 shows stain just beneath the surface of the mesomeres, arrow 9 shows some clear vesicles in the cell, possibly lysosomes where enzyme was destroyed after endocytosis. In B at arrow 3, the stain is near points of cell adhesion, as well as capping at arrow 2. In C, at arrow 5, an embryo with 8 unstained mesomeres is next to one with all mesomeres stained in a polarized manner on the external surface only. In D, at the arrow 1, unstained micromeres can be seen and at arrow 4, internal staining granules in macromeres. At arrow 2 capping of stained granules is seen. In E there is no stain without substrate. F shows enhanced cytoplasmic stain in ouabain treated assays. The bar represents 50 μm.


A
B
C
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E
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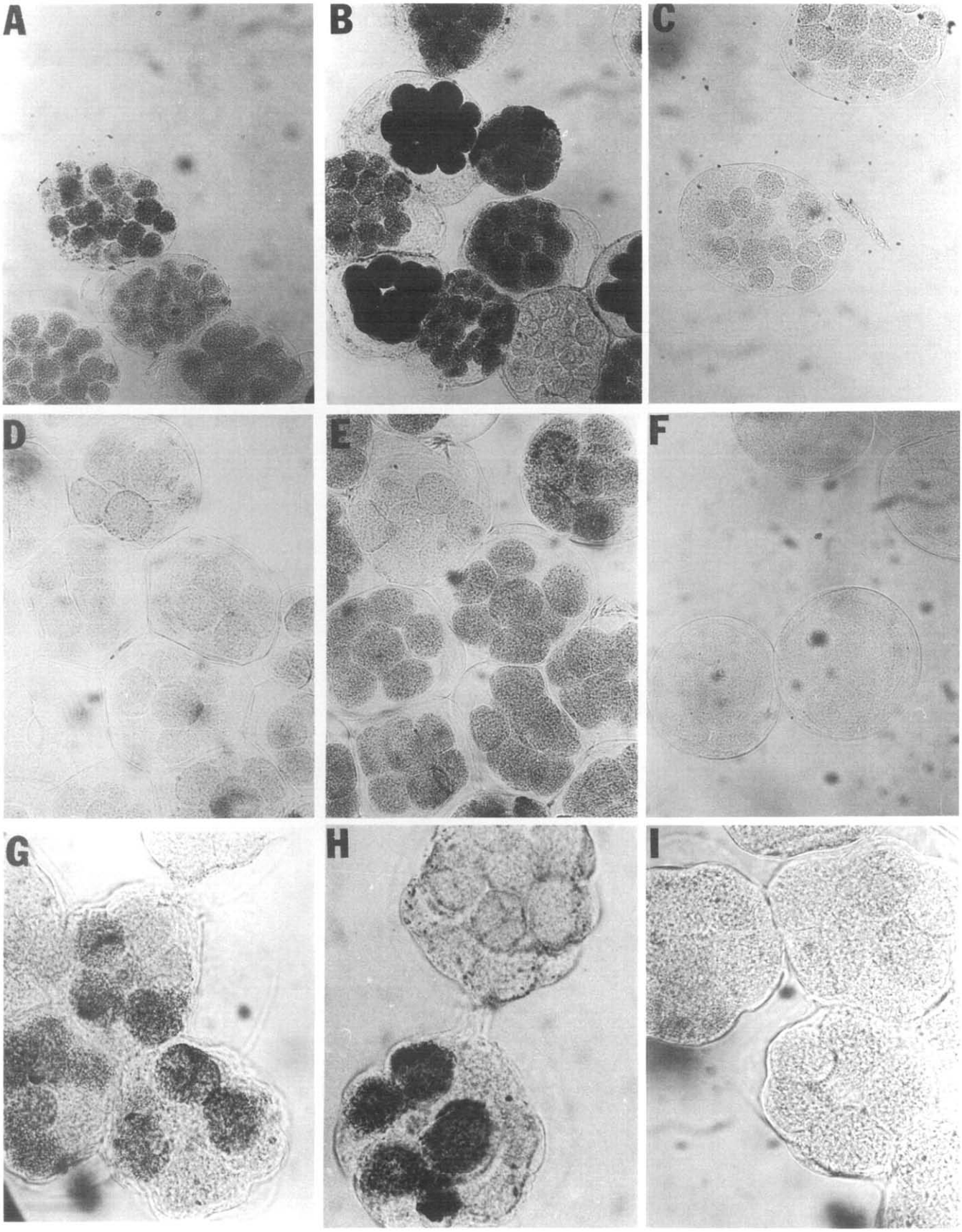
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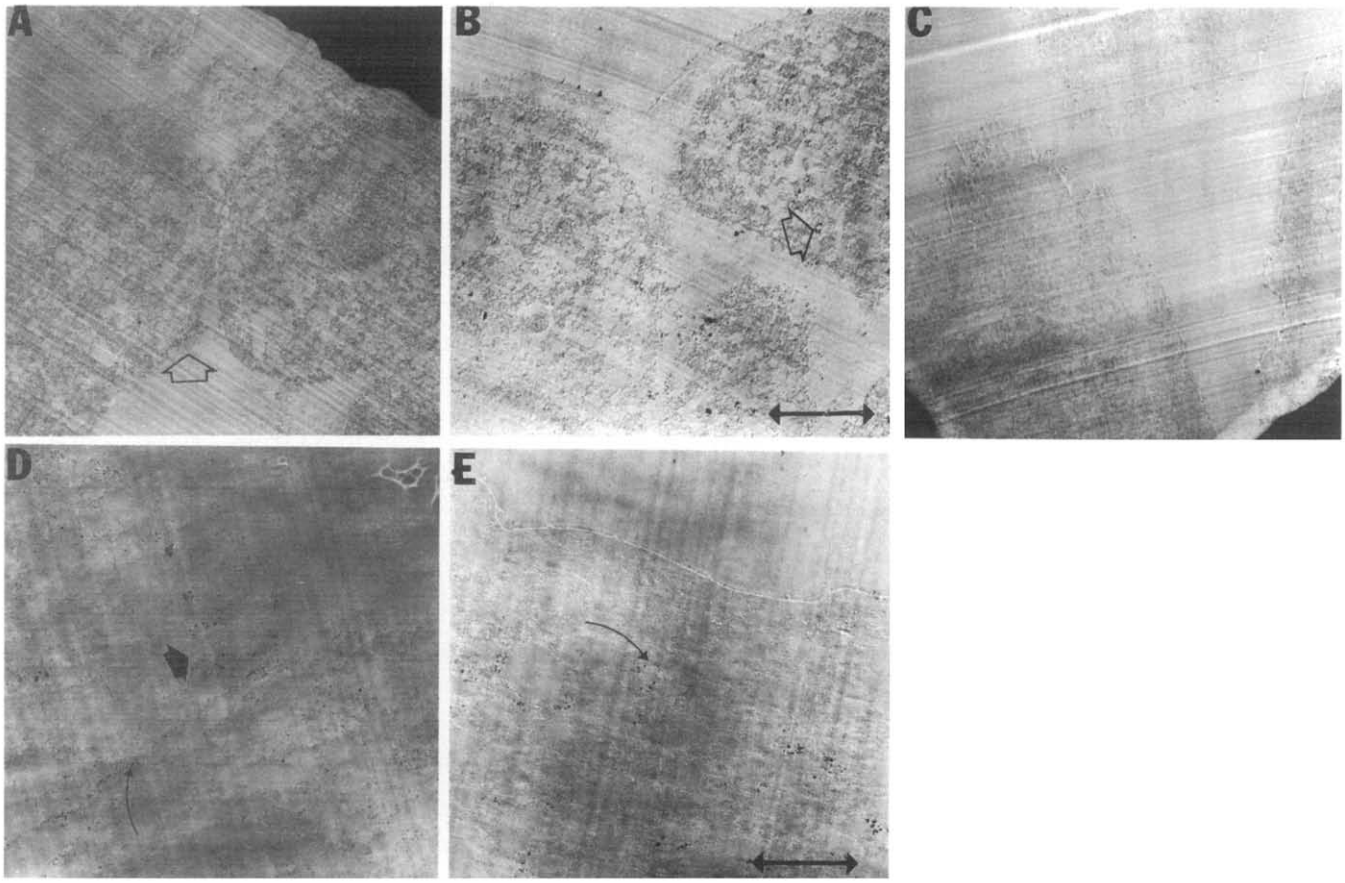

