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Studies on the gastrulation of amphibian embryos: pseudopodia in the gastrula of *Bufo bufo japonicus* and their significance to gastrulation

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SUMMARY

The course of gastrulation in embryos of *Bufo bufo japonicus* was studied by use of 1 μm Epon sections. It was observed that the cells of the presumptive pharyngeal endoderm and mesoderm form pseudopodia coinciding with their invagination. Some of the inner cells of the ectodermal layer also form pseudopodia. The cells of the endodermal mass and the surface of the embryo rarely form such structures. The observed pseudopodia seem to correspond to the hitherto reported pseudopodia formed by the dissociated cells from the gastrula of amphibians. Formation of the pseudopodia seems to suggest that the invagination of the presumptive pharyngeal endoderm and mesoderm is brought about by active migration of the individual cells along the inner surface of the ectodermal layer.

INTRODUCTION

When morphogenetic movements, such as gastrulation, occur in embryos, many factors, such as migration, adhesion, contraction and expansion of embryonic cells, are thought to play important roles. In the case of echinoderm gastrulation, cells of the invaginating archenteron protrude filopodia toward the blastocoelic wall and these filopodia play a main role in the invagination (Gustafson & Kinnander, 1956; Dan & Okazaki, 1956; Gustafson & Wolpert, 1967). During the epibolic movement in teleost embryos the deep cells form pseudopodia and migrate actively (Lenz & Trinkaus, 1967; Trinkaus & Lenz, 1967; Wourms, 1972; Trinkaus, 1973). Invaginating mesodermal cells of chick embryos, in the primitive streak and between the epiblast and hypoblast, are fibroblast-like and form filopodia (Trelstad, Hay & Revel, 1967; Granholm & Baker, 1970).

In the case of amphibian gastrulation, the cells lining the top of the invaginating archenteron resemble a bottle in shape. These cells have been termed 'bottle cells' or 'flask cells' and it was proposed that they play a main role in the invagination of the archenteron (Holtfreter, 1943*a*, *b*; Baker, 1965). Epibolic

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movement of the ectodermal layer was also thought to be involved in the gastrulation process (Holtfreter, 1944).

It has been shown that some cells dissociated from the gastrula form pseudopodia *in vitro*. It has been suggested that this pseudopodial activity may be a significant factor in the process of gastrulation (Holtfreter, 1943*b*; Sirakami, 1959, 1963).

It is not yet clear, however, whether cells in the intact embryo also form such pseudopodia and play a role in gastrulation. In this paper the existence of pseudopodia in the gastrula of *Bufo bufo japonicus* is shown and the significance of this for gastrulation is discussed.

MATERIAL AND METHODS

Fertilized eggs of *Bufo bufo japonicus* (*Bufo vulgaris*) were obtained from the natural mating of adults collected at Iwakura, Kyoto. The stages of the embryos were determined according to Baba & Okada (1932). Embryos of stage 10–14 (late blastula to late gastrula) were dejellied with fine forceps and were fixed for 2 days at 3–5 °C in a buffered (0·05 M phosphate buffer, pH 7·2) 2·5 % glutaraldehyde solution containing paraformaldehyde (2 %) and picric acid (0·1 %) (Ito & Karnovsky, 1968). They were then rinsed in the same buffer that contained sucrose (4 %), and were post-fixed for 2 h in a buffered (pH 7·2–7·4) 1 % osmium tetroxide solution that contained sucrose (2·25 %). The samples were dehydrated in a graded series of ethanol and transferred through propylene-oxide, propylene-oxide Epon mixtures and into Epon. Finally the samples were embedded in Epon containers. To facilitate orientation and to allow better fixation, the embryos were cut bilaterally before the post-fixation step. One μm sections were mounted on glass slides that had been previously coated with 0·5 % collodion (Aoki & Gutievvez, 1967) and were stained with 1 % toluidine blue (in 0·1 M phosphate buffer, pH 6·9) for 20 min at 90 °C. They were examined with a bright-field microscope.

