Morphologic aspects of adhesion and spreading behavior of amphibian blastula and gastrula cells

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SUMMARY

By means of SEM we have examined spreading and adhesive behavior of cells isolated from superficial and deep regions of germ layers from blastula to late gastrula in Rana pipiens embryos. Each of the cell populations sampled show adhesive and spreading characteristics distinctive for each region and stage which we interpret as demonstrating the following:

1. From blastula through late gastrula, cells from each region have already acquired the ability to express surface morphologic and adhesive features independently of their association with their neighbors, i.e. autonomously.

2. The distinctive spreading and adhesive characteristics for each tissue sub-population suggest kinetic properties seemingly related to their in vivo morphogenetic movements, epiboly or invagination.

3. The appearance within germ layers of two subpopulations between blastula to mid-gastrula, suggests early intratissue inductive interactions.

4. The outermost, superficial cells from each germ layer show proximal and distal surface differences which may reflect adhesive differentials as postulated by Steinberg (1970) for presumptive ectoderm cells.

5. With the exception of superficial cell proximal and distal differentiation, freshly disaggregated cells do not show morphologic characteristics seen in corresponding cells spreading for one hour.

INTRODUCTION

During gastrulation, the body plan of the amphibian embryo is established. Early experiments indicated that gastrular movements are associated with specific changes in shape and arrangement of cells (Holtfreter, 1943, 1944). Formative movements of component parts of gastrulae are partially the result of autonomous tendencies (Schechtman, 1942). Directed movements of regions appear attributable, in part, to kinetic behavior of individual cells. There are relatively few recent studies on adhesiveness, spreading and motility of isolated cells from specific regions (Johnson, 1976).

Electrophoretic studies on Rana pipiens cells showed that, with onset of

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gastrulation, cells from various presumptive (Pr) regions become electrophoretically different (Brick, Schaeffer, Schaeffer & Gennaro, 1974). Johnson (1970) indicated that changes must occur in cell contact behavior at initiation of gastrulation, in particular, cell adhesiveness. Cells from amphibian gastrulae exhibit increased surface deformability consistent with increased cell motility (Johnson & Smith, 1976). A similar situation exists with deep cells of *Fundulus* gastrulae (Tickle & Trinkaus, 1973). At gastrulation, changes in cell-surface properties apparently occur and cell-surface modulation may be important in directing and coordinating gastrular movements. Individual cells and groups of cells continually change relative positions and mutual adhesions during gastrulation.

The present study differs from previous work reported in several ways. (1) Cells from selected Pr regions of *Rana pipiens* isolated prior to, at onset of and during gastrulation adhered and spread *in vitro*. (2) Inasmuch as the amphibian blastoderm wall is multilayered, cell populations deep to the surface as well as in superficial layers were examined. (3) Steinberg (1970) suggested outer ectodermal cells of the amphibian gastrula have proximal and distal surfaces with differing adhesive values. To observe if inner and outer surfaces of these cells, as well as those from other germ regions, exhibit different morphologic characteristics, disaggregated and spreading cells from the outermost superficial layer were examined. (4) Cells were examined immediately following disaggregation to see if differences related to Pr tissue type and stage could be observed prior to cells spreading on a substratum.

The embryonic areas selected display, *in situ*, different patterns of movement during gastrulation. Pr head endoderm and notochord invaginate, Pr epidermal ectoderm undergoes epiboly, while Pr neural ectoderm undergoes convergence. Since cells can only adhere, spread and move by utilizing available equipment, behavior *in vitro* was expected to reflect spreading and adhesive behavior *in vivo*. Spreading behavior of cell types was used to assess formative tendencies specific to different Pr regions, and to detect changes in cell-surface activity of each cell type corresponding to onset and continuance of gastrular tissue translocation.

Scanning electron microscopy (SEM) provides three-dimensional images at high resolution and allows observation and quantification of fine cellular projections. Particular attention was given to type and distribution of projections formed by specific cell populations, in view of the role proposed for cell projections in adhesion and spreading (Curtis, 1964), and motility (Gustafson & Wolpert, 1961; Brick *et al*., 1974).

**MATERIALS AND METHODS**

Ovulation of *Rana pipiens* was induced and eggs fertilized according to Rugh (1962). Embryos were maintained at 13°C in spring water.

Blastula, early-, mid-, and late-gastrula stages (St) were used, St 9, 10, 11 and
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(Shumway, 1940). Embryos were brought to 23–25 °C and, following a 10–15 sec rinse in 70 % ethanol, jelly layers were removed in sterile Barth’s solution X, pH 7.5 and ionic strength of 0.09797 (Barth & Barth, 1959). All operations were under sterile conditions.

Embryos were rinsed in five changes of sterile Barth’s solution prior to vitelline-membrane removal and dissection. Demembranated embryos were transferred to fresh sterile Barth’s solution. Embryos thus demembranated developed normally.