Fig. 5. Electron micrographs of 16-cell stage embryos stained histochemically for Na^+/K^+-ATPase before sectioning, with no post stain. (A–C) at magnification 2850×, (D–E) at 7650× (A) ATP substrate, the cell is the correct diameter for a micromere; (B) ATP plus ouabain; (C) NPP; (D) ATP substrate; (E) NPP. See text for discription of stain at arrows. Bar represents 6.66 μm in (A–C) and 2.66 μm in (D–E).

ouabain not inhibit staining under our conditions, but it actually enhances the stain in the cytoplasm. Control embryos treated with everything but substrate show no intrinsic lead stain (arrow 8).

The use of pseudosubstrates such as NPP permits decreased nonspecific reactions and it is generally agreed that K-NPPase activity is a valid representation of Na^+/K^+-ATPase activity [41]. ATP served as a substrate with much more staining of the cell and ouabain enhanced this staining at some cleavage stages, including the 8–16-cell stages and the presence of Na^+ was required. The two substrates may be staining two distinct enzymes since NPP staining is mainly in the cleavage furrows [48] and some honeycomb structures capping the cell. Both ATP (and NPP, not shown) stain cells in cyclic fashion during cleavage cycles as seen in the micrographs where some embryos are stained and some are not, with percentages of stained embryos changing during the cycle (Fig. 4A and D) as shown by different stain for the 8-, 16- and 32-cell stages in these whole mounts of two cultures, with and without ouabain. However, there is one embryo in each of the ouabain treatment micrographs (Fig. 4B, E) which is not stained, showing a difference in reactivity to ouabain at different times in the cycle. Sometimes the nucleus stains, with and without ouabain as seen in Fig. 4A, B. The whole mounts of embryos from the same culture as were used to make the sections in Fig. 3 are shown in Fig. 4G, H, I. Electron micrographs of embryo sections with ATP, ouabain and NPP are shown in Fig. 5. At this stage of the cycle, the ATPase is seen along the inner cell surface and between the cells in A and D, with some granules inside the cell associated with vesicles as seen in D and E. More activity is seen in the cytoplasmic vesicles in ouabain (B and E). NPP shows little stain at this stage. These micrographs have very low

Fig. 4. Micrographs of whole mounts of embryos stained histochemically for Na^+/K^+-ATPase. (A) 16–32-cell stage (near time of division); (B) same, but with ouabain present during the assay; (C) same as A with no substrate. (D–F) are the same as (A–C) but a different batch of eggs and slightly earlier at the 8-cell stage. (G–I) are whole mounts of the same group of eggs used for Fig. 3 sections where (G–H) have ATP and I does not. (A–F) same magnification as Fig. 3, (G–I) 2× greater.

contrast since there is no poststaining, only the lead assay stain which is done on the whole embryos before sectioning. That low contrast emphasizes the knife marks which are caused by sectioning with lead particles in the block which ruins glass knives very rapidly.

Quantitation of lipid content in membranes

Proton NMR spectra of the unknown extracted lipids from the four pellet fractions of each of the MES, MAC, MIC are shown in Fig. 6A, B, C, D. Fig. 6A–C