RESULTS

Stage 10 (late blastula)

Blastomeres are closely packed and polygonal in shape (Fig. 1). Pseudopodia were not observed in any region at this stage.

Stage 11 (initial gastrula)

The first sign of invagination of the archenteron, accompanied by the appearance of bottle cells, is noticed in the area of the dorsal lip (Fig. 2A). In the marginal zone of the dorsal part, the cells of the presumptive pharyngeal endoderm and the mesoderm are the first to form pseudopodia (Fig. 2A). The pseudopodia are mostly lobopodia (Fig. 2B). Pseudopodia are also observed in the ventral part, though the bottle cells are not yet observed in the area of the presumptive ventral lip (Fig. 2C).

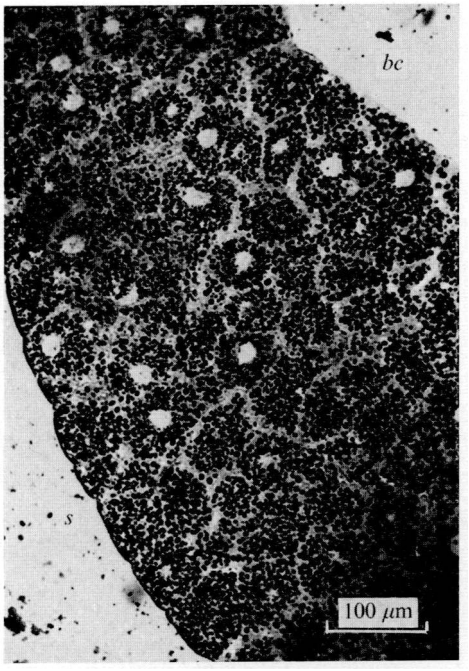

Fig. 1. Marginal zone of a mid-sagittal section of a late blastula (stage 10). *bc*, Blastocoel; *s*, surface of the embryo.

Stage 12 (early gastrula)

The archenteron is now formed, the top of which is lined by the bottle cells (Fig. 3A). Many pseudopodia are observed among the cells of the presumptive pharyngeal endoderm and mesoderm (Fig. 3B). The pseudopodia are conspicuous in the vicinity of the inner surface of the ectodermal layer (though mesodermal cells may be present in this layer at this stage) (Fig. 3B, C). The cells lining the inner surface of the ectodermal layer are tightly packed and the invaginating cells (presumptive pharyngeal endoderm and mesoderm) are more loosely packed.

Stage 13–14 (middle-late gastrula)

The archenteron elongates parallel to the inner surface of the ectodermal layer (Fig. 4A). Invagination of the endodermal and mesodermal cells is progressing in the dorsal part (Fig. 4A) and in the ventral part as well (Fig. 4C). Some cells of the endodermal mass become loosely packed and large intercellular spaces are present (Fig. 4A) but formation of pseudopodia was not observed in this region. Besides the invaginating cells, some cells of the inner surface of the ectodermal layer are observed to form pseudopodia (Fig. 4B).

Formation and distribution of the pseudopodia described above are schematically summarized in Fig. 5.

