AMER. ZOOL., 24:673–688 (1984)

# Mechanism of *Fundulus* Epiboly—A Current View[1,2]

J. P. TRINKAUS

*Department of Biology, Yale University, New Haven, Connecticut 06520 and Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

SYNOPSIS. This paper summarizes evidence for the following picture of *Fundulus* epiboly, with an eye toward laying groundwork for future investigation. The major force in epiboly is the yolk syncytial layer (YSL). Prior to epiboly, it spreads well beyond the border of the blastoderm to form the wide external YSL (E-YSL). This has contractile properties, which, however, are restrained prior to epiboly by the attached enveloping layer (EVL) of the blastoderm. Epiboly begins when the E-YSL contracts and narrows, throwing its surface into folds and pulling the internal YSL (I-YSL) and the attached EVL vegetally. When the narrowing of the E-YSL has ceased, it is postulated that its contractility continues as a circumferential wave of vegetally directed contraction that moves over the yolk toward the vegetal pole, dragging the I-YSL and the attached EVL (and blastoderm) with it. The most obvious visible manifestation of this wave is a marked marginal constriction, where the YSL joins the yolk cytoplasmic layer (YCL). As this contractile wave passes over the yolk, cytoplasm from the YCL mingles with that of the advancing E-YSL, and YCL surface adds to the already highly convoluted surface of the E-YSL. This folded surface is the site of a thin, highly localized band of rapid endocytosis that encircles the egg and passes over it with the E-YSL in a wave throughout epiboly. This internalization, which is receptor independent and therefore somehow programmed, accompanies the putative contractile wave, and accounts for the disappearance of the surface of the YCL. Since the YCL surface stands in the way of the advancing YSL, its internalization is part of the mechanism of epiboly. As the I-YSL expands in response to this marginal pull, its abundant microvilli gradually disappear, providing surface for its epiboly. The firmly attached EVL likewise expands toward the vegetal pole in response to the pull of the autonomously expanding YSL. As epiboly of the EVL progresses, it adjusts to the geometric problems posed by a sheet expanding over a sphere by active cell rearrangement within the cell monolayer. Thus, epiboly of the EVL has an active as well as a passive component. Deep cells are not causally involved in epiboly, but move about in coordinated ways in the constantly increasing space between the I-YSL and the EVL provided by epiboly and form the germ ring and the embryonic shield and eventually the embryo proper. An attempt is made to pull all of this together, and more, in order to achieve as comprehensive an understanding of epiboly as present evidence will allow.

## INTRODUCTION

Since there have been certain concrete advances in our understanding of the mechanism of teleost epiboly during the last several years, it seems appropriate at this time to summarize the state of this understanding and lay emphasis on those aspects most needing attention. The bulk of our current knowledge is based on our own studies of epiboly in *Fundulus heteroclitus*, but recently there have been other studies of other material (*e.g.*, Wourms, 1972; Van Haarlem, 1979, 1983; Long, 1980, 1984; Kageyama, 1982). This is a trend that we may hope will continue. The dimensions of the problems involved need many minds and hands and studies of epiboly in other genera of teleosts are bound to reveal aspects of this spectacular morphogenetic movement that are more amenable for study than in *Fundulus*. There is so much variation in how fishes engage in gastrulation (Ballard, 1981)!

Reduced to the simplest terms (Fig. 1), teleost epiboly involves: 1) expansion of a cohesive, monolayered sheet of cells at the surface of the blastoderm, the enveloping

[1] From the Symposium on *Gastrulation* presented at the Annual Meeting of the American Society of Zoologists, 27–30 December 1982, at Louisville, Kentucky.

[2] I wish to dedicate this small contribution to Professor Johannes Holtfreter for many reasons, but in particular because when I read his papers on the mechanism of gastrulation as a student I was inspired to think that I might one day learn how embryos gastrulate. I think that I now know a little about this seminal problem, and I am grateful to Professor Holtfreter for his stimulus and for all the fun he has given me.

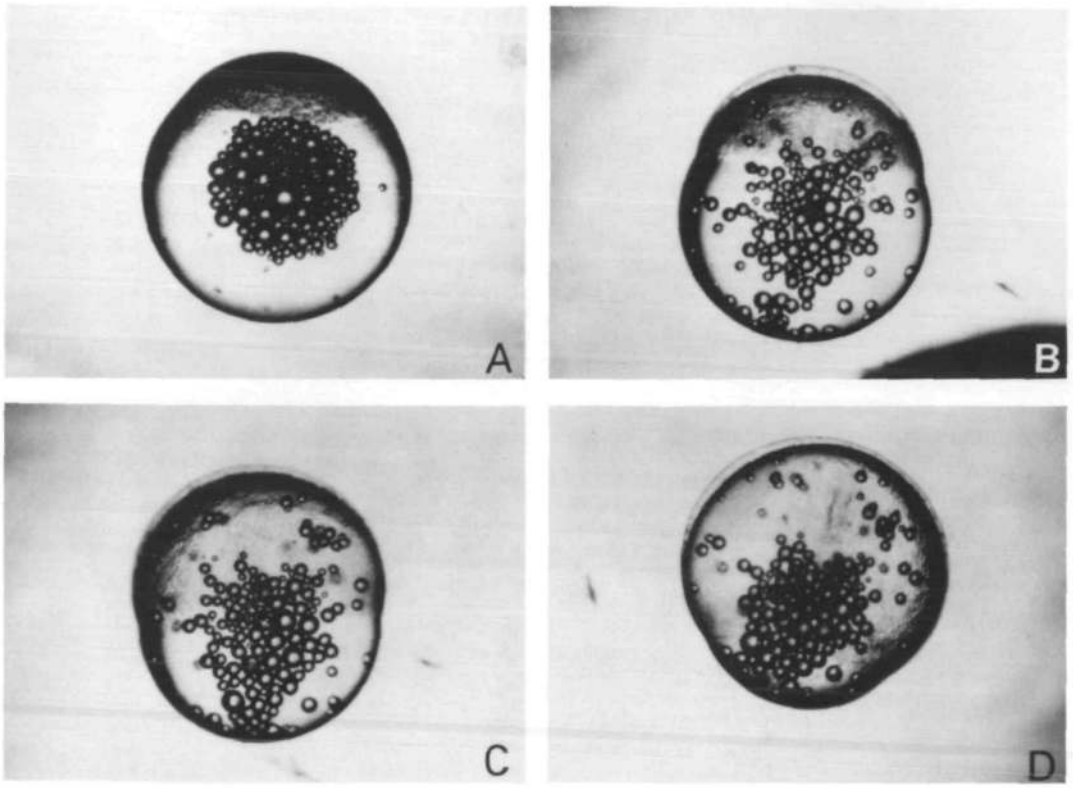


FIG. 1. Normal epiboly of the *Fundulus* gastrula. The chorion has been removed and the focus is on the margin of the egg. A. Stage 14 (early epiboly); the E-YSL, faintly visible on the right, is still narrowing (see Figs. 3 and 4). No marginal constriction is evident, only the slightly raised contour of the edge of the blastoderm. More than ¾ of the surface of the yolk sphere is enclosed by the YCL. B. Stage 15½; narrowing of the E-YSL has ceased (see Figs. 3 and 4). Note the distinct circumferential constriction at the margin of the blastoderm and the very narrow E-YSL (not shown). C. Stage 17, mid-epiboly. Note the marginal constriction. D. Stage 18½ (advanced epiboly). Note the marginal constriction. Three-quarters of the YCL has now been replaced. The axial keel of the developing embryonic shield is evident in the blastoderm. The thin, transparent enveloping layer (EVL) is partially lifted off the surface of the blastoderm in B, C, and D.

layer (EVL) or cellular envelope; 2) expansion of a syncytial layer beneath and beyond the blastoderm, the periblast or the yolk syncytial layer (YSL); 3) diminution and final disappearance of the thin cytoplasmic layer that is confluent with the YSL and covers the exposed yolk sphere, the yolk cytoplasmic layer (YCL); 4) The movement of deep cells within the expanding segmentation cavity (or subgerminal space) between the overlying EVL and the underlying YSL. I have summarized elsewhere some of the earlier work on the mechanism of teleost epiboly (Trinkaus, 1984). In this paper, I wish to concentrate on some recent instructive observations and the current state of our thinking on the matter.