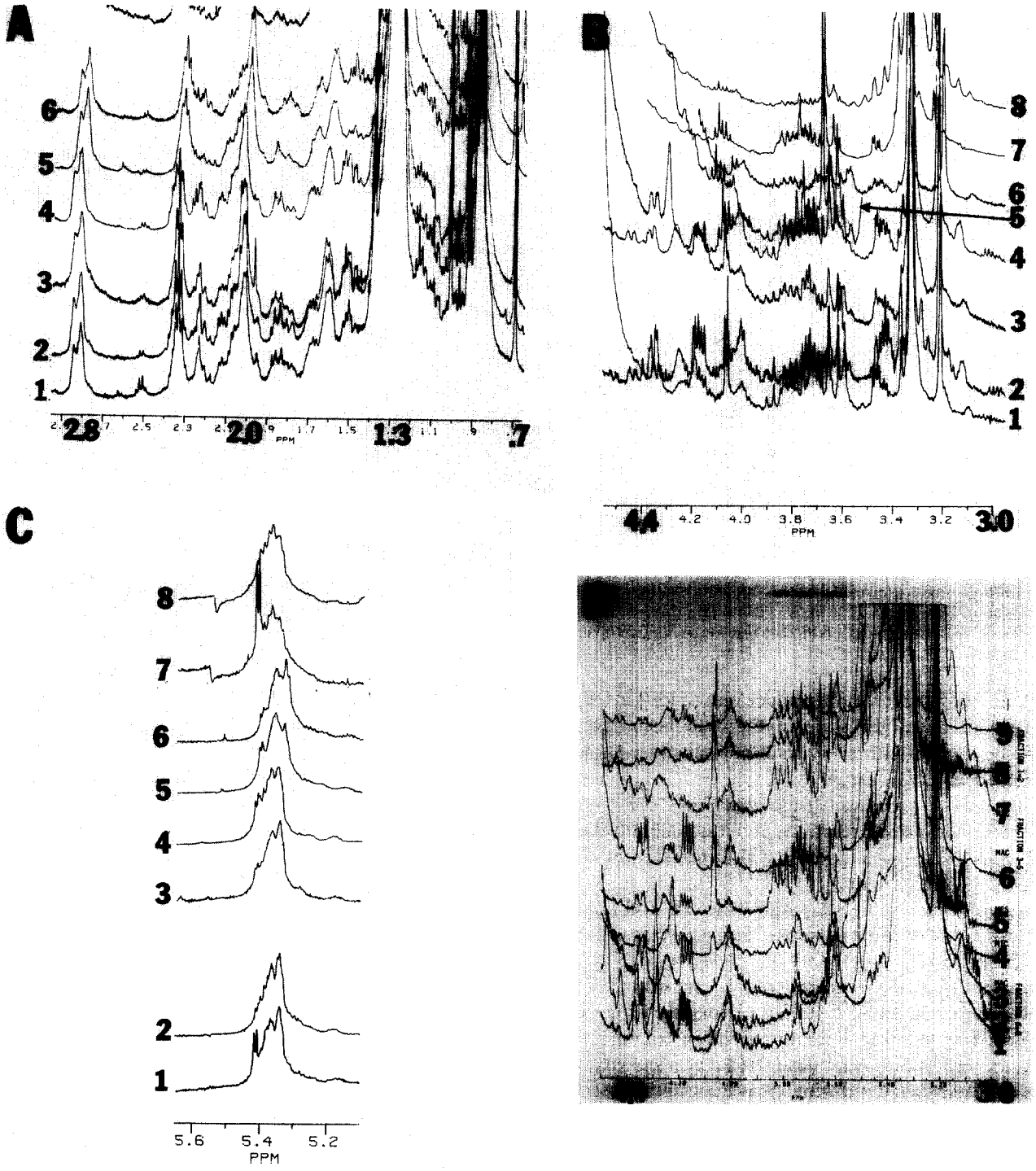

Fig. 6. Proton NMR spectra of unknown lipids extracted from the membranes from fractions 1–3 (from sucrose gradients) of the macromeres, mesomeres and micromeres of the 16-cell sea urchin embryos. The reference for 0 was tetramethyl silane (TMS). (A) acyl chain region: 1–3, MES, MAC,MIC M3; 4–6, MIC M2, MIC M1, MES M2. (B) headgroup region: 1–6 the same as (A); 7, MES M1; 8, MAC M1.(C) Double-bond region:same as (B). There is an unknown compound at higher concentration in MES fractions M1 and M3 fractions with rabbit ears at 5.4 ppm. (D) headgroup region of another set of cells: 1–3, MIC, MES, MAC M3; 4–6, MIC, MES, MAC M2; 7–9, MIC, MES, MAC M1.

are parts of the spectrum of one set of MES, MAC, MIC, with A representing the part of the spectrum including the acyl chain protons, B the region showing head group protons and C the double bond regions [32]. Fig. 6D is the headgroup region of a second group of MES, MAC, MIC, but arranged in different order from bottom to top. The spectra are different for each cell type and each membrane fraction. This reflects differences in lipid content. Spectral analysis for lipid content by LIPICK [33] confirms these differences. The variation in the choline concentration can be seen at 3.2–3.3 ppm and the ethanolamine at 3.16 ppm. Cholesterol is detected at 0.7 ppm, sphingolipids at 5.85 ppm and glycerol backbone protons are shown between 3.8 and 4.4 ppm [32]. Calculation of the relative lipid content and CH/PLIPID ratios and SPH/PC ratios and acyl chain length and level of unsaturation was done using ANALS [33]. Results are presented in detailed tables with number of runs and statistical evaluations of them, comparing all the fractions from control and treated embryos at one density and for one lipid at a time (Tables A-I, A–D.) These are essential for evaluation of the reproducibility of the results and interpretion of differences in content for each lipid in the same density fractions between different cell types and for exposing differences in relative concentration of particular lipids of MAC, MES, MIC and whole embryos. It is important to note the number (N) of NMR runs versus the frequency of detection for a particular lipid (n) in Tables A-I, A–D, to compare constant lipids and those dynamic ones which only appear sometimes in a particular density, for that cell type. Results revealed that the lipid content varies among the fractions of membranes from one cell type, but also varies between fractions which have the same density from all three cell types (Table A-I, A–D).

Comparison of MES, MAC, MIC M1–4 lipid

The average acyl chain length ranged from 12 carbons in fraction M4 of MAC to 22 for MAC fraction M2. MES had the shortest chain lengths in fractions M1–3. MIC had the longest chain lengths except in fraction M2. When the chain length is shorter, there are usually fewer unsaturations per chain. Disaturated PLIP is associated with CH, with more in the PM [49]. The CH/PLIP ratios varied from the lowest, 0.29 in fraction M1 MAC to the highest, 1.09 in MES fraction M3. Each cell type had its highest CH/PLIP domain in a different density fraction, MES in M3, MAC in M2, MIC in M1. Highest SPH/PC domains also were different: MES IN M3, MAC in M4 and MIC in M1.

Comparisons of fraction M1 lipids in MAC, MES and MIC (Table A-I, A–D) show MIC has more cholesterol (CH), sphingomyelin (SPH) and higher CH/PLIP ratio, higher SPH/PLIP and level of unsaturation and longer chain length than the other two cell types. MES have short acyl chains, more PC and PI and MAC has Con A binding, higher G6PD and more PE (MES and MIC had it only in one case each).

In fraction M2, MIC has more cerebroside (CEREB) compared to the other cell types, while MAC has more triacylglycerol (TG), a longer chain length and lacks Con-A binding, and MES has more PE and PI, binds Ruthenium red and has lower CH/PLIP. TG was detected in all three cell types in M2.

M3 shows lower CH/PLIP, less SPH (only one out of four cases) and slightly longer chain length and less unsaturation in MIC. PS is found only in fraction M3, (even then not all runs) except for MACs where it appears in one of three runs in fraction M1 (Table A-I, C). M3 from MES doesn't bind Ruthenium red.

The fraction M4 membranes have low UNSAT and considerably more CEREB in both MAC and MIC. There is more CH in MIC and higher SPH and shorter chain length in MAC. Micromeres have less PC than the other cells in fractions 1 and 4.

A comparison of Appendix Tables A-I, A–D L.P.MAC CONT lines with the MAC rows shows that the normal MAC density gradient fraction lipid content of *L. pictus* are different from those of *S. purpuratus*.

Dissociated vs. whole embryos

A comparison of 16-cell whole embryo gradient fractions (16-cell cont) and those of dissociated embryo isolated cells (MES, MAC, MIC) (Table A-I, A–D) shows that whole embryo membrane domains have a lipid content different from any of the dissociated cells or the sums of the three cell types. Whole embryo fractions have longer chain lengths, higher UNSAT, much less PI, than the isolated cells. Perhaps the dissociation process alters the membrane domains so that whole embryo membrane domains have different lipid content from any of the dissociated cells or the sums of the three cell types.

Effects of animalizing and vegetalizing agents on lipid content

Lipid content from gradient fractions of whole 16-cell treated and normal embryos of *S. purpuratus* and *L. pictus* are compared in detail in Appendix Table A-I. Some effects are similar in both species. For *S. purpuratus*, PI is found in 1/3 controls only in fraction M3, is found only in 1/3 fraction M2 in animalized (AN) and only 1/4 in vegetalized (VEG) M2 and 1/3 in VEG M3 fractions, but a considerable amount is found in VEG fraction M4. PS is found in fractions 2 and 4 in controls but some is found in M2-M4 in AN and M2 and M3 in VEG. For *L. pictus*, PI is found in fractions M1, M2 and M4 in L.P.MAC CONT and is limited to fractions M2-M4 in VEG treated and to M4 in AN embryos. PS is shifted from fraction M2 in L.P.MAC CONT to fraction M1 for AN and lost in VEG. PG is

TABLE A-IA

Lipid content of membrane fractions from 16-cell sea urchin embryos calculated from NMR spectra

N stands for number of NMR runs, *n* stands for the number of runs containing a particular lipid. Numbers in parentheses are standard deviations from the mean. Rel. conc. is the mean relative fraction.