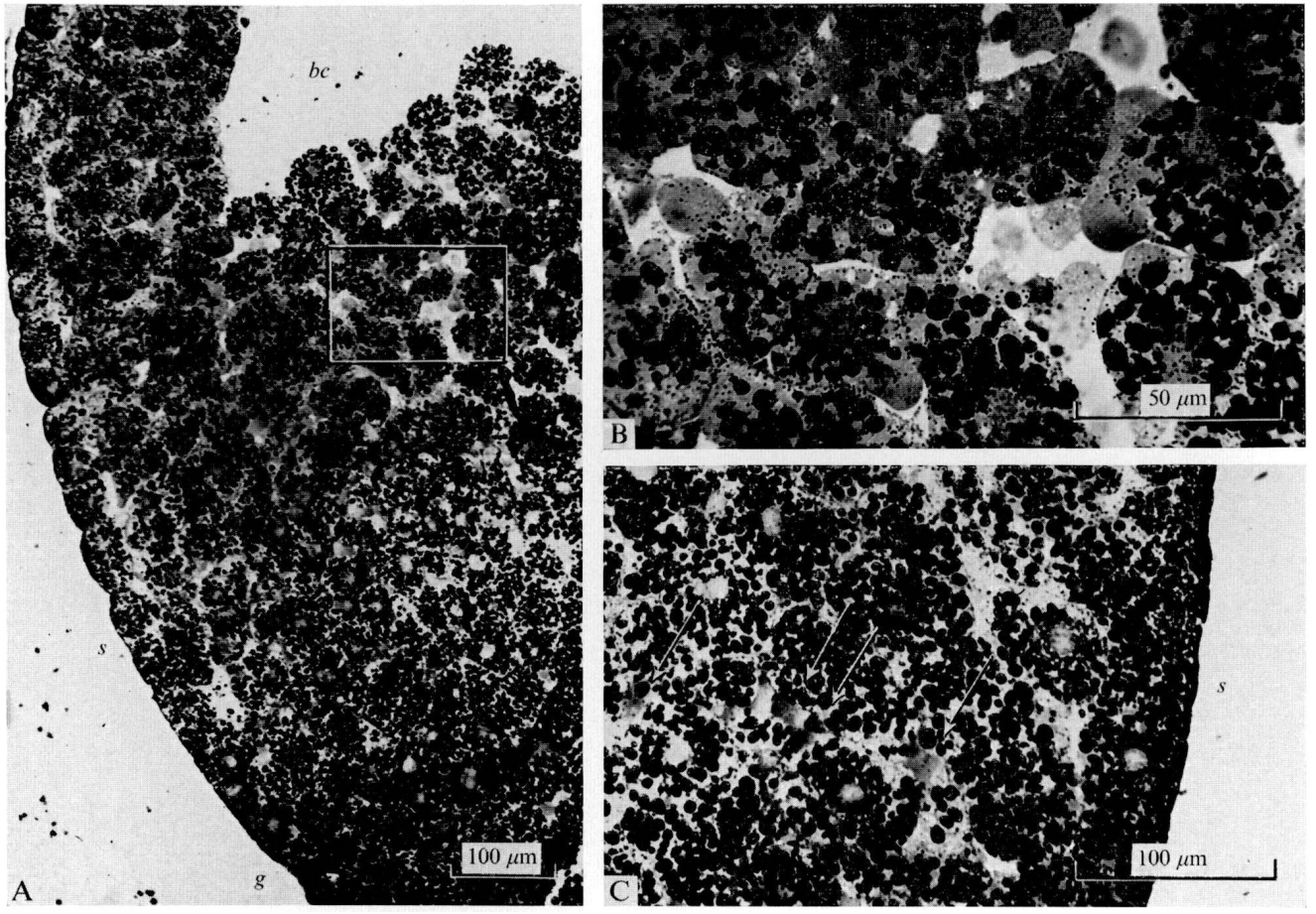

Fig. 2. Mid-sagittal section of the initial gastrula (stage 11).

(A) Dorsal part. The initial groove (*g*) of the archenteron is observed. The cells in the marginal zone form pseudopodia.

(B) An enlarged micrograph of the area indicated by the small rectangle in (A).

(C) Presumptive ventral lip region. Pseudopodia are formed among the cells (arrows). *bc*, Blastocoel; *s*, surface of the embryo.