Specific areas were dissected and transferred into modified Barth’s dissociation solution pH 8.9 (Barth & Barth, 1959), modified to contain 15 mg % of tetrasodium ethylene-diamine tetraacetate.

Pr embryonic regions utilized included: (1) Blastula (St 9) – Pr head endoderm (Pr dorsal lip); Pr notochord; Pr inner neural ectoderm; Pr inner epidermal ectoderm. (2) Early gastrula (St 10) – Pr head endoderm (dorsal lip); Pr notochord; Pr inner neural ectoderm; Pr inner epidermal ectoderm. (3) Mid-gastrula (St 11) – Pr head endoderm (leading edge of invaginating fold); Pr notochord (dorsal lip); Pr inner neural ectoderm; Pr inner epidermal ectoderm. (4) Late gastrula, mid-yolk plug (St 11ξ) – Pr head endoderm (leading edge of invaginating fold); Pr notochord (chordamesoderm, midway between anterior-posterior axis); Pr inner neural ectoderm; Pr inner epidermal ectoderm.

All cell layers disaggregated within 15 minutes. Both inner and outer ectodermal layers were excised as a unit, the tissue placed in dissociation medium and within 5 min the inner layer peeled off. The outer layer was removed (very cohesive) and the inner layer disaggregated. For superficial-cell studies, outer ectoderm was transferred to fresh dissociation medium. After disaggregation of all cell types, dissociation solution was gently pipetted off and replaced with Barth’s Ca\(^{2+}\)–Mg\(^{2+}\)-free dissociation medium without EDTA, pH 7.7. Cells remained in the rinse 5–10 minutes.

Cell-spreading experiments were made on autoclaved coverslips in sterile petri dishes. A large sitting drop of Barth’s solution was placed on coverslips and cells from Ca\(^{2+}\)–Mg\(^{2+}\)-free rinse were transferred to incubation medium. These cells spread for 60 minutes at 23–25 °C prior to fixation.

Pigmentation of superficial-layer cells provides a natural marker for orientation of isolated cells. In situ, their distal ends are heavily pigmented compared to proximal ends. Isolated cells retain the pigment asymmetry designating former proximal and distal surfaces. Light micrographs of superficial-cell preparations were made prior to coating for SEM.

Cells on coverslips were treated with 3 % glutaraldehyde in 0.03 M PIPES buffer, pH 7.4, according to Salema and Brandao (1973). Cultures in buffered fixative at least 24 h at 4–5 °C were rapidly dehydrated in an ethanol series and placed into a Denton DCP-1 carbon dioxide critical-point dryer.