Type lipid cell	Fraction M1			Fraction M2			Fraction M3			Fraction M4		
	N	*n*	rel. conc.	*N*	*n*	rel. conc.	*N*	*n*	rel. conc.	*N*	*n*	rel. conc.
Phospholipids												
PE, high in MITO [49]												
MES	2	1	0.16 (0.23)	2	2	0.26 (0.17)	4	3	0.07 (0.05)	0		
MAC	3	3	0.11 (0.003)	3	3	0.09 (0.08)	5	5	0.12 (0.06)	1	1	0.08
MIC	3	3	0.07 (0.13)	2	2	0.10 (0.002)	4	3	0.13 (0.13)	1	0	
16CELL CONT	1	1	0.11	3	3	0.14 (0.09)	3	3	0.18 (0.18)	1	0	
16CELL AN	1	1	0.20	3	3	0.10 (0.04)	3	3	0.18 (0.08)	1	1	0.20
16CELL VEG	0			4	4	0.12 (0.06)	3	3	0.12 (0.06)	1	1	0.05
L.P.MAC CONT	1	1	0.07	2	2	0.18 (0.09)	1	1	0.07	1	1	0.31
L.P.MAC AN	1	1	0.13	1	1	0.14	1	1	0.07	1	1	0.11
L.P.MAC VEG	1	1	0.01	1	1	0.23	2	2	0.19 (0.07)	1	1	0.25
PC, high in MITO, ER, PM, LYS [49]												
MES	2	2	0.27 (0.30)	2	1	0.10 (0.13)	4	3	0.11 (0.08)	0		
MAC	3	3	0.15 (0.01)	3	2	0.23 (0.23)	5	4	0.12 (0.11)	1	1	0.14
MIC	3	2	0.07 (0.06)	2	2	0.14 (0.09)	4	3	0.19 (0.17)	1	0	
16CELL CONT	1	1	0.14	3	3	0.15 (0.15)	3	3	0.08 (0.04)	1	1	0.25
16CELL AN	1	1	0.28	3	3	0.19 (0.05)	3	3	0.24 (0.11)	1	1	0.26
16CELL VEG	0			4	1	0.18 (0.10)	3	3	0.30 (0.20)	1	1	0.23
L.P.MAC CONT	1	1	0.10	2	1	0.17 (0.24)	1	1	0.29	1	0	
L.P.MAC AN	1	1	0.09	1	1	0.08	1	0		1	1	0.15
L.P.MAC VEG	1	1	0.26	1	1	0.31	2	2	0.25 (0.09)	1	1	0.33
LPC												
MES	2	0		2	0		4	0		0		
MAC	3	1	0.02 (0.04)	3	1	0.01 (0.02)	5	2	0.03 (0.06)	1	0	
MIC	3	0		2	0		4	0		1	0	
16CELL CONT	1	1	0.13	3	0		3	0		1	0	
16CELL AN	1	0		3	1	0.03 (0.05)	3	1	0.03 (0.05)	1	0	
16CELL VEG	1	0		4	2	0.06 (0.07)	3	0		1	0	
L.P.MAC CONT	1	0		2	0		1	0		1	0	
L.P.MAC AN	1	0		1	1	0.04	1	0		1	0	
L.P.MAC VEG	1	0		1	0		2	1	0.07 (0.10)	1	0	
PS, inner leaflet, PM, cytoskeleton attaches [49]												
MES	2	0		2	0		4	1	0.01 (0.03)	0		
MAC	3	1	0.03 (0.05)	3	0		5	2	0.03 (0.04)	1	0	
MIC	3	0		2	0		4	2	0.07 (0.08)	1	0	
16CELL CONT	1	0		3	3	0.06 (0.02)	3	0		1	1	0.15
16CELL AN	1	0		3	3	0.11 (0.03)	3	2	0.07 (0.06)	1	1	0.16
16CELL VEG	1	0		4	4	0.09 (0.05)	3	1	0.03 (0.05)	1	0	
L.P.MAC CONT	1	0		2	1	0.10 (0.14)	1	0		1	0	
L.P.MAC AN	1	1	0.11	1	0		1	0		1	0	
L.P.MAC VEG	1	0		1	0		1	0		1	0	
PI, high in ER and PM [49]												
MES	2	2	0.14 (0.15)	2	2	0.21 (0.14)	4	3	0.07 (0.05)	0		
MAC	3	2	0.06 (0.06)	3	1	0.06 (0.11)	5	4	0.06 (0.04)	1	0	
MIC	3	0		2	1	0.03 (0.05)	4	1	0.06 (0.11)	1	0	
16CELL CONT	1	0		3	0		3	1	0.02 (0.03)	1	0	
16CELL AN	1	0		3	1	0.05 (0.08)	3	0		1	0	
16CELL VEG	0			4	1	0.01 (0.02)	3	1	0.05 (0.09)	1	1	0.14
L.P.MAC CONT	1	1	0.06	2	1	0.05 (0.07)	1	0		1	1	0.27
L.P.MAC AN	1	0		1	0		1	0		1	1	0.10
L.P.MAC VEG	1	0		1	1	0.19	2	1	0.06 (0.08)	1	1	0.21

higher in M1 of AN treated membranes of both species and is found only in MES but is also elevated in M4 of 16-cell CONT and VEG and L.P.MAC AN.

It seemed logical that animalized embryo membranes should be more like MES membranes and vegetalized embryo membranes should be more like MAC or MIC.

TABLE A-IB

Lipid content of membrane fractions from 16-cell sea urchin embryos calculated from NMR spectra

N stands for number of NMR runs, *n* stands for the number of runs containing a particular lipid. Numbers in parentheses are standard deviations from the mean. Rel. conc. is the mean relative fraction.

Type lipid cell	Fraction M1			Fraction M2			Fraction M3			Fraction M4		
	N	*n*	rel. conc.	*N*	*n*	rel. conc.	*N*	*n*	rel. conc.	*N*	*n*	rel. conc.
Phospholipids (continued)												
PA												
MES	2	0		2	0		4	0		0		
MAC	3	0		3	0		5	1	0.02 (0.05)	1	0	
MIC	3	0		2	0		4	1	0.06 (0.11)	1	0	
16CELL CONT	1	1	0.13	3	0		3	0		1	0	
16CELL AN	1	0		3	0		3	2	0.10 (0.09)	1	0	
16CELL VEG	0			4	1	0.02 (0.03)	3	1	0.04 (0.07)	1	0	
L.P.MAC CONT	1	0		2	0		1	0		1	0	
L.P.MAC AN	1	0		1	0		1	0		1	0	
L.P.MAC VEG	1	0		1	0		2	0		1	0	
PG												
MES	2	1	0.02 (0.04)	2	0		4	0		0		
MAC	3	0		3	0		5	0		1	0	
MIC	3	0		2	0		4	0		1	0	
16CELL CONT	1	0		3	0		3	0		1	1	0.21
16CELL AN	1	1	0.23	3	1	0.06 (0.10)	3	0		1	0	
16CELL VEG	0			4	1	< 0.001	3	2	0.11 (0.10)	1	1	0.19
L.P.MAC CONT	1	0		2	0		1	0		1	0	
L.P.MAC AN	1	1	0.11	1	0		1	0		1	1	0.13
L.P.MAC VEG	1	0		1	0		2	0		1	0	
Spingolipids												
SPH (also PLIP)												
MES	2	0		2	0		4	3	0.24 (0.17)	0		
MAC	3	1	0.14 (0.24)	3	1	0.08 (0.13)	5	4	0.20 (0.15)	1	1	0.36
MIC	3	3	0.21 (0.12)	2	1	0.03 (0.05)	4	1	0.09 (0.18)	1	0	
16CELL CONT	1	0		3	2	0.09 (0.08)	3	2	0.18 (0.16)	1	0	
16CELL AN	1	0		3	1	0.11 (0.19)	3	0		1	0	
16CELL VEG	0			4	2	0.14 (0.16)	3	1	0.02 (0.03)	1	0	
L.P.MAC CONT	1	1	0.24	2	0		1	0		1	0	
L.P.MAC AN	1	1	0.03	1	1	0.12	1	1	0.25	1	1	0.39
L.P.MAC VEG	1	0		1	1	0.02	2	0		1	0	
Glycosphingolipids (no P)												
CEREB, GANG, SULF												
MES	2	2	0.07 (0.01)	2	0		4	2	0.11 (0.09)	0		
MAC	3	2	0.13 (0.12)	3	1	0.04 (0.07)	5	0		1	1	0.18
MIC	3	2	0.06 (0.07)	2	2	0.21 (0.08)	4	0		1	1	0.68
16CELL CONT	1	0		3	2	0.16 (0.14)	3	0		1	0	
16CELL AN	1	0		3	0		3	0		1	0	
16CELL VEG	0			4	1	0.1 (0.03)	3	0		1	0	
L.P.MAC CONT	1	0		2	0		1	1	0.38	1	0	
L.P.MAC AN	1	1	0.08	1	1	0.14	1	1	0.13	1	0	
L.P.MAC VEG	1	1	0.36	1	0		2	0		1	0	