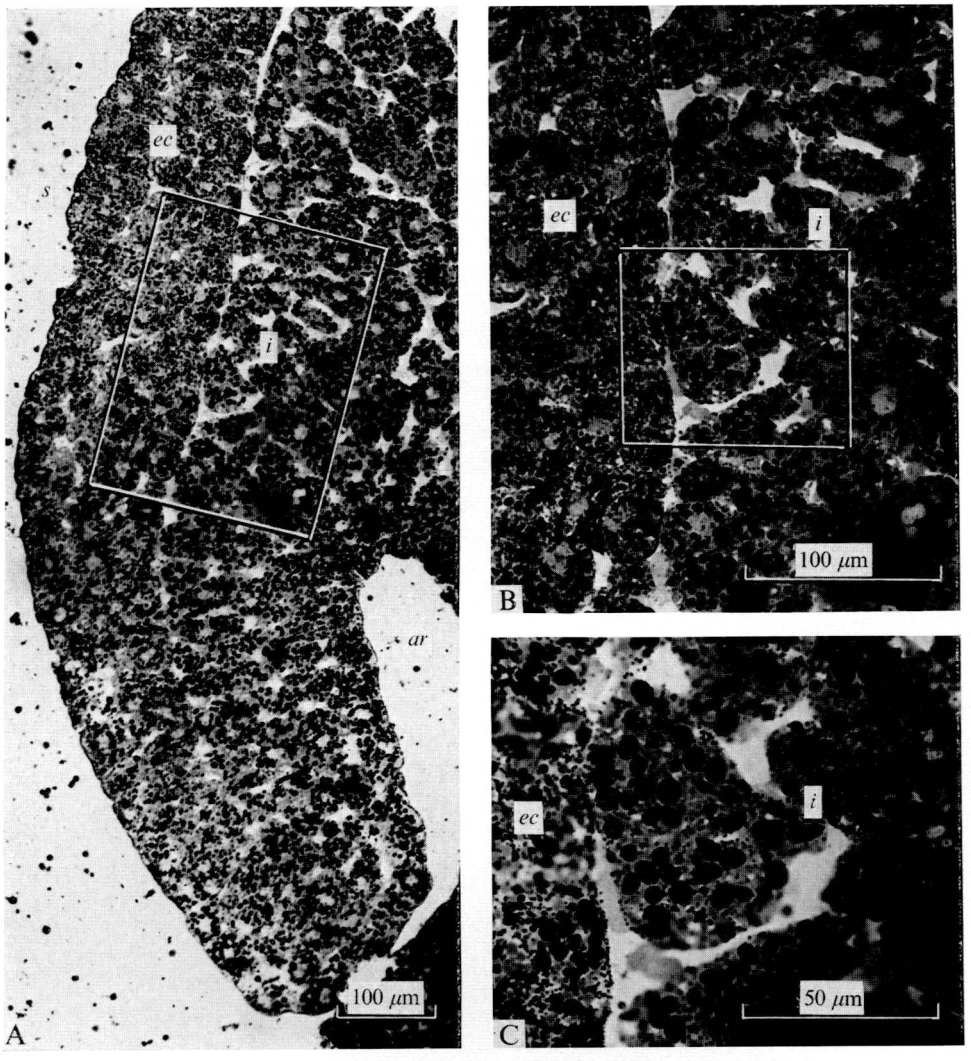

FIGURE 3

Mid-sagittal section of the early gastrula (stage 12).

(A) Dorsal lip.

(B) An enlarged micrograph of the area indicated by the rectangle in (A).

(C) An enlarged micrograph of the area indicated by the rectangle in (B).

ar, Archenteron; *ec*, ectodermal layer; *i*, invaginating cells (presumptive pharyngeal endoderm and/or mesoderm); *s*, surface of the embryo.