Following critical-point drying, preparations were coated with carbon and gold-palladium alloy (60 % gold, 40 % palladium) in a Denton DV-502 vacuum
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pr inner neural ectoderm cells</th>
<th>Pr inner epidermal ectoderm cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage: blastula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell shape</td>
<td>Spherical</td>
<td>Spherical</td>
</tr>
<tr>
<td>Adhesion/spreading</td>
<td>Variable width peripheral filopodia, some with periodic swellings</td>
<td>Variable width peripheral filopodia</td>
</tr>
<tr>
<td>Flattening</td>
<td>Minimal</td>
<td>Attached region extremely flattened</td>
</tr>
<tr>
<td>Unattached surfaces</td>
<td>Few projections</td>
<td>Few blebs, few short filopodia</td>
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<tr>
<td>(Fig. 1)</td>
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<td>(Fig. 3)</td>
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<tr>
<td>Stage: early gastrula</td>
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<tr>
<td>Cell shape</td>
<td>No change</td>
<td>Spherical</td>
</tr>
<tr>
<td>Adhesion/spreading</td>
<td>No change</td>
<td>Peripheral thick, uniform filopodia</td>
</tr>
<tr>
<td>Flattening</td>
<td>No change</td>
<td>Distal filopodia tips flattened</td>
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<tr>
<td>Unattached surfaces</td>
<td>No change</td>
<td>Few projections</td>
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<tr>
<td>(Fig. 4)</td>
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<td>(Fig. 5)</td>
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<tr>
<td>Stage: mid-gastrula</td>
<td>Two populations</td>
<td></td>
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<tr>
<td>Cell shape</td>
<td>Similar to above</td>
<td>One end elongated</td>
</tr>
<tr>
<td>Adhesion/spreading</td>
<td>Similar to above</td>
<td>Mainly at elongate end by stem protrusion, some cells at both ends</td>
</tr>
<tr>
<td>Flattening</td>
<td>Similar to above</td>
<td>Minimal</td>
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<tr>
<td>Unattached surfaces</td>
<td>Similar to above</td>
<td>Extensive at webbing &amp; filopodia at distance from cell base</td>
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<tr>
<td>(Fig. 2)</td>
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<td>(Fig. 5)</td>
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<tr>
<td>Stage: late gastrula</td>
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<tr>
<td>Cell shape</td>
<td>Same as above</td>
<td>Spherical</td>
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<tr>
<td>Adhesion/spreading</td>
<td>Same as above</td>
<td>One end lamellipodium with microspikes, other regions filopodia</td>
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<tr>
<td>Flattening</td>
<td>Same as above</td>
<td>Entire cell flattened</td>
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<tr>
<td>Unattached surfaces</td>
<td>Same as above</td>
<td>Few projections</td>
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<td>(Fig. 7)</td>
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<td>(Fig. 7)</td>
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<td>Table 1 (cont.)</td>
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<tr>
<td><strong>Pr head endoderm cells</strong></td>
<td><strong>Pr notochord cells</strong></td>
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<tr>
<td>Outer, superficial cells</td>
<td>Inner, more proximal cells</td>
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<tr>
<td>Spherical, annular constriction, small distal, large proximal end</td>
<td>Spherical</td>
<td></td>
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<tr>
<td>Proximal end peripheral filopodia, some lobopodia</td>
<td>Fine filopodia</td>
<td></td>
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<tr>
<td>Slight</td>
<td>Minimal</td>
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<tr>
<td>Scattered filopodia proximally, blebs distally (Fig. 8)</td>
<td>Fine filopodia</td>
<td>Evenly distributed microvilli, short filopodia</td>
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<tr>
<td>Same as above</td>
<td>Spherical</td>
<td></td>
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<tr>
<td>Same as above</td>
<td>Fine filopodia, more than blastula (finer than both ectoderm)</td>
<td></td>
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<tr>
<td>Same as above</td>
<td>Minimal</td>
<td></td>
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<tr>
<td>Same as above</td>
<td>Flatter than blastula</td>
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<tr>
<td>Same as above</td>
<td>Fine filopodia (Fig. 9)</td>
<td>Few projections (Fig. 12)</td>
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<td><strong>Two populations</strong></td>
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<tr>
<td>Dumbell, one flat, one spherical end</td>
<td>Spherical</td>
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<tr>
<td>Extended periphery at flat end, spherical end short filopodia</td>
<td>Long fine filopodia extending some distance</td>
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<tr>
<td>Considerable at one end</td>
<td>Minimal</td>
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<tr>
<td>Smooth surface flat end, extensive blebbing spherical end (Fig. 10)</td>
<td>Microvilli randomly distributed</td>
<td></td>
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<td></td>
<td>Few projections</td>
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<td>Few, if any, projections</td>
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<td>(Fig. 13)</td>
<td>(Fig. 14)</td>
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<tr>
<td>Same as above</td>
<td>Same as above</td>
<td>Spherical with elongate end</td>
</tr>
<tr>
<td>Same as above</td>
<td>Same as above</td>
<td>Broad stem protrusion, no filopodia</td>
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<tr>
<td>Same as above</td>
<td>Same as above</td>
<td>Only at distal end of protrusion</td>
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<tr>
<td>Same as above</td>
<td>Same as above</td>
<td>Few, if any, projections</td>
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<td>(Fig. 16)</td>
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Prior to Invagination | Invaginating | After Invagination |
<table>
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<tbody>
<tr>
<td>Spherical, one end tapered</td>
<td>Central broad protrusion with filopodia from above and at glass</td>
<td>Broad adhesion, frequently adhere both ends, no filopodia</td>
</tr>
<tr>
<td>Tapered end narrow stem protrusion with distal filopodia</td>
<td>At distal ends of variable width filopodia</td>
<td>Minimal</td>
</tr>
<tr>
<td>Only at distal ends of protrusions</td>
<td>Few projections</td>
<td>Few projections</td>
</tr>
<tr>
<td>Microvilli randomly distributed</td>
<td>Few projections</td>
<td>Few, if any, projections</td>
</tr>
</tbody>
</table>
evaporator. Cells were examined in an AMR 1000 scanning electron microscope at 20 kV.

Cells for observation following a 15-min disaggregation, were transferred to 3 % glutaraldehyde in 0.03M PIPES buffer, pH 7.4, for at least 24 h at 4–5 °C. Cells were transferred onto a flotronic filter immersed in 30 % ethanol in a vacuum filtration funnel, dehydrated through an ethanol series and critical-point dried. The rest of procedure was the same as for cultures.

For each Pr cell type at the various stages, at least two cultures were made, and in most cases, more than two. In each culture, cells from all areas on the substratum were observed, the number of examined cells generally ranging from 20–60. Descriptions of a Pr cell type at a particular stage are characteristic of typical surface behavior observed in those cells unless otherwise noted.