## MATERIALS AND METHODS

The materials for these studies were blastulae and gastrulae of the teleost *Fundulus heteroclitus,* from mid-blastulae (stage 11) throughout epiboly up to closure of the yolk plug (stage 20), staged according to the series of Armstrong and Child (1965). Eggs were dechorionated prior to all observations and experiments (Trinkaus, 1967) and were routinely cultured in double-strength Holtfreter's solution. When the behavior of the exposed internal syncytial

layer (I-YSL) was studied (after removal of the blastoderm), eggs were cultured in the tissue culture medium Leibowitz L-15 (GIBCO) (without serum) at 21°C. Fixation for ultrastructural studies was always in glutaraldehyde (3%), followed by post-osmication.

## Results and Discussion

### *Expansion of the yolk syncytial layer*

Observation of normal development, both in living and fixed intact eggs and in dissected living material, shows that the EVL and YSL both engage in epiboly and do so largely together (*e.g.*, Wilson, 1889; Trinkaus, 1951*a*; Long, 1980). Prior to the onset of epiboly, the YSL extends well beyond the margin of the EVL in a broad exposed belt known as the external yolk syncytial layer (E-YSL) (Figs. 1, 3 and 4). During the first stages of epiboly, this belt narrows rapidly, as the EVL expands more rapidly than the YSL (Trinkaus, 1951*a*; Betchaku and Trinkaus, 1978). However, the margin of the EVL never moves beyond the margin of the E-YSL; indeed, it never quite reaches it (Betchaku and Trinkaus, 1978). A narrow belt of E-YSL precedes the margin of the EVL throughout epiboly. Clearly, normal epiboly of the EVL, and therefore of the whole cellular blastoderm, is associated with its tight marginal adhesion to the underlying expanding YSL. Moreover, since the YSL of *Fundulus* continues to expand in epiboly when the blastoderm is removed (Trinkaus, 1951*a*), epiboly of the YSL is independent of the blastoderm and therefore of the EVL. This suggests that epiboly of the EVL depends on that of the YSL. All of this indicates that epiboly of the YSL deserves careful investigation.

Three important questions face us: 1) the source of surface for the enormous expansion of the YSL during epiboly; 2) the stimulus for the onset of YSL epiboly and the motive force for its continued expansion; and 3) how the YCL is replaced as the YSL expands.

*Source of surface for expansion of the YSL.* Since the surface area of the YSL increases approximately 12-fold during epiboly of *Fundulus*, there must be a ready source of cell surface. This could either be extrinsic or intrinsic. A possible extrinsic source is the yolk cytoplasmic layer (YCL). As the YSL spreads in epiboly, ultimately to encompass the entire yolk sphere, the surface of the YCL, with which it is confluent (Lentz and Trinkaus, 1967), diminishes and ultimately disappears. Does the YSL spread by a progressive transformation of the surface of the YCL, such that transformed YCL surface is constantly added to the margin of the YSL? There appear to be two possible intrinsic sources of cell surface—addition of new plasma membrane from below by exocytosis or by unfolding of protuberances present on the surface of the YSL at the beginning of epiboly (Lentz and Trinkaus, 1967; Betchaku and Trinkaus, 1978).

I first tried to test these hypotheses in a simple but revealing way by attaching carbon particles to the surfaces of the YCL and internal YSL (I-YSL) of eggs whose blastoderm had been removed and then following their fate during epiboly by means of camera lucida tracings (Trinkaus, 1971). If the YCL surface is continually transformed into YSL surface where they join, marks placed on the YCL should come to lie on the YSL with the progress of epiboly. They do not. They are approached and ultimately reached by the advancing margin of the YSL, to be sure (Fig. 2) (see also Fig. 3, Trinkaus, 1951*a*), but then are carried vegetalward by it, as the margin of the YSL advances in epiboly. Since this result does not support the hypothesis of extrinsic addition of cell surface, it shifts attention to the possibility that expansion of the YSL is due to intrinsic factors. If this hypothesis is valid, the distance between marks on the surface of the I-YSL should increase (Fig. 2) and marks placed on or very near the I-YSL margin should not recede from the margin as epiboly progresses (Fig. 4). Both of these conditions were met. Clearly epibolic expansion of the YSL depends on factors intrinsic to it.

Current evidence is against the possible insertion of new surface from the cytoplasm by exocytosis or other means (see below). However, since the YSL cytoplasm

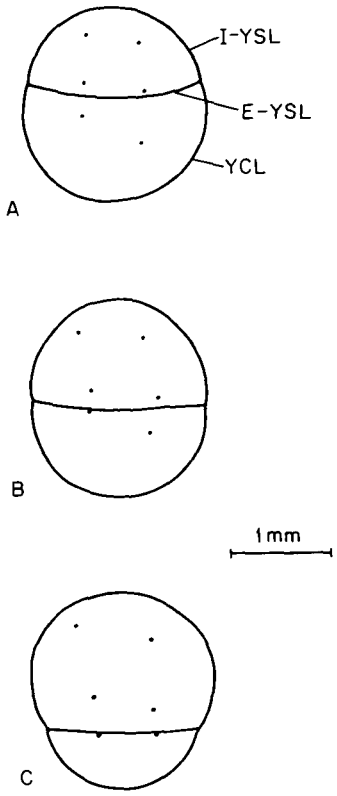


FIG. 2. Camera lucida tracings, showing the behavior of carbon marks placed on the YCL and the exposed I-YSL of an early mid-gastrula (stage 16). The position of the E-YSL is indicated, but it is too narrow at this stage to be observed as a separate entity at this low magnification. A = stage 16, 0 time; B = stage 17⅓, after an elapse of 2 hr; C = stage 18, after an elapse of 4 hr. Note that marks on the YCL are stationary and are approached by the margin of the YSL as it progresses in epiboly. When each mark is reached by the YSL margin, it is carried vegetally by it. The marks on the YCL do not come to lie on the surface of the YSL. In contrast, marks placed on the surface of the I-YSL at various distances from its margin are not stationary; they move away from one another, especially in the animal-vegetal axis, and away from the margin of the I-YSL at a steady rate, as the I-YSL expands in epiboly.

possesses elaborate Golgi (Lentz and Trinkaus, 1967), it has part of the machinery for manufacturing new membrane. In consequence, this is still a possibility. The other possible intrinsic source of cell surface—from surface already there at the beginning, in the form of protuberances—rests on strong evidence. At the onset of epiboly, the surface of the I-YSL is covered with a forest of long, branched microvilli of an approximately uniform size (Lentz and Trinkaus, 1967; Betchaku and Trinkaus, 1978). In their aggregate, these microvilli add substantially to the total surface of the YSL. Indeed, Betchaku and I (1978) have estimated that at the beginning of epiboly, the I-YSL has enough surface, taking these microvilli into account, to accommodate most of epiboly, if the surface stored in the microvilli were made available for general surface expansion. Significantly, these long microvilli are steadily replaced by shorter microvilli as epiboly progresses. Also, when a denuded I-YSL of an early gastrula is treated with cytochalasin B, which disrupts the insertion of the microfilaments of the microvilli on the plasma membrane, the I-YSL expands within a few minutes to a surface area about the same as its *total* surface area (taking microvilli into account). These facts are obviously consistent with the conclusion that the long microvilli of the I-YSL of an early gastrula serve as a reserve of cell surface that is gradually made available for epibolic expansion by unfolding.