However, for the PI and PS content which really change with dissociation and teratogen treatment, this does not appear to be so. Including all the four fractions, MAC and MIC and 16-cell CONT have low PI and PS content whereas MES have higher PI and PE content. 16-cell VEG also contain more PI and less PS as does L.P.MAC VEG. 16-cell AN has low PI and more PS. The teratogen treatment and dissociation may trigger the IP3 signaling system [50] and disrupt the PI content.

The CH/PLIP is highest in fraction M2 in the controls of both species. Treated cell fractions have considerably lower CH/PLIP except for fraction M3 for the L.P. compared to control.

Although there are changes in other lipids, they do not behave in a similar way in the two species: (1) SPH is found in fractions 2 and 3 in 16-cell CONT and VEG and only in fraction M2 of AN. SPH is in fraction M1 in L.P.MAC CONT but is shifted to all fractions in AN

TABLE A-IC

Lipid content of membrane fractions from 16-cell sea urchin embryos calculated from NMR spectra

N stands for number of NMR runs, *n* stands for the number of runs containing a particular lipid. Numbers in parentheses are standard deviations from the mean. rel. conc. is the mean relative fraction.

Type lipid cell	Fraction M1			Fraction M2			Fraction M3			Fraction M4		
	N	*n*	rel. conc.	*N*	*n*	rel. conc.	*N*	*n*	rel. conc.	*N*	*n*	rel. conc.
Nonpolar lipids												
CH												
MES	2	2	0.33 (0.18)	2	2	0.28 (0.05)	4	4	0.39 (0.12)	0		
MAC	3	3	0.31 (0.02)	3	3	0.28 (0.09)	5	5	0.42 (0.05)	1	1	0.24
MIC	3	3	0.44 (0.09)	2	2	0.35 (0.12)	4	4	0.34 (0.13)	1	1	0.32
16CELL CONT	1	1	0.20	3	3	0.41 (0.01)	3	3	0.50 (0.05)	1	1	0.29
16CELL AN	1	1	0.29	3	3	0.35 (0.08)	3	3	0.39 (0.04)	1	1	0.38
16CELL VEG	0			4	4	0.37 (0.12)	3	3	0.34 (0.14)	1	1	0.36
L.P.MAC CONT	1	1	0.42	2	2	0.35 (0.18)	1	1	0.26	1	1	0.33
L.P.MAC AN	1	1	0.39	1	1	0.47	1	1	0.39	1	1	0.13
L.P.MAC VEG	1	1	0.37	1	1	0.25	2	2	0.27 (0.04)	1	1	0.21
DOL, DOLP												
MES	2	0		2	0		4	0		0		
MAC	3	1	0.03 (0.06)	3	0		5	0		1	0	
MIC	3	2	0.06 (0.07)	2	0		4	0		1	0	
16CELL CONT	1	0		3	0		3	0		1	0	
16CELL AN	1	0		3	0		3	0		1	0	
16CELL VEG	0			4	0	3	0	1	1	0.04		
L.P.MAC CONT	1	1	0.11	2	1	0.15 (0.22)	1	0		1	1	0.09
L.P.MAC AN	1	0		1	0		1	0		1	0	
L.P.MAC VEG	1	0		1	0		2	0		1	0	
TG												
MES	2	0		2	1	0.16 (0.22)	4	0		0		
MAC	3	0		3	3	0.20 (0.18)	5	0		1	0	
MIC	3	0		2	1	0.11 (0.16)	4	1	0.9 (0.19)	1	0	
16CELL CONT	1	1	0.24	3	0		3	0		1	0	
16CELL AN	1	0		3	0		3	0		1	0	
16CELL VEG	0			4	0		3	0		1	0	
L.P.MAC CONT	1	0		2	0		1	0		1	0	
L.P.MAC AN	1	0		1	0		1	1	0.16	1	0	
L.P.MAC VEG	1	0		1	0		2	1	0.16 (0.22)	1	0	
FA												
MES	2	0		2	0		4	0		0		
MAC	3	0		3	1	0.01 (0.02)	5	0		1	0	
MIC	3	0		2	1	0.03 (0.04)	4	0		1	0	
16CELL CONT	1	1	0.5	3	0		3	1	0.05 (0.08)	1	1	0.09
16CELL AN	1	0		3	0		3	0		1	0	
16CELL VEG	0			4	0		3	0		1	1	0.08
L.P.MAC CONT	1	0		2	0		1	0		1	0	
L.P.MAC AN	1	1	0.05	1	0		1	0		1	0	
L.P.MAC VEG	1	0		1	0		2	0		1	0	

fractions and a small amount is in VEG M2. (2) PC in controls is in all 16-cell fractions but in AN is increased in M1-M3 and increased in fraction M3 in VEG. In L.P. fractions, PC is elevated in VEG and lowered in M2 and M3 of AN. (3) CEREB is found only in M2 of 16-cell CONT is greatly reduced or lost in treated. The position of CEREB in the gradients is shifted from L.P.MAC CONT fraction M3 to M1-M3 in L.P.MAC AN and M1 in VEG treated embryos. (4) PA and LPC appear only in fraction M1 in 16-cell CONT but in treated embryos they are found in fractions 2 and 3. PA does not appear in L.P. fractions and LPC does not appear in L.P.MAC CONT but appears in AN M2 and VEG M3. There is an effect on chain length by AN-VEG treatment in both species. Chain length is decreased in 16-cell VEG fraction M3 and increased in 16-cell AN fraction M4. L.P. AN and VEG treatments specifically reduce the acyl chain length and degree of unsaturation in all but fraction L.P.MAC AN M3.

Comparison of TLC and NMR results

The TLC results confirmed which major lipids were present, but showed little difference between the cell types. Spots were difficult to see on the TLC plates for

TABLE A-ID

Lipid properties of membrane fractions from 16-cell sea urchin embryos calculated from NMR spectra

N stands for number of NMR runs. Numbers in parentheses are standard deviations from the mean. Rel. conc. is the mean relative fraction.