RESULTS

(1) One hour cell-spreading studies

Cell populations from specific regions from late blastula through gastrula demonstrate differences in spreading tendencies, and in number and type of projections produced. As a specific area undergoes formative gastrular movements, there are corresponding changes in the in vitro cell surface, in respect to form, size and distribution of surface projections and spreading behavior. Morphologic and adhesive features of cell types at all stages are summarized in Table 1.

(A) Presumptive inner neural ectoderm: Spreading and adhesive features of late blastula (Fig. 1) and early gastrula cells are similar but at mid- and late gastrula two cell populations are seen. In one group, morphology and spreading are similar to earlier stages. In the other group there is a considerable reduction in the number of filopodia projecting to the substratum and cells are polarized with respect to morphology and adhesive sites (Fig. 2). In some cases adhesion occurs at both ends, in others only at the stem-like projection.

(B) Pr inner epidermal ectoderm: Blastula Pr inner epidermal ectodermal cells, while similar to blastula Pr inner neural ectodermal cells, are more flattened (Fig. 3). The most distinctive change in morphology and adhesion occurs at mid-gastrula when a cytoplasmic webbing is present between filopodia (Fig. 5), a feature not seen earlier in these cells and never seen in Pr inner neural ectoderm cells. Late gastrula cells are more flattened (Fig. 7) than at earlier stages and than Pr inner neural ectoderm cells at any stage. Furthermore, spreading is now along one axis and attachment in some regions is via lamellipodia bearing distal microspikes, in other regions via filopodia. In Fig. 6, a group of Pr inner epidermal ectoderm cells extend varying length lamellipodia from their underside, some of which contact the glass. Small bleb-like formations are also seen.

(C) Pr head endoderm: Pr head endoderm cells are located in different regions
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Fig. 1. Pr inner neural ectoderm cell isolated from blastula and spreading on glass for 1 h. × 1272. Scale mark equals 7.9 μm.

Fig. 2. Pr inner neural ectoderm cell isolated from mid-gastrula and spreading on glass for 1 h. × 1288. Scale mark equals 7.8 μm.

Fig. 3. Pr inner epidermal ectoderm cell isolated from blastula and spreading on glass for 1 h. × 1248. Scale mark equals 8.0 μm.

Fig. 4. Pr inner epidermal ectoderm cell isolated from early gastrula and spreading on glass for 1 h. × 1710. Scale mark equals 5.9 μm.

At the various stages studied. Initially, at late blastula, these cells are at the Pr dorsal lip. At this stage, and at early gastrula the outer, superficial-layer cells and the inner proximal cells reveal different morphologic and spreading features. Cells from the outer layer have a circumferential indentation constricting the cells into two unequal parts (Fig. 8); the larger segment facing, in situ, proximally. These superficial cells adhere and spread by their proximal ends.

At mid- and late gastrula, Pr head endoderm cells are located at the leading edge of the invaginating fold migrating along the dorsal blastocoel wall. Two types of activity are seen in attachment and spreading (Table 1). In Fig. 11,
Fig. 5. Pr inner epidermal ectoderm cell isolated from mid-gastrula and spreading on glass for 1 h. × 2182. Scale mark equals 4-6 μm.

Fig. 6. Pr inner epidermal ectoderm cells isolated from late gastrula and spreading on glass for 1 h. × 3000. Scale mark equals 3-3 μm.

Fig. 7. Pr inner epidermal ectoderm cell isolated from late gastrula and spreading on glass for 1 h. × 2600. Scale mark equals 3-9 μm.

Fig. 8. Superficial Pr head endoderm cell (from Pr dorsal lip of blastopore) isolated from blastula and spreading on glass for 1 h. × 1094. Scale mark equals 9-1 μm. (D = distal end; P = proximal end).

Fig. 9. Inner Pr head endoderm cell (from dorsal lip of blastopore) isolated from early gastrula and spreading on glass for 1 h. × 1332. Scale mark equals 7-5 μm.
**Fig. 10.** Pr head endoderm cell (from leading edge of invaginating fold) isolated from mid-gastrula and spreading on glass for 1 h. $\times 2376$. Scale mark equals 4.2 $\mu$m.

**Fig. 11.** Pr head endoderm cells (from leading edge of invaginating fold) isolated from mid-gastrula and spreading on glass for 1 h. $\times 3000$. Scale mark equals 3.3 $\mu$m.

**Fig. 12.** Pr notochord cell isolated from early gastrula and spreading on glass for 1 h. $\times 1680$. Scale mark equals 6.0 $\mu$m.

**Fig. 13.** Pr notochord cells (from marginal area adjacent to dorsal lip of blastopore) isolated from mid-gastrula and spreading on glass for 1 h. $\times 1080$. Scale mark equals 9.3 $\mu$m.