Although each increase in surface area must of course be examined for its own merits in relation to its own situation, this proposal for the expansion of the I-YSL of *Fundulus* is analogous to that for several other unrelated cases, where spreading also appears to occur without an absolute increase in cell surface. A case in point is the ubiquitous fibroblast (Erickson and Trinkaus, 1976; for a review, see Trinkaus, 1980), where rounded cells are covered with blebs and microvilli and spread cells possess a smooth surface. In all instances, however, the analysis has stopped short of an explanation of how this unfolding might occur. Since all microvilli are filled with contractile microfilaments that insert on the plasma membrane, it is universally thought that these microfilaments must somehow control the unfolding. Just how they might do this, however, remains a mystery.

Although the probability is high that all or almost all of the epiboly of the I-YSL of *Fundulus* can be accounted for by an unfolding of cell surface already present at

its inception, this does not preclude some addition of new cell surface from beneath. It does suggest, however, that there is little need of it.

*The stimulus for the onset of YSL epiboly and the motive force for its continued expansion*

What causes the YSL to begin its epibolic expansion? An interesting experiment suggests that factor(s) intrinsic to the YSL itself are at play and that the blastoderm actually has an inhibitory role. If a *Fundulus* blastula is placed in tissue culture medium (Leibowitz L-15) and the blastoderm removed well before epiboly of the I-YSL has begun, at about stage 11 when there is a wide band of external YSL, the I-YSL begins to expand precociously within a few minutes after the operation and continues epiboly at an accelerated rate, reaching stage 16 (early mid-epiboly) before the I-YSL and the blastoderm of intact control eggs have expanded to stage 15 (Fig. 3). Clearly, the presence of the blastoderm inhibits the onset of epiboly of the I-YSL prior to the time it normally commences. What then causes an exposed I-YSL to begin epiboly precociously, once the inhibiting blastoderm is removed? The answer appears to lie in the external YSL. Significantly, the wide E-YSL begins narrowing precociously at the same time as the exposed I-YSL begins expanding, and the subsequent rate of expansion of the I-YSL up to about stage 15–15½ (the first phase of epiboly) corresponds roughly to the rate of narrowing of the E-YSL (Fig. 3). This arresting result leads to four conclusions. 1) The narrowing of the E-YSL that occurs during the first phase of normal epiboly and results in its becoming highly folded (Trinkaus, 1951*a*; Betchaku and Trinkaus, 1978) is not the result of pushing by an actively expanding blastoderm (EVL). Quite the contrary. Since it takes place in the absence of the blastoderm, it must be due to active contraction of the E-YSL itself (Fig. 4). 2) The normal expansion of the I-YSL and of the blastoderm during the first phase of normal epiboly must, therefore, be due to this active contraction of the E-YSL. 3) The normal delay in the onset of normal epiboly of the blastoderm and I-YSL is due to inhibitory restraint exercised by the EVL, whose margin is firmly attached to the YSL. 4) Onset of epiboly of the blastoderm and I-YSL of an intact egg at the normal time (stage 13) must, therefore, be due to a weakening of this inhibitory restraint, possibly because of a reduction in the tension of the EVL.

One other point should be made. A big remaining unknown is how the YSL expands normally beyond the margin of the blastoderm to form the wide E-YSL in the first place (compare 0:00 and 1:16 of Fig. 3, and Fig. 4A and B of Trinkaus, 1951*a*). This occurs well before normal epiboly commences.

Although these results provide a neat explanation for the commencement of epiboly of the I-YSL and the blastoderm during the first phase of epiboly, *i.e., ca.* ¼ of epibolic expansion, they do not explain the continuation and completion of epiboly after narrowing of the E-YSL has ceased. The motive force for continued epiboly of the YSL after this first phase could possibly reside in whatever factors are responsible for the unfolding of the microvilli of the I-YSL. However, much of this unfolding has already occurred before mid-epiboly (see Fig. 30 of Betchaku and Trinkaus, 1978). Moreover, such a mechanism would probably result in constant expansion of the I-YSL per unit area, which would cause an exponential increase in area with time. This is not what happens (Milkman and Trinkaus, 1953; Betchaku and Trinkaus, 1978). Rate of increase of area accelerates at first, but then declines, whereas, in contrast, rate of linear advance of the margin increases slowly but steadily throughout epiboly (Betchaku and Trinkaus, 1978, Table 2). This points to the marginal region as a possible site of the propelling force, as postulated by Devillers (1950) and myself (Trinkaus, 1951*b*) some years ago.

Certain salient features of the marginal region are apropos. 1) As soon as the E-YSL has narrowed maximally (Figs. 3 and 4) (about stage 15½), the marginal region of the YSL and of the blastoderm (Fig. 1) becomes markedly constricted circumferentially and remains so throughout the rest

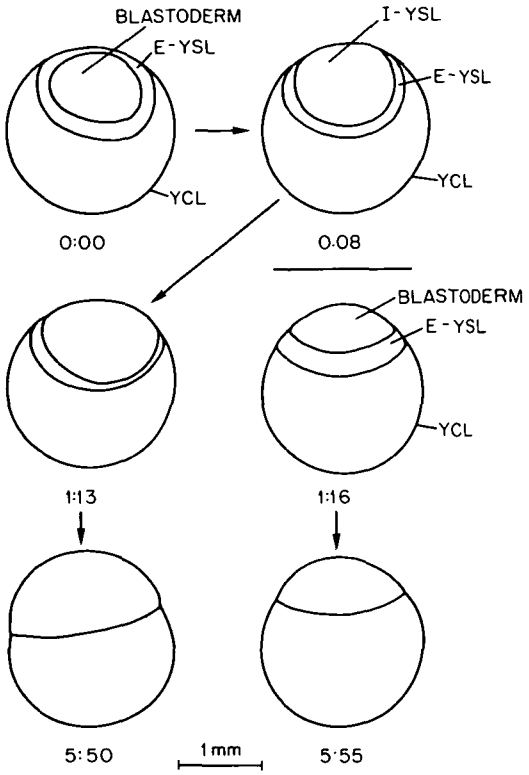


FIG. 3. Camera lucida tracings, showing the effect of removal of a blastoderm in a blastula stage, well before epiboly has normally begun, on the epiboly of the YSL. Time in hours and minutes. 0:00. An egg at stage 11 with a wide external yolk syncytial layer (E-YSL) just prior to removal of the blastoderm. At this stage the E-YSL is still widening (see 1:16). 0:08. The same egg, the blastoderm having been removed 6 minutes previously. Note the spectacular expansion of the now exposed internal yolk syncytial layer (I-YSL), accompanied by a corresponding narrowing of the E-YSL. The outer margin of the E-YSL has moved very slightly toward the vegetal pole. 1:13. The I-YSL of the experimental continues to expand rapidly, accompanied by continual but uneven narrowing of the E-YSL. The outer margin of the E-YSL has continued to move slowly but perceptively toward the vegetal pole. 1:16. An intact control egg, which was at the same stage as the experimental egg at 0:00. The width of the E-YSL has increased markedly during the interval and the blastoderm has expanded slightly. 5:50. The exposed I-YSL of the experimental has now reached stage 16, almost mid-epiboly. The E-YSL has now narrowed so much that it is no longer visible as a distinct entity at this low magnification. 5:55. The blastoderm of the control egg has now expanded, accompanied by almost full narrowing of the E-YSL, which can not be seen at this magnification. The egg is slightly tilted away from the viewer, giving the impression that the blastoderm has expanded somewhat less than it really has. Actually it is in stage 15. Note that both the experimental and