Type lipid cell	Fraction M1		Fraction M2		Fraction M3		Fraction M4	
	N	rel. conc.	*N*	rel. conc.	*N*	rel. conc.	*N*	rel. conc.
CH/PLIP								
MES	2	0.37 (0.34)	2	0.47 (0.16)	4	1.09 (0.50)	0	
MAC	3	0.29 (0.08)	3	1.05 (1.02)	5	0.72 (0.46)	1	0.32
MIC	3	0.74 (0.22)	2	0.67 (0.73)	4	0.51 (0.42)	1	0.47
16CELL CONT	1	0.40	3	1.16 (0.35)	3	1.08 (0.65)	1	0.47
16CELL AN	1	0.42	3	0.68 (0.46)	3	0.50 (0.35)	1	0.61
16CELL VEG	0		4	0.59 (0.47)	3	0.31 (0.36)	1	0.59
L.P.MAC CONT	1	0.89	2	1.47 (1.86)	1	0.35	1	0.57
L.P.MAC AN	1	0.68	1	0.89	1	0.88	1	0.15
L.P.MAC VEG	1	0.59	1	0.34	2	0.88 (0.59)	1	0.27
SPH/PC								
MES	2	0/0.27	2	0/0.1	4	2.18	0	
MAC	3	0.92	3	0.34	5	1.66	1	2.57
MIC	3	3.0	2	0.21	4	0.47	1	0.0
16CELL CONT	1	0/0.14	3	0.69	3	2.21	1	0/0.25
16CELL AN	1	0/0.28	3	0.58	3	0/0.24	1	0/0.26
16CELL VEG	0		4	0/0.18	3	0.07	1	0/0.18
L.P.MAC CONT	1	2.4	2	1.6	1	0/0.29	1	0.0
L.P.MAC AN	1	0.33	1	1.5	1	0.25/0	1	2.6
L.P.MAC VEG	1	0/0.26	1	0.06	2	0/0.25	1	0/0.33
Chain length								
MES	2	14.77	2	17.64	4	17.06	0	
MAC	3	19.08	3	21.81	5	17.26	1	11.93
MIC	3	21.07	2	17.69	4	19.39	1	17.42
16CELL CONT	1	32.8	3	27.5	3	47.14	1	13.83
16CELL AN	1	30.47	3	24.5	3	24.4	1	40.9
16CELL VEG	0		4	21.94	3	15.2	1	18.8
L.P.MAC CONT	1	26.7	2	30.3	1	17.6	1	25.4
L.P.MAC AN	1	14.3	1	14.5	1	17.6	1	15.3
L.P.MAC VEG	1	14.9	1	14.5	2	13.41	1	13.4
UNSAT								
MES	2	1.17	2	1.05	4	0.92	0	
MAC	3	1.49	3	1.06	5	0.94	1	0.57
MIC	3	1.73	2	0.81	4	0.89	1	0.85
16CELL CONT	1	2.32	3	2.39	3	3.49	1	0.48
16CELL AN	1	3.04	3	2.30	3	1.59	1	3.17
16CELL VEG	0		4	1.84	3	0.92	1	1.26
L.P.MAC CONT	1	2.23	2	3.29	1	0.54	1	1.46
L.P.MAC AN	1	0.89	1	0.89	1	0.88	1	0.25
L.P.MAC VEG	1	0.61	1	0.64	2	0.80	1	0.80

the embryo fractions using the quantities of lipid available after the NMR (half for each of the neutral and polar plates.) Tracings of absorbance used for quantitation may read values which represent trailings of other lipids between spots rather than real spots. Consistently higher FA and TG readings were measured from the plates where they were found in all fractions in all cell types as compared to NMR results, but spots were present. The differences between PS and PI are not seen in TLC because the PI and PS spots cannot easily be distinguished using this solvent mixture unless both are present in higher quantities and two spots are present.

Differences in NMR spectra for the fractions as seen in Fig. 6 suggest that real differences must occur. A comparison of NMR and TLC errors on known mixtures showed that the NMR results were as good or better than the TLC for higher concentrations [32]. For the low concentrations in these unknowns, the NMR may be better and also lacks the acid and oxygen effects of the TLC method.

Cilia as an apical membrane domain

Apical sea urchin cell membrane domain could be represented by the cilia of the blastula stage. Table IV shows the unique lipid character of isolated cilia compared to deciliated embryos or intact embryos. The cilia membranes have less UNSAT, long chains, low PE, no TG, high SPH/PC and low Ch/PLIP. Such domains

TABLE IV

Relative mole fraction of lipid of S. purpuratus blastula, calculations from NMR spectra

	Cilia	Deciliated embryos	Whole embryos
CH, CE	0.297	0.251	0.183
PE	0.032	0.163	0.105
SPH	0.432		
PC	0.166	0.221	0.172
PS			0.080
DOL			
PI			0.099
PA			0.212
CEREB			
FA			
PG			
TG		0.366	0.150
LPC	0.073		
SULF			
C/PLIP	0.423	0.654	0.274
CHN LN	47.9	39.9	41.8
UNSAT	2.2	2.96	3.14
SPH/PC	2.6	0/0.22	0/0.17

are not at any uniform density for the three cell types at the 16-cell stage. Deciliated embryos lost PI,PA, PS, again showing changes with introduction of stress on the embryos.

Discussion

MES,MAC,MIC membrane preparations are different

Based on data from 16 different preparations of MES, MAC, MIC, we conclude that each of the three cell types at the 16-cell stage has a distinct density gradient distribution of surface markers and a unique though variable density distribution of enzyme activities and relative lipid content (CH/PLIP, SPH/PC). The differences lead to a conclusion that already at the 16-cell stage, there has been differentiation of the surface domains in the three cell types. It should not be surprising to find that three different cell types, with membrane parcelled out during cleavage from totally different regions of the original zygote which already possesses some polarity, would have different membrane domains and probably different attached cell coats and cytoskeletal elements and different densities.

Membrane domains containing a particular enzyme or surface marker or lipid are not restricted to one density in the gradients. MAC and MIC each contain PM domains of more than one density which contain high specific activities of Na^+/K^+-ATPase and which do not have the same lipid content in the two cell types. The same holds true for MES, MAC and MIC in regard to Con A receptors, for glycolipids or glycoproteins which bind Ruthenium red and for cytomembrane marker enzymes: they are not limited to one density and the distribution is different for the three cell types.

Each of the four density gradient fractions studied contained surface markers from at least one cell type. The same kinds of comparable membrane domains do not appear to be present at the same density in all three cell types. Density does not define a certain kind of membrane. There is no correlation between density of membrane and CH/PLIP ratio or SPH/PC ratio, since for any particular density the ratios vary with the three cell types. Highest CH/PLIP is not necessarily correlated with highest SPH/PC ratios.

Comparison of MAC membrane fractions between species (*S. purpuratus* and *L. pictus*) shows they have density gradient fractions that differ in lipid content. There doesn't seem to be a MAC membrane lipid pattern distinct from the other cell types which is generalized between species even though their development is similar.

AN-VEG teratogens perturb membrane fractions

The membrane domain densities and lipid contents are perturbed upon exposure of embryos to teratogenic (animalizing and vegetalizing) agents which produce abnormal development. This suggests an alteration of the pattern of membrane domains present as a result of the treatments which may be related to abnormal development. In particular, the content of PI and PS is altered in treated cell fractions, suggesting possible effects in the IP_3 signaling pathway [50] and effects which could alter attachment of cytoskeleton to the membrane [49].

Ongoing investigations using other drugs which disrupt cytoskeletal elements or membrane or metabolism are being completed on early cleavage embryos before membrane isolation and lipid analyses. These studies which attempt to determine the mechanism of change in enzymes or lipid domain density and content during development will be reported later.