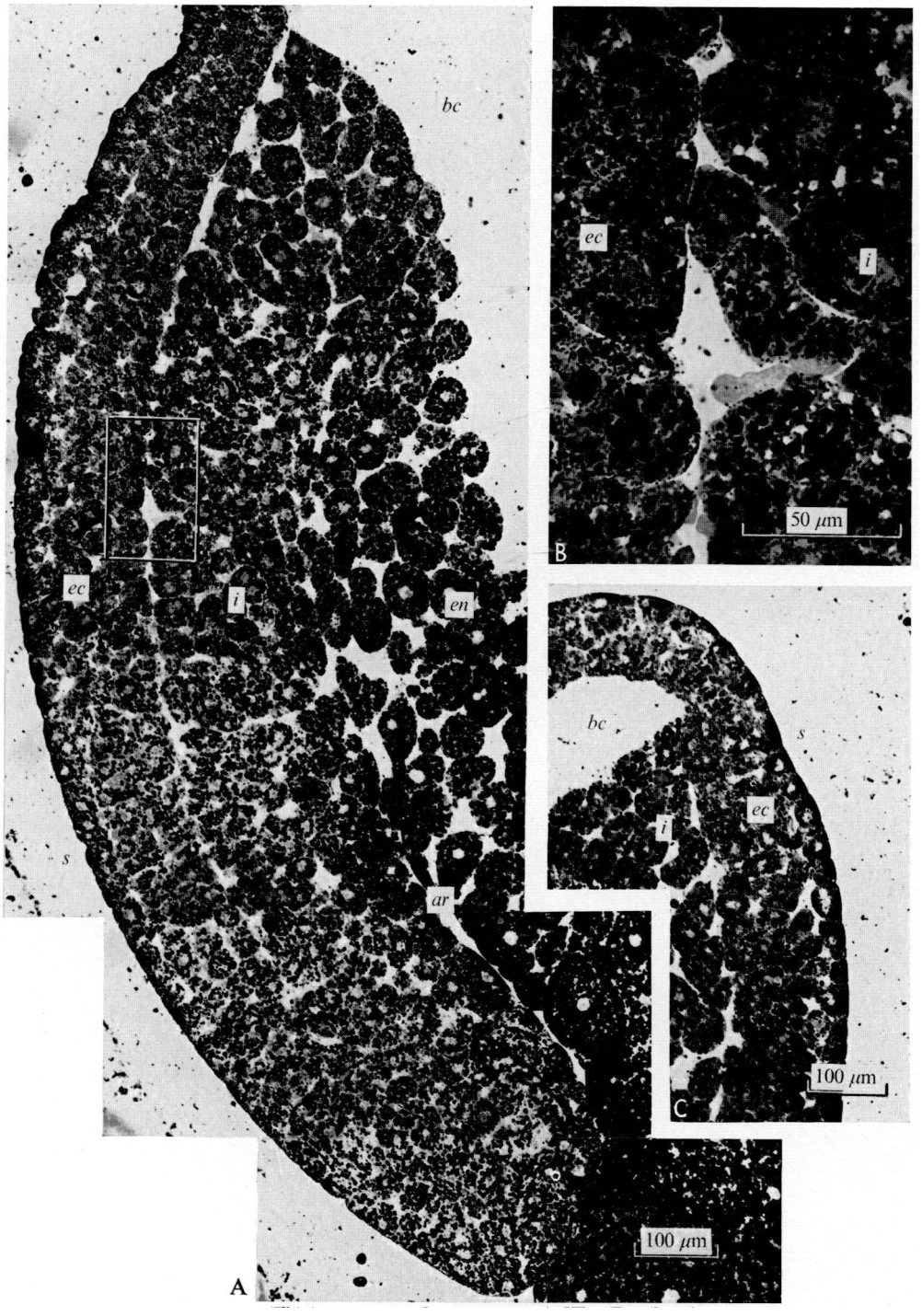

FIGURE 4

Mid-sagittal section of the middle-late gastrula (stage 13-14).

(A) Dorsal part.

(B) An enlarged micrograph of the area indicated by the small rectangle in (A).

(C) Ventral part.

ar, Archenteron; *bc*, blastocoel; *ec*, ectodermal layer; *en*, endodermal mass; *i*, invaginating cells; *s*, surface of the embryo.