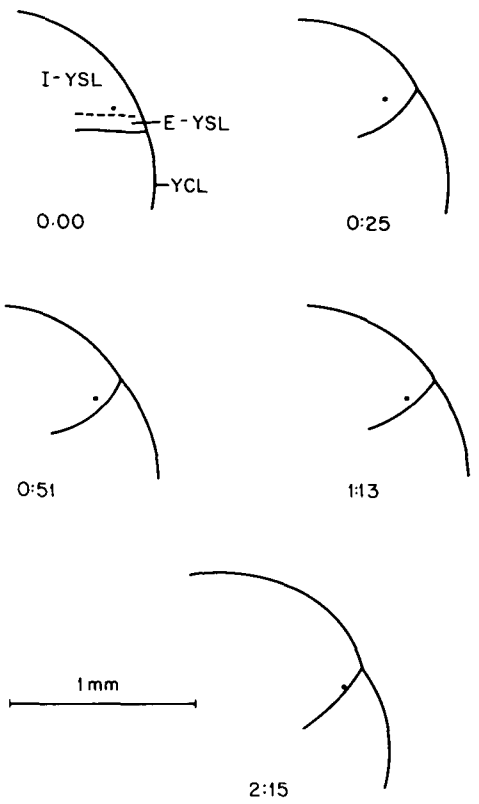


FIG. 4. Camera lucida tracings, showing the behavior of a carbon mark on an exposed I-YSL almost at its margin, *i.e.*, very close to its junction with the E-YSL, soon after epiboly has begun. The inner boundary of the wide E-YSL, where the margin of the EVL was attached prior to the operation, is indicated by a dashed line. Although the blastoderm was removed at stage 14, the I-YSL expanded to about stage 14⅔ during the interval of about 10 min between the operation and affixing the carbon mark (see Fig. 3). Time in hours and minutes. As the E-YSL continues to narrow, and the I-YSL continues to expand, the mark is drawn toward the outer margin of the E-YSL. Finally, when narrowing of the E-YSL is essentially complete, at stage 15½ (between 1:13 and 2:15) the mark has moved almost to the margin of the now very narrow E-YSL (which is invisible at 2:15 at this low magnification). Note that although there is a slight circumferential constriction of the margin while the E-YSL is narrowing and its margin is progressing slowly toward the vegetal pole (0:00–1:13) the degree of this constriction increases markedly when narrowing of the E-YSL has ceased. This shows well in 2:15, at stage 17, but it is actually clearly evident before that, at stage 15½–16 (see Fig. 3).

←

the control eggs now show a marked constriction at the margin of the YSL, more so in the case of the experimental.

of epiboly (Fig. 1) (see also Oppenheimer, 1937). Significantly, this constriction is often accentuated upon removal of the blastoderm, especially in advanced epiboly, when it can be truly dramatic (Trinkaus, 1951*a*, Fig. 6). These observations suggest that the constricting force lies within the marginal region of the YSL, and that it is greater than expressed during normal epiboly, when the blastoderm is present. 2) The marginal constricted region of the YSL, the peripheral region of the I-YSL and the entire E-YSL, is much thicker than the rest of the YSL and remains so to the very end of epiboly. (This has been observed many times in the past, of course, in a number of species [*e.g.*, Van Bambeke, 1872; Klein, 1876]. It shows nicely in Fig. 6 of the famous paper by Agassiz and Whitman published just 100 years ago [1884].) In contrast to this thickening of the marginal region, the rest of the YSL constantly thins with epibolic advance, accentuating the marginal thickening (Betchaku and Trinkaus, 1978; Figs. 55–63). 3) The cortical cytoplasm of this thick marginal region is packed with microfilaments (Betchaku and Trinkaus, 1978, Fig. 28). If these are contractile, which seems probable (they certainly are from stage 13–15½, when the E-YSL is narrowing), they could be exerting contractile force. If so, and if the force within the periphery of the YSL is exerted in a vegetal direction, as it is during narrowing of the E-YSL, and moves toward the vegetal pole in a wave over the surface of the yolk sphere (being fed at its front constantly by cytoplasm from the YCL), it would tend to pull the YSL in a vegetal direction. Further, such a postulated contractile wave would explain the circumferential marginal constriction of the YSL, the thickness of this region (contractile activity should pull it together), the constant folding of the surface of the E-YSL throughout the remainder of epiboly, and the high concentration of microfilaments in it. Finally, it seems hardly a coincidence that the marginal constriction becomes much more evident precisely when the narrowing of the E-YSL has terminated, at the end of the first phase of epiboly, at about stage 15½ (Figs. 3 and 4; see also Fig. 4E of Trinkaus, 1951*a*). Since narrowing of the E-YSL as such can no longer be a force in epiboly, another force must come into play. I suggest that this force is not a new force but merely the continued directional contraction of the E-YSL, now moving over the yolk as a contractile wave, whose most obvious visual manifestation is this impressive marginal constriction. Since this marginal constriction persists throughout the rest of epiboly (Fig. 1), it constitutes strong support for the hypothesis that the major motive force for epiboly of the YSL, and probably of the blastoderm as well (see pp. 683–684), during the bulk of epiboly (from stage 15½ onward to closure of the yolk plug at the end of epiboly) is this postulated contractile wave. Now we must find ways of testing this hypothesis.

*What happens to the yolk cytoplasmic layer as it is replaced by the yolk syncytial layer?* This is a matter of considerable intellectual interest, for we are speaking of the disappearance of a very thin cytoplasmic entity that during all of its existence has the important role of helping contain the viscous fluid yolk in a sphere (or, more accurately, an oblate spheroid), with all the resistance to the force of gravity that this implies (Fig. 1). But, in addition, this layer stands in the way of epiboly. If it did not disappear at its junction with the YSL (see p. 686), epiboly of the YSL would presumably stop, and with it epiboly of the blastoderm. The question before us, therefore, is how does it disappear? Presumably, its cytoplasm joins and intermingles with the cytoplasm of the YSL, with which it is constantly confluent. But what about its surface? Since it seemed improbable that it disappears into thin air, Betchaku and I searched for a biological mechanism. Examination of our thin sections of the E-YSL suggested that it might occur by internalization. There are many vesicles in the cytoplasm of the E-YSL (Betchaku and Trinkaus, 1978; Figs. 28, 38, 41–45). We decided to test this hypothesis by placing normal gastrulae in full course of epiboly in the fluorescent dye lucifer yellow (CH) for 5 min, followed by a wash in 2× Holtfreter's solution and then examining them in a fluorescent microscope (Betchaku and

Trinkaus, 1982). What we observed was truly spectacular! Such an exposed egg is completely dark except for a thin band of tiny, bright, fluorescent yellow vesicles precisely localized in the E-YSL encircling the egg (see Fig. 12-8 in Trinkaus, 1984). Clearly, endocytosis takes place very rapidly and continually where the YCL joins the YSL during all of epiboly, once the YSL begins expanding. Although the entire egg was exposed to lucifer yellow, internalization occurred *only* in this region and *only* during epiboly; prior to epiboly there is no internalization. This explains the disappearance of the surface of the YCL during epiboly. It is internalized where the advancing YSL surface joins the YCL. Since it thus makes way for the advance of the YSL, this internalization must be considered part of the mechanism of epiboly. Moreover, since it occurs in Holtfreter's solution or sea water (or even in distilled water), it is clearly independent of macromolecules in its environment and therefore is not receptor-mediated. It is intrinsically controlled. What we are dealing with here is a programmed internalization of cell surface, proceeding as a wave in a narrow circumferential band over the surface of a gastrula 1.8 mm in diameter during its epiboly. This is the first example we know of endocytosis playing a part in the mechanism of a morphogenetic movement.

Now then, a number of questions arise. How does this endocytosis occur? Is it mediated by coated pits? Precisely where in the E-YSL does it occur? After internalization, what is the fate of the endocytotic vesicles? Do they, for example, join the I-YSL by exocytosis and thus contribute to its expansion?