**Fig. 14.** Pr notochord cell (from dorsal lip of blastopore) isolated from mid-gastrula and spreading on glass for 1 h. $\times 1831$. Scale mark equals 5.5 $\mu$m.

**Fig. 15.** Pr notochord cell (from chordamesoderm) isolated from mid-gastrula and spreading on glass for 1 h. $\times 1152$. Scale mark equals 8.7 $\mu$m.
Fig. 16. Pr notochord cell (from chordamesoderm) isolated from late gastrula and spreading on glass for 1 h. ×1284. Scale mark equals 7.8 μm.

Fig. 17. Light micrograph of Pr outer neural ectoderm cells isolated from blastula, in fixative solution. ×126. Scale mark equals 79.4 μm. (D = distal pigmented end; P = proximal end.)

Fig. 18. Light micrograph of Pr outer neural ectoderm cells isolated from blastula, critical-point dried. ×161. Scale mark equals 62.1 μm. (D = distal pigmented end; P = proximal end).

Fig. 19. Pr outer neural ectoderm cell isolated from blastula and spreading on glass for 1 h. ×762. Scale mark equals 13.1 μm (D = distal end; P = proximal end).

Fig. 20. Freshly dissociated Pr inner neural ectoderm cells isolated from blastula. ×564. Scale mark equals 17.7 μm.

Fig. 21. Freshly dissociated superficial Pr head endoderm cell isolated from blastula. ×2052. Scale mark equals 4.9 μm. (D = distal surface; P = proximal surface).
long filopodia are seen in the intervening space between neighboring cells, a feature seen in all other cell types.

(D) Pr notochord: Pr notochord cells, late blastula, are spherical with the area of adhesion narrower than cell width. Cells at this stage and at early gastrula (Fig. 12) spread and attach via filopodia. At mid-gastrula, Pr notochord cells from the dorsal lip and chordamesoderm were examined. Dorsal-lip cells spread differently depending on whether they are from the marginal region just dorsal to the blastopore (Fig. 13) or from the blastopore (Fig. 14) and both of these differ from notochord cells from the chordamesoderm (Fig. 15). Pr notochord cells from the marginal region just dorsal to the blastopore are polarized; this was also observed in cells from the marginal region lateral to the blastopore (Pl somite cells).

(E) Superficial-ectoderm-cell studies: When dissecting embryos, the tight cohesiveness of superficial-layer cells (single layer of outer cells) and the elasticity of this layer is apparent. This layer is the most difficult to disaggregate. Resistance to disaggregation is age-dependent, becoming increasingly more difficult to dissociate with advancing age.

Outer-ectodermal-layer cells during disaggregation reveal a circumferential indentation segmenting the cells into unequal portions (Figs. 17, 18). The highly pigmented small end represents the distal end in situ; the larger, less pigmented end faces proximally. The last region to separate is at the indentation. Epithelial fragments tend to curl so the highly pigmented ends of the cells face one another, with the larger, less pigmented ends facing the periphery.

In Fig. 19, a blastula Pr outer neural ectoderm cell has ends with different surface topographies. The outer, smaller end is relatively smooth with microvilli-like buds; the inner, larger end is covered by filopodia, many projecting to the glass. Superficial cells from Pr epidermal ectoderm, Pr head endoderm and Pr notochord behave similarly.

(2) Cells fixed immediately following dissociation

Pr ectodermal cells, neural and epidermal, fixed immediately after disaggregation, at all stages, are basically spherical with relatively smooth surfaces (Fig. 20). Filopodia may be present on cells from both ectoderms at all stages except blastula. When blebs are present, they are small; there is almost no surface infolding and underlying cortical structures are delineated slightly. Pr ectodermal cells contrast markedly to Pr head endoderm and Pr notochord cells.

Superficial Pr head endoderm cells, blastula and early gastrula (forming dorsal lip), differ at proximal and distal ends (Fig. 21). The distal end has microvilli; the proximal end has filopodia. SEM observations on intact embryos show cell surfaces in the forming blastopore area with numerous microvilli and elevated folds, some cells exhibiting dense arrays of microvilli (LeBlanc and Brick, in preparation). Pr head endoderm cells maintain a good proportion of their projections during the 15-min disaggregation.
Fig. 22. Freshly dissociated inner Pr head endoderm cell isolated from early gastrula. \( \times 1260 \). Scale mark equals 7-9 \( \mu m \).

Fig. 23. Freshly dissociated Pr head endoderm cell (from leading edge of invaginating fold) isolated from late gastrula. \( \times 2400 \). Scale mark equals 4-2 \( \mu m \).

Fig. 24. Freshly dissociated Pr notochord cell (from chordamesoderm) isolated from late gastrula. \( \times 2268 \). Scale mark equals 4-4 \( \mu m \).