Differences between whole embryos and dissociated cells

Disruption of cell adhesion may also affect membrane domains and their isolation since adhesion organelles have attached cytoskeleton and help to segregate receptors, pores, channels, enzymes and lipid domains to various sides of cells [54,69]. Zonula adherons or desmosomes remain attached to adherant membrane domains from two different cell types in liver homoginates and membrane isolation [4]. Dissociation may release this segregation and allow mixing of domains in a short time: 40 min for mixing of apical into lateral domains, 80 min for apical into basolateral frog bladder cells [54]. Fluorescent markers specifically attached to the apical PM start to move to the lateral membranes only after removal of Ca^{2+} and tight junctions [69].

The differences noted between whole 16-cell embryos and the dissociated cells are probably due to altered membrane adhesion and attached cytoskeleton due to divalent cation removal even though adhesion organelles are incomplete.

Comparison with lipids from other cytomembranes and plasma membranes

Evidence has been accumulating to make us cautious about expecting to find clear differences between cytomembranes (ER,Golgi,nuclear membranes) and PM in any one particular cell type. There is a great deal of overlap in density and lipid content as shown in Tables V and VI for various cytomembranes and cell membranes. In the cases of cytomembranes 21/25 had CH/PLIP ratios less than 0.51. Comparisons are made of PM preparations from Table VI and 16 additional references tabulated in Ref. 51. The majority of PM domains (38/58) have CH/PLIP above 0.5, but the domains with ratios less than that have values comparable to cytomembranes. It does appear to hold true that from the same cell type there is a membrane fraction containing surface markers which has a higher CH/PLIP than the cytomembranes from that cell type. However, if only that fraction is studied as the PM, other PM domains will be ignored. Using more than one surface marker shows that different PM domains of the same cell type are quite different in Ch/PLIP and SPH/PC ratios [40,52,10]. In general, PM has fewer than half the cases (14/31) with ratios of SPH/PC less

TABLE V

Comparison of lipid content [a] of various cell cytomembranes

Source	Density [b]	CH/PLIP	SPH/PC	Ref.
Intracellular membranes				
Nuclear membrane				
Rat embryos	2 fractions	0.31, 0.38		72
Bovine liver		0.255	0.11	73
Mitochondria				
Rat liver		0.1	0 /0.49	74
Rat embryos		0.48, 0.46		72
Rat liver	1.19, 1.21			75
Rat liver		0.12		76
Microsomes				
Rat embryo		0.47, 0.52		72
Rat parotid			0.16	77
Rat liver		0.255	0.11	73
Rat liver		0.07	0.07	76
Rough ER				
Rat liver		0.04	0.06	53
GP pancreas		0.12	0.10	78
Smooth ER				
GP pancreas		0.47	0.54	78
Rat liver		0.24	> than micro	76
Sarcoplasmic reticulum				
Rabbit muscle		0.02	0.08	13
Calcium pump				
Platelets		0.42		79
Intracellular membranes				
Human platelet		0.29	0.04	52
Golgi				
Bovine liver		0.14	0.27	73
Bovine liver	1.05–1.09			80
Rat intestine		0.49	0.39	81
Secretory granules				
Brain		0.29		82
Neuromuscular junction		0.48		82
Zymogen granules				
GP pancreas		0.55	1.09	78
Lysosomes				
Rat kidney		0.92	0.51	5
Pinocytotic vesicles				
Rat kidney		0.61	0.58	5

[a] Molar ratios, values taken from or calculated from references listed.
[b] Densities from references listed or calculated from % sucrose using Isco Tables, 4°C.

than 0.3, whereas cytomembranes have the majority (11/17) less than 0.3. Apical PM domains have much higher SPH/PC ratios than other PM domains [10, 5] as do the cilia from the sea urchin blastula studied here, which represent apical membrane at that stage. These differing ratios could make the apical PM more or less fluid and more or less amenable to insertion of particular proteins. The densities of PMs isolated from various

TABLE VI

Comparison of lipid content [a] of isolated plasma membranes

Source	Density [b]	CH/PLIP	SPH/PC	Ref.
Rat liver		0.76	0.53	76
		0.21	0.47	73
		0.6	0.89	74
		1.17	0.4	75
Rabbit small int. BBM		0.5	0.38	83
Rabbit ileum BLM		0.61	0.67	84
Rat intest BBM		0.82	0.5	11
Rat kidney BBM		0.9	2.12	10
BLM		0.5	0.35	10
Rat kidney BLM		0.73	0.91	5
BBM	microvilli	0.80	1.53	5
Rat kidney BBM	1.16			85
Sheep platelet	1.09–1.12	0.56		86
Pig platelet	1.15			86
Human platelet	1.06			86
Human platelet	1.18	0.59		79
Human platelet 2 fractions		0.61, 0.74	0.37, 0.47	52
Bovine brain		0.11	0.22	13
Duck salt gland		0.13	0.36	13
Lymphocytes		0.58	0.14	87
Thymocytes		0.58	0.09	87
Thymoma		0.44	0.08	87
L1210 cells		0.55	0.32	87
Lymphocyte		0.74	0.15	88
Leukemic lymphocyte		0.32	0.02	88
GP pancreas		0.51	0.78	78
Luteal cells-ewe			0.20	89
Calf thymus fraction	1.06	0.34		40
	1.09	0.46		40
	1.16	0.49		40
Hamster embryo fibroblasts				
surface coat, LETS	1.25			36
PM enzymes	1.18			36
Sea urchin oocyte		1.2	0.02	66
Sea urchin egg ghosts				
unfertilized		0.41	0.05	19
fertilized		0.52	0.18	19
Immature starfish oocyte		0.41		90
Mature starfish oocyte		0.25		90
Rat embryo				
2 fractions	1.0–1.05	0.85, 0.69		72
Ram spermatozoa		0.36	0.24	91
LM fibroblasts ConA-bound		0.63	0.17	92
non-bound		0.70	0.15	92
Myoblasts-undifferentiated		0.73	0.65	93
differentiated		1.04	0.41	93
Rat brain synaptic membrane		0.51		94
7-day embryo synaptic membrane		0.29		94
Ascites cell vesicles	1.10	0.45	1.11	59
sheets	1.16	0.30	0.59	59
INDUCED SHED VESICLES leukocytes		0.88	0.18	88
leukemic leukocytes	1.1		9x > SPH than PM	88

[a] Molar ratios, values taken from or calculated from references listed.

[b] Densities from references listed or calculated from % sucrose using Isco Tables [95], 4°C.

kinds of cells, identified by surface markers or morphology, range through the most freguently used density gradient from 1.05–1.18 or from 12 to 40% sucrose.

The conclusion must be made that there is extensive overlap of surface membrane and cytomembrane fractions in terms of density and lipid content. Quite often, investigators try to isolate only one part of the membrane, so the use of only one fraction is desirable. Generalizations cannot be made regarding other PM domains or other cell types (Table VI), or even the same cell type in a different species (observe the different platelets in Table VI.) High CH/PLIPID (40] ratios and SPH/PC ratios [53,52] have in the past suggested presence of PM, whereas low values suggest presence of cytomembranes. We are suggesting some caution with this generalization. It has already been established in adult cells that membrane domains facing the outside with microvilli may have a different density from the part between cells where adhesion may develop, or the basal area with its basement membrane [4]. Differences between isolated PM domains with different densities can be due to presence or absence of cell coats or LETS protein [36,54], attached cytoskeleton [6,8,9], different surface marker enzymes [55,36,9] or lipid relative concentrations [10].