We do not yet know whether this wave of endocytosis is mediated by coated pits, as occurs so generally (Steinman *et al.*, 1983), for although our fixation method has not revealed coated pits or coated vesicles, it may have been inadequate for the purpose. But two important observations weigh against this possibility (Betchaku and Trinkaus, 1985). 1) When internalized vesicles of lucifer yellow are viewed in living gastrulae, just after removal from the dye, with the ultraviolet microscope, rod-shaped vesicles with a maximum dimension of 3.5 $\mu$m can be seen fusing to form disc-shaped vesicles. Coated vesicles are much too small to be seen at this magnification. 2) Internalization is confined to the region of the E-YSL where folding is occurring (Betchaku and Trinkaus, 1978). The size of these folds and particularly of the valleys between them, where endocytosis seems to be occurring, are much too large to be in the refined category of coated pits. Continuing research is obviously needed to settle this important question.

The fate of the internalized cell surface was studied with TEM of gastrulae fixed at different intervals after immersion in electron dense markers (native and cationized horse spleen ferritin) (Betchaku and Trinkaus, 1982). These markers were readily endocytosed (like lucifer yellow) and vesicles containing them quickly sank to the bottom of the YSL, where they came to rest on the surface of the huge yolk sphere. Interestingly, very little migration of these vesicles was observed beyond the precise region of their entry. The margins of the I-YSL and the EVL simply move over these sunken vesicles as epiboly progresses. In complete confirmation of our suggestion that expansion of the I-YSL depends entirely or almost entirely on utilization of surface already present in microvilli at the beginning of epiboly (see p. 676), none of these endocytosed vesicles was observed near the surface of the I-YSL or joining it.

This is where work on this important aspect of *Fundulus* epiboly stands. We now know how and where the yolk cytoplasmic layer disappears during epiboly. But many questions remain. How is this programmed internalization of cell surface controlled (the most basic question and probably the most elusive)? Since this internalization of the YCL is necessary for epiboly of the I-YSL (and with it of the blastoderm) and therefore part of the mechanism of epiboly, inhibition of it should stop epiboly. This will probably not be easy, for two important reasons: 1) the exceedingly low permeability of the surface of the *Fundulus* egg (Bennett and Trinkaus, 1970; Dun-

ham *et al.*, 1970) (intact eggs, for example, undergo full, normal epiboly in the presence of cytochalasin B), and 2) the unsatisfactory results of efforts to find specific total inhibitors of endocytosis (Marsh and Helenius, 1980). Nonetheless, an effort to inhabit endocytosis of the YCL in various ways must be high on the list of future priorities. Finally, we do not know important details of the internalization process itself, whether for example, it is mediated by coated pits and coated vesicles; and we do not yet know the ultimate fate of the endocytotic vesicles.

*Expansion of the enveloping layer*

The enveloping layer (EVL) of *Fundulus* is a typical epithelium, a monolayer of cells tightly joined to each other by circumferential, occluding tight junctions, desmosomes, gap junctions and long stretches of zonulae adherentes, where the apposed plasma membranes are separated by a gap of approximately 15 nm. The extent and complexity of these junctions increases during epiboly, as the EVL is placed under greater and greater tension (Lentz and Trinkaus, 1971). Its marginal cells are joined to the underlying YSL by tight junctions, whose width also increases with the progress of epiboly (Betchaku and Trinkaus, 1978). Consistent with this fine-structure, the EVL has extraordinarily low permeability,—as measured both electrophysiologically (Bennett and Trinkaus, 1970) and by uptake of $^3H_2O$ (Dunham *et al.*, 1970).

As with the YSL, I shall organize my treatment of the epiboly of the EVL around a number of questions. 1) Is cell division necessary? 2) What is the source of the large increase in surface area? 3) Is there cell rearrangement within the EVL, as its margin increases in extent in pre-equatorial epiboly and decreases in sub-equatorial epiboly? 4) Do the marginal cells move actively, as do cells of an expanding epithelial sheet in culture or in wound closure, or passively, in response to the pull of the YSL? Is EVL epiboly active or passive?

*Cell division.* Increase in cell number is certainly not necessary for epiboly of the EVL of *Fundulus*. The cell number at the beginning, about 5,000, changes very little during epiboly (Betchaku and Trinkaus, 1978) and when one inhibits what little cell division there is by colchicine (Kessel, 1960), epiboly continues willy-nilly. Here, then, as in essentially all morphogenetic movements of short duration (Trinkaus, 1984), cell division is not a force.

*Source of increase in cell surface area.* Since the surface area of the EVL of *Fundulus*, like that of the YSL, increases approximately 12-fold during epiboly, and since there is little or no cell division, the surface area of individual cells of the EVL perforce must increase enormously. Morgan observed such an increase in area many years ago (1895) and noted that, with this, the cells become exceedingly thin (compare Figs. 3 and 4 and 46 of Betchaku and Trinkaus, 1978). Most of the non-nuclear region of each EVL cell of *Fundulus* in advanced epiboly, in fact, is less than 1 $\mu$m thick (Betchaku and Trinkaus, 1978; Figs. 50 and 51). An obvious possible source of cell surface for this expansion might be in protuberances of the surface of each cell (as for the I-YSL). And, indeed, the surfaces of most EVL cells prior to epiboly are highly folded (Betchaku and Trinkaus, 1978; Figs. 4 and 5). Moreover, most of these folds disappear quickly after epiboly begins, remaining only near the cell boundaries (Betchaku and Trinkaus, 1978; Figs. 26 and 46). This suggests that these folds serve as a reserve source of cell surface, at least during the earliest stages of epiboly. But since this postulated reserve is almost entirely exhausted soon after epiboly has begun, it cannot account for the continued increase in surface area during the rest of epiboly. Further, since this presumed store is in the form of irregular folds, rather than microvilli with rather precise dimensions, there is no way of estimating with any pretention of accuracy the amount of extra cell surface provided. At any rate, since most of the epiboly of the EVL occurs after most of the folds have disappeared, most expansion of the cells of the EVL must come from another source, presumably from the synthetic apparatus of each cell. But we have no evidence on this.

*Cell rearrangement during epiboly.* Since the advent of the electron microscope, we have come to base the tight cohesion of cells within an epithelial sheet on circumferential tight junctions or septate junctions, desmosomes, gap junctions and zonulae adherentes. It has long seemed reasonable, therefore, to assume that cells within such a sheet do not displace or exchange neighbors as the sheet spreads. Now, however, we have indisputable evidence against this assumption for cells of the leg imaginal disc of *Drosophila* (Fristrom, 1976, 1982) and superficial cells of the *Xenopus* gastrula (Keller, 1978), and, more to the point, a suggestion that cell rearrangement also occurs during epiboly of the EVL of the teleost *Oryzias latipes* (Kageyama, 1982). Following the lead of these investigations, Keller and I have recently studied the possibility that cells of the *Fundulus* EVL also rearrange during epiboly, in spite of their tightly joined state (Keller and Trinkaus, 1982, and in preparation). Staining cell boundaries with the ancient silver nitrate technique (Kageyama, 1980) quickly revealed that the number of cells at the margin of the EVL decreases continuously during subequatorial epiboly, from 105 ± 4.5 at mid-epiboly (stage 17) to <20 at closure of the yolk plug (stage 20). Hypothetically, this decrease could be due to a) cell death, b) cell fusion, c) ingression into the interior, or d) cell rearrangement within the EVL. We have never observed cell death or cell fusion in numerous time-lapse films and electron micrographs of the EVL. To determine whether there is cell ingression or rearrangement required direct observation of the exceedingly thin marginal cells in living gastrulae. We accomplished this with the aid of the high resolution Zeiss planapochromat 63× objective N.A. 1.4, photomicrography, and cinemicrography. We observed no ingression. Rearrangement, on the contrary, was obvious and continuous. The advancing border of a marginal cell becomes tapered and then recedes, its place being taken by the adjacent marginal cells (see Fig. 12-12 in Trinkaus, 1984). Although this recession takes place quite slowly (0.5–1.0 μm/min), it is fast enough for the overall linear rate of epiboly at these stages (>3.0 μm/min) (see Table 2 of Betchaku and Trinkaus, 1978). Not unexpectedly, submarginal cells also rearrange. We consider this cellular rearrangement, and others like it, to be noteworthy, in view of the tight attachment of these cells to each other and to the YSL beneath. Without question, such cells are able to move relative to one another without disrupting this high resistance barrier. This suggests that tight junctions are dynamic. It is not known how this dynamism is expressed structurally, by partially breaking and instantly remaking in a kind of steady state, or by sliding, or whatever (see Fristrom, 1982). Finally, it has not escaped our notice that these results for the *Fundulus* enveloping layer, and the other rearrangements cited, may have significance for the many epithelial systems that have the dual function of maintaining a high resistance barrier and engaging in distortions necessary for morphogenesis.