Fig. 25. Cells of the blastopore groove area in a hemisected early gastrula embryo which are invaginating into the blastocoel. \( \times 324 \). Scale mark equals 30-9 \( \mu m \).

The inner, deeper Pr head endoderm cells, blastula and early gastrula, of the dorsal lip are basically spherical. At early gastrula, cells show more surface blebbing, blebs in some cases being large. Also, some highly elongate ‘sausage-like’ cells are seen (Fig. 22).

Superficial Pr notochord cells, blastula and early gastrula, have distal ends with few microvilli in contrast to Pr head endoderm cells. Notochord cells at these stages are from the marginal area located dorsal to the forming dorsal lip. In intact embryos, this area has fewer microvilli than the blastopore (LeBlanc and Brick, in preparation). Proximal surfaces of these cells bear filopodia. The
inner, deeper cells from the notochord region are spherical, but also exhibit a change from blastula to early gastrula, i.e. more extensive blebbing.

When Pr head endoderm and Pr notochord invaginate, the surfaces of cells disaggregated from these tissues change. Pr head endoderm cells, isolated from the leading edge of the invaginating fold at mid-gastrula, exhibit surfaces slightly infolded delineating cortical structures. By late gastrula, the cell surfaces are highly infolded and clearly outline cortical structures (Fig. 23). Small pit-like areas are seen but these may be membrane inpocketings.

Pr notochord cells excised during invagination, at mid-gastrula exhibit blebbing, frequent cell elongation, and delineation of cortical structures. At late gastrula, in vivo, these cells are in the chordamesoderm as part of the forming archenteron roof. Now, following 15 min of disaggregation, the entire surface of these cells show extensive blebbing, with many intervening infoldings (Fig. 24). Disaggregated Pr notochord cells, as Pr head endoderm cells, exhibit very different surfaces when lying deep within the embryo at later gastrulation, as compared to when they lie more superficially during blastula and early gastrula.

**DISCUSSION**

Holtfreter (1943, 1944) observed that isolated cells from germ regions of the amphibian gastrula adhere to, spread and move on glass in distinctive patterns. Our SEM data agree with and extend these observations in that these regionally specific processes, in vitro, undergo stage-specific changes coincident with the onset and continuance of gastrular tissue translocation. Individual cell behavior appears to play a major role in gastrulation, suggested by our observations that isolated cells have acquired autonomous behavior and morphologic expressions consistent with the various *in situ* morphogenetic movements.

Isolated Pr inner neural ectoderm cells exhibit marked differences in spreading from blastula to late gastrula. By mid-gastrula two populations of Pr inner neural ectoderm are present which may be related to *in vivo* events. By mid-gastrula, Pr notochord tissue constitutes the mid-dorsal archenteron roof. The decisive condition in neural-plate determination is apposition of Pr neural ectoderm and archenteron roof. Sirakami (1959), noted 'tug cells' in the neural plate with long protoplasmic stalks, and suggested they might participate in neural-tube formation. Holtfreter's (1948) isolated neural-plate cells maintain their elongate shape and further elongate in culture.

It is a reasonable inference that elongate cells in our cultures are forerunners of elongated cells described in the neural plate (*op. cit.*). These morphologically similar cells in our mid- and late-gastrula Pr inner neural ectoderm cultures may result from the determinative interaction between archenteron roof and Pr neural ectoderm. Therefore, these cells, not elongate *in situ* at these stages, may result from induction between early and mid-gastrula. No elongate cells were seen in blastula and early-gastrula Pr inner neural ectoderm cultures.
A characteristic morphogenetic movement of amphibian gastrulation is epiboly of Pr epidermal ectoderm, a process including locomotor advance of this epithelium over the surface, flattening of its individual cells and intercalation of deeper Pr inner epidermal ectoderm cells into the peripheral layer (Holtfreter, 1943). Pr inner neural ectoderm and Pr inner epidermal ectoderm cells, morphologically and behaviorally similar in blastula cultures, show increasing divergence in both respects when derived from progressively later stages. The character of the divergence is consistent with the different in situ morphogenetic behavior of each cell type. Stage-specific changes of Pr inner epidermal ectoderm cells in culture appear related to epiboly. These cells, at all stages, were more flattened and extended than Pr inner neural ectoderm cells. Holtfreter (1947) observed that isolated Pr epidermal ectoderm cells spread more frequently than Pr neural or mesodermal cells. Enhanced spreading may be related to greater adhesiveness and cohesiveness, a possible requirement for epiboly.