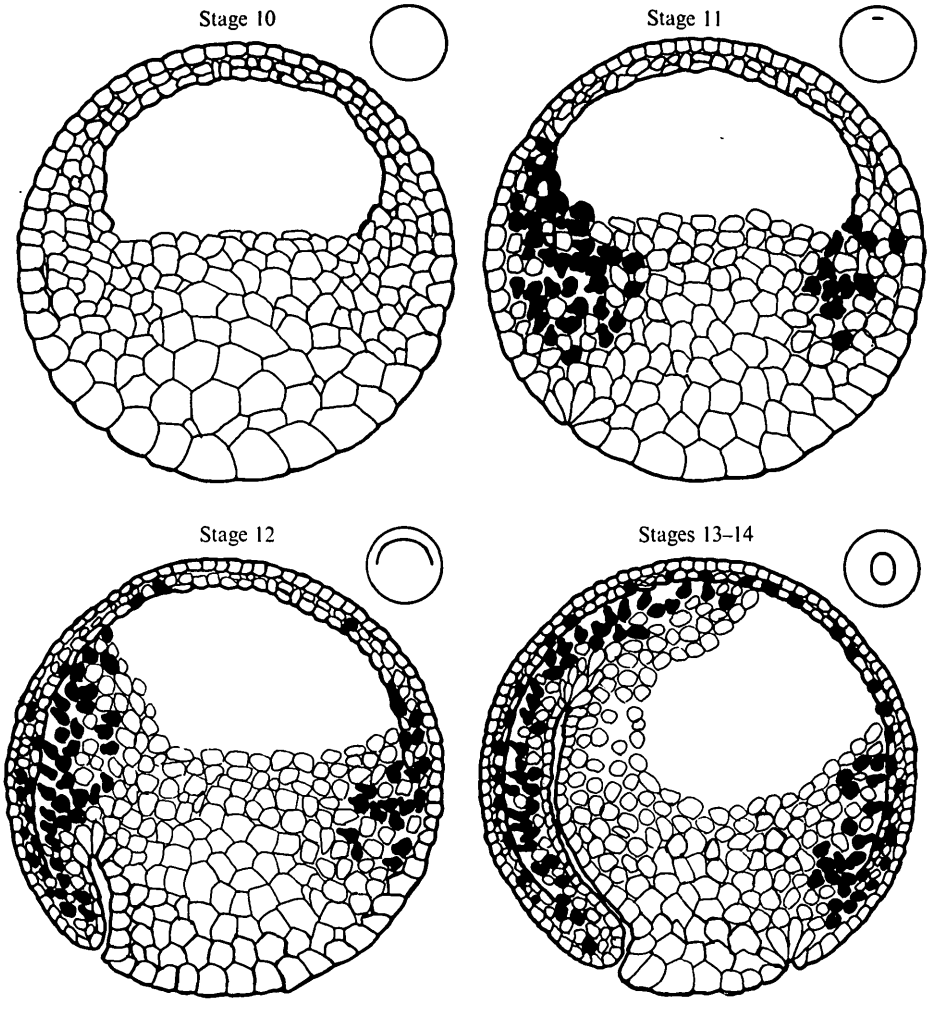

Fig. 5. Schematic mid-sagittal sections of the late blastula and three stages of the gastrula. Cells observed to have the pseudopodia are drawn black.

DISCUSSION

The sinking-in of the bottle cells into the endodermal mass and epibolic movement of the ectoderm are processes that have been proposed as the major factors that cause gastrulation (Holtfreter, 1943*b*, 1944; Baker, 1965). The sinking-in activity of the isolated bottle cells was clearly shown by stepwise observation of their migration into an endodermal mass (Holtfreter, 1944). In the gastrula of *Bufo bufo* the bottle cells are present among the endodermal and mesodermal cells, and these cells move toward the animal pole during gastrulation.

Mesodermal cells seem to originate from the internal marginal zone of the blastula as in other anurans such as *Xenopus laevis* (Nieuwkoop & Florschütz, 1950). These cells become displaced toward the blastoporal lip, then turn the

direction of their displacement toward the animal pole by rolling around the internally situated lip, and come to underlie the ectodermal layer. It is not clear how such a displacement of the mesodermal cells is regulated.