We are happy to have settled the question of rearrangement within the EVL of *Fundulus,* but we have no idea how this orderly rearrangement of cells takes place within the plane of the monolayer. Here, as in *Drosophila* imaginal discs (Fristrom, 1976), the cells rearrange without any overt motile activity of their surfaces. In time-lapse films, they seem quiescent and, because of the maintenance of the permeability barrier, there cannot be any intercellular spaces into which they might move. They just seem to slip by each other. This is an absolutely unexplored mode of cell locomotion that deserves attention in future research.

*Mechanism of expansion of the enveloping layer.* Inasmuch as the EVL of *Fundulus* is in many ways a typical epithelial sheet and such sheets are routinely able to spread when given a free edge due to the locomotory activity of their marginal cells, it was reasonable to suspect that the EVL of *Fundulus* spreads in epiboly by the same mechanism, using as its substratum the YSL, which conveniently spreads beneath it and thus provides a constant substratum right to the end of epiboly. Moreover, when part or all of the marginal attachment of the EVL to the YSL is severed, it will fre-

quently reattach to the YSL (now the I-YSL because of the retraction of the EVL), and spread over it like any epithelial sheet (Trinkaus, 1951*a*). The EVL will also close wounds, by spreading of the free margin of the wound (Trinkaus, 1949). Clearly,the EVL has the capacity to spread actively, when artificially provided with a free edge. Nonetheless, we have proposed that the normal epibolic expansion of the EVL does not depend on the active spreading of its free margin, but rather on the firm attachment of that free margin to the actively spreading YSL. In short, we believe that epiboly of the enveloping layer of *Fundulus* is passive (Betchaku and Trinkaus, 1978). Since this unusual hypothesis has been discussed at length elsewhere (Betchaku and Trinkaus, 1978; Trinkaus, 1984), it will suffice here simply to summarize the evidence.

The advancing edge of marginal EVL cells is morphologically quiescent during all of epiboly. Filming with Nomarski differential interference optics at all stages of epiboly reveals no lamellipodial activity or other deformations of the margin usually associated with actively spreading cells (see also Van Haarlem, 1983). This quiescence is not surprising, if one considers the fine-structural relationship between the edge of a marginal EVL cell and its YSL substratum. In contrast to other cells known to be actively moving *in vivo*, such as *Fundulus* deep cells (Trinkaus and Lentz, 1967; Hogan and Trinkaus, 1977) and *Xenopus* epidermal cells (Radice, 1980), EVL cells are attached to their substratum by tight junctions that are physiologically occluding and increase steadily in width during the course of epiboly. Indeed, in late epiboly this junction is approximately 2 μm wide and the leading edge of each marginal cell is actually embedded in the YSL, instead of resting on its surface, as we would expect were it spreading actively over that surface (see Fig. 48 in Betchaku and Trinkaus, 1978). It may be objected that two of us have just shown that tight junctions are no impediment to cell movement; cells within the EVL rearrange during epiboly (Keller and Trinkaus, 1982). This is a legitimate objection and one reason why our conclusion of passive EVL expansion is still tentative. It should be emphasized, however, that cell rearrangement within the EVL is much slower than the rate of advance of the margin of the EVL during sub-equatorial epiboly—0.5–1.0 μm/min as compared with >3.0 μm/min. This suggests that different mechanisms may be involved.

If the motive force for EVL epiboly is indeed the actively expanding YSL to which it is attached, we would expect epiboly of the YSL to accelerate when it is relieved of the burden of a firmly attached blastoderm that is under ever increasing tension as epiboly progresses. This is precisely what occurs when the marginal attachment of the EVL to the YSL is severed during full epiboly, either totally, all around the circumference (Betchaku and Trinkaus, 1978, p. 416), or partially, along about 10% of the circumference (Trinkaus, 1971). Indeed, I have recently learned that if a larger segment of the attached margin of the EVL is severed, say 30–50% of the circumference, the expansion of the freed border of the YSL now accelerates so far ahead of the remaining border still attached to the EVL that it actually passes over the vegetal pole long before the attached margin reaches it and does not stop until it meets the E-YSL of the still attached region on the other side of the egg. In other words, during the last part of its epiboly, the detached border of the YSL is actually expanding *toward the animal pole,* instead of in the usual vegetal direction.[3] Further (as fully described above, p. 677), even the onset of epiboly of the I-YSL can be accelerated, if the blastoderm is removed prior to epiboly. These various experiments have certainly provided strong support for the hypothesis.

Finally (again, as fully described above, p. 677), narrowing of the external YSL also occurs in the absence of the blastoderm, and occurs more rapidly. This indicates that

[3] Incidentally, this result is also consistent with the hypothesis that internalization of the YCL surface constitutes an additional epibolic force. When the YCL surface is completely internalized, epiboly stops, wherever that might be.

narrowing of the E-YSL is an active contractile process that is completely independent of the blastoderm and strongly suggests that the initial epibolic expansion of the EVL (so-called first phase) depends entirely on this contractile narrowing of the E-YSL.

All in all, the hypothesis that the motive force for epiboly of the EVL (and therefore of the whole cellular blastoderm) resides in the YSL rests on strong and varied evidence. It is certainly the best working hypothesis we have and thus provides a solid basis for further research.

Although the linear movement of the margin and hence the overall expansion of the EVL in epiboly seems to be passive, much active cellular movement occurs within the EVL, as it adjusts to the geometric problems imposed by the expansion of a sheet over a sphere—first to the steady increase in its marginal region during the first half of epiboly and then to its steady decrease during the second half. The EVL accomplishes this, as we now know, by constant, orderly cellular rearrangement within the monolayer. EVL epiboly clearly has an active as well as a passive component.

In addition to its special interest for the mechanism of *Fundulus* epiboly, the probable passive nature of the linear progress of the EVL in epiboly has general interest for everyone interested in mechanisms of morphogenesis, in any embryo. If this hypothesis turns out to be valid for *Fundulus*, as certainly seems likely, it will constitute one more case of a lengthening list of morphogenetic cell movements that have a strong passive aspect, depending for their movement wholly or in part on their attachment to other cells or tissues that are moving actively (see Trinkaus, 1982). Passive cell movement has clearly emerged as a major means of morphogenesis.