We can adduce considerable suggestive evidence for greater cohesiveness of neural and epidermal ectoderm compared to other Pr tissues. Townes and Holtfreter (1955), Steinberg (1970), suggested that amphibian gastrular ectoderm is the most cohesive of the germ layers Brick et al. (1974), inferred from electrokinetic data that Pr inner and superficial neural ectoderm cells are the most cohesive among Pr tissues tested, however, they made no electrokinetic determinations on epidermal ectoderm. In our experiments, ectodermal layers required the longest disaggregation times increasing with embryonic age, results in agreement with Johnson (1970). A direct relationship may exist between disaggregation rate and cohesiveness (Johnson 1970) and in accordance with this criterion Pr inner neural ectoderm and Pr inner epidermal ectoderm cells are more cohesive than cells of other Pr tissues. It is reasonable to assume that strength of adhesion of cells to a substratum would increase progressively as area of contact with the substratum increases and, therefore, extent of spreading is a possible indicator of relative adhesive strength. On this basis, mid- and late-gastrula Pr inner epidermal ectoderm cells are more adhesive than Pr inner neural ectoderm cells, and both are more adhesive than Pr head endoderm and Pr notochord.

Given morphologic spreading and adhesive characteristics of Pr inner epidermal ectoderm cells, these properties may be related to epibolic translocation in two ways; (1) epiboly results, in part, from spreading potency residing in each cell, and (2) since this is a movement of an epithelium, sufficient intercellular cohesion would be required to maintain its integrity. Pr inner epidermal ectoderm cells from late gastrula produced many intercellular contacts, more than in cultures of other Pr tissues. These are not residual contacts, as only cells from completely disaggregated tissues were used. Formation of such contacts among Pr inner epidermal ectoderm cells, suggests a greater potential for these cells, compared to others, to reestablish epithelial organization.

Pr head endoderm cells exhibit characteristic features and stage-specific
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changes in spreading and adhesion different from both types of Pr ectodermal cells. Pr head endoderm at blastula and early gastrula, in vivo, are located at the site of dorsal-lip formation and the progression of in vitro changes can be correlated with invagination. Our observations on these cells at the dorsal-lip site included those cells lying at the embryo surface and those lying deeper, proximal to the blastocoel, the former invaginating into the latter. The outstanding feature of isolated superficial cells is their distinctive proximal and distal ends. Adhesion to glass occurred only at the proximal end. These cells may be the in situ bottle cell (Holtfreter, 1943; Baker, 1965). Microvilli on apical surfaces of invaginating bottle cells, as on these in vitro cells, have been demonstrated by SEM in vivo by Brick et al. (1974) and by Baker (1965) in TEM. The apical annular constriction demonstrated may result from constriction of the cortical microfilaments beneath apical and lateral cell surfaces (Perry & Waddington, 1966). Furthermore, circumferential microfilaments in these superficial cells occur at the level of apical junctions which, in vivo, bind these cells together (Perry, 1975).

Isolated inner, more proximal cells, of the dorsal lip exhibit increasing numbers of filopodia projecting to the glass from blastula to early gastrula. This may be correlated with the role of these cells in invagination. Trinkaus and Lentz (1967) have correlated increased blebbing and filopodia with morphogenetic movements in Fundulus. Gustafsson & Wolpert (1961) demonstrated in sea-urchin embryos, protrusion of microprojections to the blastocoel wall suggesting involvement in gastrula morphogenesis. SEM of hemisected amphibian embryos (Le Blanc and Brick, in preparation), at blastopore groove initiation, show cells in the invaginating region protruding into the blastocoel. The surface of these cells facing the blastocoel bear fine filopodia in contact with adjacent cells (Fig. 25). In the current study, cells of the dorsal-lip region (early gastrula), both superficial and deep, bear similar filopodia at their proximal ends which in vivo, would be facing the blastocoel.

At mid- and late-gastrula, Pr head endoderm cells constitute the leading edge of the invaginating fold. Cells from these stages display two kinds of spreading behavior. The smaller number of polarized cells projecting a lamellipodium or lobopodium may be from the leading edge. SEM of frontally hemisected embryos (Brick et al., 1974), and time-lapse films of similarly hemisected living embryos (Brick and Weinberger, in preparation) show cells at the anterior edge of the invaginating fold flattened against the blastocoel roof, attaching by means of lamellipodia. Both cell types bear very few projections at their unattached ends, a situation consistent with observations that as cells invaginated their surfaces became free of microvilli (Monroy, Baccetti & Denis-Donini, 1976).

Pr notochord cells also exhibit marked differences in spreading between blastula and late gastrula, differing from cells of other Pr tissues. Their in vitro behavior correlates with their behavior and location in vivo, i.e. those moving toward the dorsal lip; those in the process of invaginating; and those already in
the archenteron roof. Between blastula and early gastrula, filopodia extending to the glass become more numerous, a change corresponding with onset of invagination. At mid-gastrula those Pr notochord cells which *in situ* are still at the surface are polarized with respect to *in vitro* adhesion and spreading. These cells *in vivo*, as well as those in the marginal lateral zone, are stretched in the direction of the groove (Holtfreter, 1944). The *in vitro* polarization of these cells is in striking contrast to invaginating Pr notochord cells.