The cells of the presumptive pharyngeal endoderm and mesoderm move along the inner surface of the ectodermal layer toward the animal pole. These cells are loosely packed and observed to form pseudopodia coinciding with their invagination. The pseudopodia are conspicuous in the vicinity of the ectodermal layer. Some cells lining the inner surface of the ectodermal layer also form pseudopodia. The formation of the pseudopodia and the shape and arrangement of the cells seem to suggest that the invagination of the presumptive pharyngeal endoderm and mesoderm is brought about by the active migration of the individual cells along the inner surface of the ectodermal layer.

Movement of embryonic cells during gastrulation is dependent on the pseudopodia in other species such as in echinoderms and fishes (Gustafson & Kinnander, 1956; Dan & Okazaki, 1956; Gustafson & Wolpert, 1967; Trinkaus, 1973; Wourms, 1972). The migrating cells in these cases move along the inner surface of the cell layer that envelops the embryo. This is also suggested for *Bufo bufo* as described above. The pseudopodia observed in the gastrula of *Bufo bufo* are morphologically similar to those observed in teleost embryos (Lenz & Trinkaus, 1967; Trinkaus & Lenz, 1967; Trinkaus, 1973; Wourms, 1972) and could also be responsible for the movement of the cells.

Dissociated cells from the gastrula of amphibians were observed to form pseudopodia (Holtfreter, 1944, 1946, 1947; Sirakami, 1959, 1963). This seems to have a correlation with the pseudopodia observed in the gastrula of *Bufo bufo*.

By cell electrophoresis, an increase in the negative charge on the surface of cells from the dorsal lip region of *Rana pipiens* embryos, was shown to occur at the beginning of gastrulation (B. E. Schaeffer, Schaeffer & Brick, 1973). This change of the surface charge might be related to pseudopodial activity, because the increase of pH that increases the negative charge on the cell surface (H. E. Schaeffer, Schaeffer & Brick, 1973), activates the formation of the pseudopodia in the dissociated cells from the amphibian embryos (Holtfreter, 1943*b*, 1944, 1946) and accelerates movement of the cells from the chick embryo *in vitro* (Taylor, 1962).

The whole process of gastrulation must be looked on as the integration of many factors. The sinking-in activity of the bottle cells and constriction of the fibrillar layer, observed in the apical end of the bottle cells (Baker, 1965; Perry & Waddington, 1966), seem to bring about the initial formation of the blastoporal groove. Spreading of the ectodermal layer then engulfs the endodermal mass. In anuran amphibians, at least, the invagination of the presumptive pharyngeal endoderm and mesoderm seems mainly due to the active migration of the individual cells along the inner surface of the ectodermal layer. It is possible that the active migration of these cells causes the elongation of the archenteron.

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REFERENCES

AOKI, A. & GUTIEVVEZ, L. S. (1967). A simple toluidine blue–basic fuchsin stain for spermatozoa in epoxy sections. *Stain Technol.* **42**, 307–310.

BABA, K. & OKADA, Y. (1932). *Development of the frog* (Bufo vulgaris). Tokyo, Japan: Iwanami Publishing Co.

BAKER, P. A. (1965). Fine structure and morphogenetic movements in the gastrula of the tree frog, *Hyla regilla*. *J. Cell Biol.* **24**, 95–116.

DAN, K. & OKAZAKI, K. (1956). Cyto-embryological studies of sea urchins. III. Role of the secondary mesenchyme cells in the formation of the primitive gut in sea urchin larvae. *Biol. Bull. mar. biol. Lab., Woods Hole* **110**, 29–42.

GRANHOLM, N. H. & BAKER, J. R. (1970). Cytoplasmic microtubules and the mechanism of avian gastrulation. *Devl Biol.* **23**, 563–584.