*Epiboly of the deep cells*

Epiboly of *Fundulus*, and presumably of teleosts generally, does not depend on the deep cells. Regardless of the progress of the morphogenetic movements of the deep cells, epiboly moves inexorably to completion. This is illustrated normally during the development of small *versus* large eggs (see below), and experimentally as well. When *Fundulus* gastrulae are treated with colchicine, cytokinesis and much movement of deep cells are inhibited, grossly interfering with their morphogenetic movements (Trinkaus and Erickson, 1983), nevertheless epiboly proceeds normally to completion. Epiboly of the whole blastoderm depends rather on epiboly of the enveloping layer, which, in turn, seems to depend on the yolk syncytial layer, with the cooperation of the diminishing yolk cytoplasmic layer. The deep cells participate in epiboly, to be sure, moving in directed ways between the I-YSL and EVL, constantly filling new submarginal space as it is added, but they do not play a causal role. Deep cells participate by coordinating their movements with epibolic expansion so that the formation and convergence of the germ ring, the formation of the embryonic shield and its antero-posterior axial extension, and the subsequent formation of the embryo (Pasteels, 1936; Oppenheimer, 1936; Ballard, 1973*b*) all take place normally. Nor are the I-YSL and the EVL neutral in this. Both provide deep cells with substrata for movement (Trinkaus, 1973; Wourms, 1972; Kageyama, 1977; Van Haarlem, 1979; Trinkaus and Erickson, 1983). And, in addition, Long (1980, 1984) has recently provided evidence that the movements of dorsal convergence of deep cells may depend on the underlying expanding I-YSL; its cytoplasm also converges toward the mid-dorsal line. Finally, since many deep cells adhere to the underside of the EVL and the upperside of the I-YSL (as they must if they are to move on them), a vegetalward bias is added to their movements (Van Haarlem, 1979). While moving about actively in directed ways to form the embryo and various constituents of the yolk sac, some deep cells are also being carried passively toward the vegetal pole.

Although this extraordinarily ordered coordination of the movements of the deep cells with the epiboly of the EVL and YSL is certainly one of the wondrous features of teleost gastrulation, the variation in how this coordination relates to the extent of epiboly in different species is also of inter-

est. The coordination of these processes varies enormously with the size of the egg. In small eggs, like those of *Brachydanio* and *Serranus* (Wilson, 1889), for example, epiboly is complete long before embryo formation begins. In medium sized eggs, like those of *Fundulus* and *Oryzias,* the beginnings of embryo formation more or less coincide with the end of epiboly (Fig. 1; also see Armstrong and Child, 1965). In contrast, in large eggs, like those of the trout (Pasteels, 1936; Ballard, 1973*a*), embryo formation is well-advanced well before epiboly is complete. It would seem that the morphogenetic movements of the deep cells and epiboly of the EVL and YSL take place at about the same relative rate, regardless of the size of the egg, the former being less advanced at the relatively rapid completion of epiboly in small eggs and more advanced at the relatively delayed completion of epiboly in large eggs. This intriguing relationship seems not to have been explored in detail.

Also, I have often observed for *Fundulus* (as have no doubt others for other species) that making a larger egg smaller accidentally, by wounding the YCL and causing a major loss of yolk, appears not to impede embryo formation. In such cases, much of the early morphogenesis involved in embryo formation must take place *after* closure of the yolk plug, as occurs normally in small eggs, and is, therefore, completely independent of epiboly. This important phenomenon likewise seems not to have been investigated. A combined comparative and experimental study, with careful marking à la Ballard, would no doubt yield information that bears both on post-epibolic morphogenesis and the normal role of epiboly in gastrulation.

## Epilogue

At the risk of reiteration, perhaps it would be useful to pull these thoughts together and summarize the main features of our current understanding of *Fundulus* epiboly, laced here and there with some modest speculation.

During the so-called blastula stage, the yolk syncytial layer extends far beyond the margin of the blastoderm to form a broad syncytial band that we have named the external yolk syncytial layer. Details of the morphology of this process have not been studied. This wide E-YSL develops impressive contractile properties that eventually lead to its narrowing at the commencement of epiboly. These contractile properties remain latent prior to normal epiboly because of the restraint of the overlying firmly attached enveloping layer. During this period, the contractile E-YSL is "straining at the leash," as it were, for it contracts immediately when the blastoderm is removed. Eventually, the resistance of the EVL diminishes (or the contractile properties of the E-YSL increase) and the E-YSL begins to narrow. As the E-YSL contracts, epiboly of the internal YSL and the EVL (and, of course, the rest of the blastoderm) begins, the rate of their expansion corresponding roughly to the rate of contraction of the E-YSL. Clearly, the first phase of epiboly depends on directional contraction of the E-YSL. When this ceases and the E-YSL is reduced to a very narrow band, this phase of epiboly comes to an end.

With decrease in the width of the E-YSL no longer an epibolic force, a new mechanism must come into play. I suggest that no new force is required, merely a continuation of the vegetally directed contraction of the E-YSL as a contractile wave that moves over the yolk sphere toward the vegetal pole, fed by cytoplasm from the YCL which is being constantly replaced, pulling the I-YSL and the attached EVL with it. Actually, the margin of the E-YSL already begins moving vegetally while the whole E-YSL is still narrowing, an observation that supports the suggestion that we are dealing with the same contractile force. After narrowing of the E-YSL is essentially maximal, of course, this contractile wave becomes the major epibolic force, revealing itself visually by a marked circumferential constriction of the peripheral region of the YSL and the attached EVL.

The contraction of the E-YSL during the first phase of epiboly causes its wide, smooth surface to buckle, throwing it into complex folds. Also, as the E-YSL margin begins to move vegetally, replacing the yolk cyto-

plasmic layer, the surface of the YCL is added to the E-YSL, along with its cytoplasm, increasing the folding. In spite of the constant addition of new surface from the YCL, however, the degree of folding remains roughly uniform, because of the rapid endocytosis of surface that accompanies the folding throughout epiboly. This is how the YCL surface is disposed of. The folding of the E-YSL surface, therefore, serves two purposes: 1) temporary accommodation of the compressed surface of the originally wide, smooth-surfaced E-YSL and of the new YCL surface being added, and 2) internalization of all this extra surface, the valleys between the folds serving as sites of endocytosis. Eventually, when epiboly is complete, the surfaces of both the E-YSL and the YCL are completely replaced, both having been internalized in this programmed wave of highly localized endocytosis that constantly precedes the advancing margins of the I-YSL and the EVL. By removing surface that stands in the way of advance of the I-YSL, and thus of the EVL, this wave of internalization is an essential part of the machinery of epiboly. This, it seems, is how the margin of the YSL advances in epiboly—by a wave of marginal contraction, resulting in circumferential constriction and folding of the surface of the E-YSL, contributed to by the cytoplasm and surface of the YCL, as its territory is occupied, and accompanied by a wave of surface internalization in the folded region of the E-YSL that disposes of the added surface at the same rate as it is added. Marginal contraction pulls the I-YSL and EVL toward the vegetal pole and marginal internalization of YCL surface clears the way.

Perhaps this contractile folding of cell surface induces the internalization. Perhaps folding and internalization are simply two aspects of the same process. If so, all that is needed basically to account for epiboly is propagation of the circumferential contractile wave. It has been suggested that such a contractile wave could even be self-propagating, once it begins, like a brush fire, the constant addition of fresh cytoplasm from the YCL providing the fuel. And, then, once there is no more fuel, when the YCL is completely gone, the contractile wave—and epiboly—should stop. They do.

In the meantime, the internal YSL must adjust to the peripheral pull imposed by the steady progress of its margin. By having previously manufactured a great amount of cell surface, which is stored in numerous long microvilli, the I-YSL has sufficient total surface for the expansion imposed on it. Gradually, as need requires, this surface is distributed throughout by diminution and eventual disappearance of its microvilli.

Contractile cortical microfilaments, which insert on the plasma membrane, are no doubt crucial to all these processes: disappearance of the microvilli of the I-YSL, the contractile narrowing of the E-YSL and its consequent folding, the postulated contractile wave in the periphery of the YSL, with the addition of YCL surface and continued folding, and the internalization of cell surface in the region of folding. But we have no firm ideas as to how. Accordingly, this will necessarily be one of the major preoccupations of those interested in these problems in the future (as in the field of cell motility generally).