Pr notochord cells after invagination are located internally against the dorsal, posterior blastocoel wall (mid-gastrula). When isolated, they are slug-like, reminiscent of Holtfreter's (1947) 'creeping mesoderm cell'. These cells *in vivo* are also stretched along one axis.

Pr notochord cells isolated from chordamesoderm midway along anterior-posterior axis (late gastrula) adhere by a 'foot-like' process, many cells being elongated. Holtfreter (1947) stated that, while most amphibian embryonic cells are endowed with monaxial polarity, the migrating mesoderm cell had the strongest tendency. Brick *et al.* (1974) proposed that translocation of Pr head endoderm and chordamesoderm is a result of active migratory behavior of cells comprising the epithelium. In *Xenopus*, Keller & Schoenwolf (1977) suggest endoderm is dragged passively by migrating mesoderm cells. Nakatsuji (1976), in TEM, observed chordamesoderm cells projecting filopodia, blebs, or lobopodia to the internal surface of the blastocoel wall and in many cases the processes were flattening against this internal surface. In our study, these cells did not produce such projections, at least not after one hour. Adhesive properties of cells and substratum, however, play a major role in cell morphology and their processes (Elsdale & Bard, 1972; Bard & Hay, 1975). The broad 'foot-like' projection of late-gastrula chordamesoderm on glass may be transformed into filopodia when contacting other mesoderm or ectoderm cells; in 24 h cultures of chordamesoderm (LeBlanc and Brick, in preparation), cells extended filopodia to the surface of contacted cells.

Superficial ectoderm cells may have proximal and distal surfaces with different adhesive properties (Steinberg, 1970). We have shown that these surfaces differ morphologically and, therefore, might be expected to differ with respect to molecular organization and physical properties. Holtfreter (1943, 1944), considered the outer cell layer to be non-adhesive. Steinberg (1970), presented a different view of the adhesive characteristics of this layer to explain amphibian gastrulation. Distal cell surfaces would be slightly adhesive compared to very highly adhesive proximal surfaces. Proximal and distal morphologic differences of superficial ectoderm cells may reflect this proposed adhesive differential. In fact, the adhesive behavior of superficial cells from all Pr germ layers suggests adhesive differentials between proximal and distal ends. Superficial cells, from Pr neural ectoderm, Pr epidermal ectoderm, Pr head endoderm and Pr notochord, although exhibiting their own characteristic spreading behavior, have distinctive distal and proximal surfaces. In all of these cells, *in vitro*, adhesion
was via their proximal ends. In a few cases attachment occurred at both ends but in no cases did any of these cells adhere by distal ends alone.

Cells were fixed immediately after disaggregation to determine if differences related to Pr tissue type and stage could be observed prior to spreading. Data interpretation can be grouped into two categories. (1) Basically similar proximal and distal cell surface features and differences are seen in superficial cells, at each of the stages, as are seen in cells spreading for one hour. This suggests that in vitro distal and proximal surface specializations represent in vivo morphologic surface differences and are not a consequence of spreading. (2) Cell-surface morphology of Pr head endoderm and Pr notochord cells varied when these cells were disaggregated and fixed at the time their respective tissues were on the embryo surface or when they had moved into the interior. This situation is in contrast to Pr inner neural ectoderm and Pr inner epidermal ectoderm cells fixed immediately after disaggregation, which in situ remain at the surface throughout gastrulation, and show little change from blastula to late gastrula. In general, with the exception of superficial-layer cells, spreading characteristics are not reflected in freshly disaggregated cells.

We suggest that Pr head endoderm and Pr notochord results occur because invaginated cells are exposed to a new environment, i.e. blastocoel fluid, the composition of which differs from the external medium and might be expected to affect plasma membrane behavior (Monroy et al., 1976). Increases in concentration of Ca$^{2+}$, Na$^+$ and K$^+$, and increases in pH have been demonstrated in blastocoel fluid during gastrulation (Stableford, 1967). Holtfreter (1943) had shown that in vitro cell-surface motility increases with increased pH, and Schaeffer, Schaeffer & Brick (1973) reported increased cell-surface negativity with increased pH.

Surface features of freshly disaggregated Pr head endoderm cells vary from blastula to early gastrula. Coincident with onset of invagination these cells show increased surface blebbing and some were markedly elongated suggesting changes in surface fluidity and deformability. Trinkaus (1976) suggested that gastrula cells, in general, display greater surface activity than blastula cells. Tickle and Trinkaus (1973) related membrane fluidity and deformability to bleb formation. Blebs have been observed on cell surfaces in vivo prior to onset of locomotion (Trinkaus & Lentz, 1967). Blebbing seen in our freshly disaggregated cells may, therefore, correlate with morphogenetic movements in vivo.

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