GUSTAFSON, T. & KINNANDER, H. (1956). Microaquaria for time-lapse cinematographic studies of morphogenesis in swimming larvae and observations on sea urchin gastrulation. *Expl Cell Res.* **11**, 36–51.

GUSTAFSON, T. & WOLPERT, L. (1967). Cellular movement and contact in sea urchin morphogenesis. *Biol. Rev.* **42**, 442–498.

HOLTFRETER, J. (1943*a*). Properties and function of the surface coat in amphibian embryos. *J. exp. Zool.* **93**, 251–323.

HOLTFRETER, J. (1943*b*). A study of the mechanics of gastrulation: Part I. *J. exp. Zool.* **94**, 261–318.

HOLTFRETER, J. (1944). A study of the mechanics of gastrulation: Part II. *J. exp. Zool.* **95**, 171–212.

HOLTFRETER, J. (1946). Structure, motility and locomotion in isolated embryonic amphibian cells. *J. Morph.* **79**, 27–62.

HOLTFRETER, J. (1947). Observations on the migration, aggregation and phagocytosis of embryonic cells. *J. Morph.* **80**, 25–55.

ITO, S. & KARNOVSKY, M. J. (1968). Formaldehyde-glutaraldehyde fixative containing trinitro compounds. *J. Cell Biol.* **39**, 168a–169a.

LENZ, T. L. & TRINKAUS, J. P. (1967). A fine structural study of cytodifferentiation during cleavage, blastula and gastrula stages of *Fundulus heteroclitus*. *J. Cell Biol.* **32**, 121–138.

NIEUWKOOP, P. D. & FLORSCHÜTZ, P. A. (1950). Quelques caractères spéciaux de la gastrulation et de la neurulation de l'œuf de *Xenopus lævis* Daud. et de quelques autres Anoures. 1. Etude descriptive. *Archs Biol., Liège* **61**, 113–150.

PERRY, M. M. & WADDINGTON, C. H. (1966). Ultrastructure of the blastopore cells in the newt. *J. Embryol. exp. Morph.* **15**, 317–330.

SCHAEFFER, B. E., SCHAEFFER, H. E. & BRICK, I. (1973). Cell electrophoresis of amphibian blastula and gastrula cells; the relationship of surface charge and morphogenetic movement. *Devl Biol.* **34**, 66–76.

SCHAEFFER, H. E., SCHAEFFER, B. E. & BRICK, I. (1973). Electrophoretic mobility as a function of pH for disaggregated amphibian gastrula cells. *Devl Biol.* **35**, 376–381.

SIRAKAMI, K. I. (1959). Cyto-embryological studies of amphibians. II. On 'tug-cells' and their bearings upon the gastrulation and some other morphogenetic movements. *Mem. Fac. Liberal Arts and Education Yamanashi Univ.* **10**, 125–127.

SIRAKAMI, K. I. (1963). Cyto-embryological studies of amphibians. IV. Behaviour of isolated ectodermal cells from blastula stage embryos of *Bufo vulgaris*. *Mem. Fac. Liberal Arts and Education Yamanashi Univ.* **14**, 132–140.

TAYLOR, A. C. (1962). Responses of cells to pH changes in the medium. *J. Cell Biol.* **15**, 201–209.

TRELSTAD, R. L., HAY, E. D. & REVEL, J.-P. (1967). Cell contact during early morphogenesis in the chick embryo. *Devl Biol.* **16**, 78–106.

TRINKAUS, J. P. & LENZ, T. L. (1967). Surface specialization of *Fundulus* cells and their relation to cell movements during gastrulation. *J. Cell Biology* **32**, 139–154.

TRINKAUS, J. P. (1973). Surface activity and locomotion of *Fundulus* deep cells during blastula and gastrula stages. *Devl Biol.* **30**, 68–103.

WOURMS, J. P. (1972). The developmental biology of annual fishes. II. Naturally occurring dispersion and reaggregation of blastomeres during the development of annual fish eggs. *J. exp. Zool.* **182**, 169–200.

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