We cannot tell what kind of membrane is in a fraction by lipid content and density alone. Fractions from 16-cell embryos with CH/PLIP ratios higher than 0.5 are found at different densities in the three cell types. Domains with an identifiable high CH/PLIP or SPH/PC are also shifted with drug treatment, so that it appears that associations of proteins or sugars with these domains are altered during development or drug treatment. Further work will be required to identify the types of membranes present at different densities in sea urchin embryos. Precise timing in membrane isolation may be very important for repeating results.

Dynamic changes in ATPase domains and their cell polarity during division

The concept of heterogeneity in PM domains at the 16-cell stage is supported in this study as well by the cytochemical Na^+/K^+-ATPase lead stain. A polarity of the Na^+/K^+-ATPase (or a nucleotidase) is already present at this stage, but it is unstable and dynamic. It is not the same in all three cell types and varies with the cell cycle and the location in the embryo. Factors known to stabilize and isolate PM domains of adult epithelial cells (tight junctions, zonula adherons or belt desmosomes) [54, 16] are not yet well developed.

Membrane domains containing Na^+/K^+-ATPase have been shown to also have attached ankyrin and spectrin or fodrin which may be involved in the segregation at the time cell polarity is established for the enzyme [9].

Division may make membranes more dynamic. An association of altered Na^+/K^+-ATPase activity with administration of regulators of cell division (phorbol myristate acetate, phytohemagglutinin) and inhibition of cell division by ouabain has previously been reported [56]. Cortical ATPase showed altered ouabain sensitivity during meiosis and first cleavage also in *R. pipiens* [57]. This may mean that regular changes in enzyme activity occur due to normal cleavage cycle regulators. Na^+/K^+-ATPase was highest in mouse fibroblasts after cell cycle activation in G1 [58]. Changes in density distribution of cell surface subclasses containing surface enzymes also occurs during division [59].

The lability or dynamic nature of the membrane sends traffic between PM and cytomembrane fractions [60,12,49]. This is probably the result of endocytosis and exocytosis of vesicles between the PM and the cytomembranes (Golgi, ER). This can result in proteins and lipids selective removal or insertion if only certain domains can participate because of their association with cytoskeletal elements. However, these vesicles may only store these membrane elements to later replace them at the surface. That means that the lipids and proteins removed will appear in another cytomembrane category, having a different density due to the attached cytoskeletal elements and selective internalization of particular lipid domains. It also means that various cytomembranes can be very similar to each other at times of high membrane turnover between fractions. This would explain the high degree of overlap between densities, lipid ratios and enzyme content, among different membrane categories. The amount of Golgi, or ER, or NM, or pinocytotic vesicles (PV), or lysosome membrane may vary during cell cycle and cell differentiation.

Since the Na^+/K^+-ATPase histochemical stain appears inside some of the cells of the 16-cell stage (associated with membrane vesicles) depending on the cell cycle, (and this is even more obvious during the first three cleavages (Sparling, unpublished)), endocytosis of the membrane domains containing the enzyme is suspected. Cytoplasmic vesicles have been noted in sea urchin embryos [61] and such vesicles, if they are formed by endocytosis would be outside PM inward and unless broken by detergent might sequester membrane components and cause negative enzyme assays of isolated membrane fractions. However, in the cytochemical assays, the 20% DMSO makes the PM permeable to assay chemicals, so the enzyme could be detected. PV have previously been shown to contain ATPase in differentiated cells [5] or to contain externally applied ouabain which binds to that enzyme [62]. There are three cycles of endocytosis of ATPase per cell generation, with cell cycle fluctuations of ion pumping [63]. Most PM proteins have a much longer halflife than the ATPase which disappears once it is inside the cell (in the lyso-

somes) [63]. The density of the membrane domain which has the ATPase could vary due to the stage of internalization or externalization since PV containing horseradish peroxidase are less dense than PM or lysosomes [5]. This may explain some of the variability in our results.

The abberant ouabain effect

The question of why ouabain does not inhibit the enzyme in the cytochemical stain in this study may be due to the proportions of Na^+ and K^+ and Mg^{2+}, since high Na^+ and Mg^{2+} together alter the binding of ouabain to the enzyme [67]. Ouabain stimulation of the enzyme has been noted previously in other tissues, but usually at lower concentrations [68]. Ouabain accelerated fluid accumulation in the polar 16-cell mouse embryos for blastocoel formation at 10 μM, but inhibited it at 100 μM [96] showing the importance of concentration.

We cannot incontrovertably say there is polarity of NA,K-ATPase which is dynamic and changes during cleavage since the usual test for it is inhibition by ouabain. We can say that there is some ATPase present which is polarized and dynamic, appearing on the surface, then on cytoplasmic membranes during cell division with cycles differing in the three cell types.

Comparison to other studies

In contrast to our studies, Na^+/K^+-ATPase detected by antibody instead of the lead stain does not become polarized in mouse embryos until after compaction where cell adhesion becomes stabilized in the morula stage, but its appearance doesn't seem to be tied to cell division [70]. It is seen in the cytoplasm first, then at the membrane and becomes restricted to cells outside the inner cell mass.

A comparison of our membrane fractions with the work of others shows that previous attempts to isolate sea urchin PM have produced variable results. Others have encountered difficulties in isolating sea urchin embryo membranes during early development, since the specific activities of ATPase and NPPase "failed to fit any meaningful pattern" [64]. They also found no inhibition of ATPase by ouabain or omission of Na^+. They concluded that the value of cholesterol and the ATPase and NPPase enzymes as PM markers was uncertain in sea urchins [64]. Several fractions containing membrane marker enzymes were isolated from swimming blastulas at five interfaces in discontinuous gradients [65] but they chose to pick one of the fractions (at 38% sucrose, 1.17 density) which lacked enzymes of cytoplasmic particulates. The other fractions also had 5′nucleotidase and phosphodiesterase and may be other domains of PM [65]. Previous work in our laboratory revealed changes in ATPase activity and lipid content in cortical hulls of two species of sea urchins at fertilization [19]. In another laboratory, large sheets of membrane were isolated from unfertilized eggs and high levels of PE and PI and CH were found [66]. Their membrane contained ouabain-sensitive ATPase. Extraction with KI removed a surface coat and caused vesiculation of the membrane and decreased the density of the membrane [66].

There is much dynamic action in the membrane fractions at the 16-cell stage: rapid cleavage, rapid setting of embryonic axes and differentiation. This helps to explain past problems with membrane isolation and variation amongst different preparations in early embryos.

Quite in contrast to this picture of dynamic change in the membrane is the recent report [71] in starfish embryos of stable domains associated with the original external surface, which are involved in embryo polarity and cannot become involved in cell adhesion. These regions are revealed by cortical factors (nile blue sulfate granules). If that part of one of the blastomeres at the two cell stage is rotated toward the other blastomere, you get twin embryos since it can't adhere. This cortical area remains affected up to the 256-cell stage, so it appears to be very stable. There may be one set of factors which is not changed and maintains a memory of original polarity and another set which changes all the time as division occurs, setting up new polarities, which may be the case for the ATPase observed here.

Further work is needed to elucidate the functions of the various membrane domains present in each cell type and the mechanisms involved in the lipid and enzyme differentiation between the three cell types, in dissociated cells and in drug-treated cells.

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