Now what about the blastoderm, whose gastrulation, after all, is really the embryological point of it all? The deep cells have nothing to do with the mechanism of epiboly. In a sense, they simply utilize the increasing space and substratum surface provided by epiboly to go about their business of forming an embryo. They move about in highly coordinated, very efficient ways, of course, coordinated with one another and with the expanding I-YSL below and the EVL above, but they are not essential to the mechanism of epiboly. The essential component is the enveloping layer. By virtue of the firm attachment of its marginal cells to the underlying YSL (just at the juncture of the I-YSL and E-YSL, thus defining them), the EVL moves with the expanding I-YSL in epiboly. Its linear movement to the vegetal pole seems to be entirely passive, dependent on its attachment to the actively moving YSL. But it adjusts to the geometric problems imposed by expansion of a sheet over a sphere in a very active way, by constant rearrangement of its cells within the plane of the

monolayer. The mechanism of EVL epiboly clearly has both active and passive components.

Obviously all of these separate processes must be exquisitely coordinated, if epiboly is to proceed normally. The force of contraction of the E-YSL during the first phase of epiboly must be attuned to the resistance of the EVL. The force of the postulated contractile wave of the peripheral YSL beginning during the first phase and continuing during the rest of epiboly must be attuned to the rate of disappearance of the microvilli of the I-YSL, the resistance of the EVL, which is placed under ever increasing tension with the progress of epiboly, the rate and pattern of cell rearrangement within the EVL, and the rate of internalization of the YCL (and finally also of the E-YSL). We have no tested ideas at present as to how all of these really very elegant coordinations are achieved. But now that we have reached some understanding of the individual activities on which epiboly depends, by all this analytical work, we are finally in a position to synthesize, to ruminate on the classical embryological problems of interaction and overall coordination. I have suggested that feedback mechanisms may be involved in some of this. May I also suggest that quite possibly these, or other mechanisms, will turn out to be much less complex than we are often wont to think.

## Acknowledgments

I am indebted to Teiichi Betchaku and Raymond Keller for fruitful collaboration and to the consortium of embryologists at the University of California at Berkeley for helpful discussions. The work reported in this paper, both unpublished and recently published, was supported by PHS Grant number USPHS-CA-22451 awarded by the National Cancer Institute, DHHS to J. P. T. and NSF grant PCM81-10985 awarded to R. E. Keller.

## References

Agassiz, A. and C. O. Whitman. 1884. On the development of some pelagic fish eggs. Preliminary notice. Proc. Am. Acad. Arts and Sci. 20:23–75.

Armstrong, P. B. and J. S. Child. 1965. Stages in the normal development of *Fundulus heteroclitus*. Biol. Bull. 128:143–168.

Ballard, W. W. 1973*a*. Normal embryonic stages for salmonid fishes, based on *Salmo gairdneri* Richardson and *Salvelinus fontinalis* (Mitchill). J. Exp. Zool. 184:7–26.

Ballard, W. W. 1973*b*. Morphogenetic movements in *Salmo gairdneri* Richardson. J. Exp. Zool. 184: 27–48.

Ballard, W. W. 1981. Morphogenetic movements and fate maps of vertebrates. Amer. Zool. 21:391–399.

Bennett, M. V. L. and J. P. Trinkaus. 1970. Electrical coupling of embryonic cells by way of extracellular space and specialized junctions. J. Cell Biol. 44:592–610.

Betchaku, T. and J. P. Trinkaus. 1978. Contact relations, surface activity, and cortical microfilaments of marginal cells of the enveloping layer and of the yolk syncytial and yolk cytoplasmic layers of *Fundulus* before and during epiboly. J. Exp. Zool. 206:381–426.

Betchaku, T. and J. P. Trinkaus. 1982. Membrane internalization plays an important role in teleostean epiboly. J. Cell Biol. 95:96a.

Betchaku, T. and J. P. Trinkaus. 1985. Programmed endocytosis during epiboly of *Fundulus heteroclitus* Amer. Zool. (In press)

Devillers, Ch. 1950. Mécanisme de l'épibolie gastruléene. C. R. de l'Acad. des Sci. 230:2232–2234.

Dunham, P. B., A. Cass, J. P. Trinkaus, and M. V. L. Bennett. 1970. Water permeability of *Fundulus* eggs. Biol. Bull. 139:420–421.

Erickson, C. A. and J. P. Trinkaus. 1976. Microvilli and blebs as sources of surface membrane during cell spreading. Exp. Cell Res. 99:375–384.

Fristrom, D. K. 1976. The mechanism of evagination of the imaginal discs of *Drosophila melanogaster*. III. Evidence for cell rearrangement. Devel. Biol. 54:163–171.

Fristrom, D. K. 1982. Septate junctions in imaginal discs of *Drosophila*: A model for the redistribution of septa during cell rearrangement. J. Cell Biol. 94:77–87.

Hogan, J. C. and J. P. Trinkaus. 1977. Intercellular junctions, intramembranous particles and cytoskeletal elements of deep cells of the *Fundulus* gastrula. J. Embryol. Exp. Morphol. 40:125–141.

Kageyama, T. 1977. Motility and locomotion of embryonic cells of the medaka, *Oryzias latipes*, during early development. Develop., Growth and Differ. 19:103–110.

Kageyama, T. 1980. Cellular basis of epiboly of the enveloping layer in the embryo of medaka, *Oryzias latipes*. I. Cell architecture revealed by silver staining method. Develop., Growth and Differ. 22:659–668.

Kageyama, T. 1982. Cellular basis of epiboly of the enveloping layer in the embryo of the medaka, *Oryzias latipes*. II. Evidence for cell rearrangement. J. Exp. Zool. 219:241–256.

Keller, R. E. 1978. Time-lapse cinemicrographic analysis of superficial cell behavior during and prior to gastrulation in *Xenopus laevis*. J. Morphol. 157:223–248.

Keller, R. E. and J. P. Trinkaus. 1982. Cell rearrangement in a tightly-joined epithelial layer during *Fundulus* epiboly. J. Cell Biol. 95:325a.

Kessel, R. G. 1960. The role of cell division in gastrulation of *Fundulus heteroclitus.* Exp. Cell Res. 20:277–282.

Klein, E. 1876. Observations on the early development of the common trout (*Salmo fario*). Quart. J. Micr. Sci. 16:113–131.

Lentz, T. L. and J. P. Trinkaus. 1967. A fine structural study of cytodifferentiation during cleavage, blastula, and gastrula stages of *Fundulus heteroclitus.* J. Cell Biol. 32:121–138.

Lentz, T. L. and J. P. Trinkaus. 1971. Differentiation of the junctional complex of surface cells in the developing *Fundulus* blastoderm. J. Cell Biol. 48: 455–472.

Long, W. L. 1980. Analysis of yolk syncytium behavior in *Salmo* and *Catostomus.* J. Exp. Zool. 214: 323–331.

Long, W. L. 1984. Cell movements in teleost fish development. BioScience 34:84–88.

Marsh, M. and A. Helenius. 1980. Adsorptive endocytosis of Semliki Forest virus. J. Mol. Biol. 142: 439–454.

Milkman, R. and J. P. Trinkaus. 1953. Site of action of epibolic forces in the egg of *Fundulus heteroclitus.* Anat. Rec. 117:558–559.

Morgan, T. H. 1895. The formation of the fish embryo. J. Morphol. 10:419–472.

Oppenheimer, J. M. 1936. Processes of localization in developing *Fundulus.* J. Exp. Zool. 73:405–444.

Oppenheimer, J. M. 1937. The normal stages of *Fundulus heteroclitus.* Anat. Rec. 68:1–15.

Pasteels, J. 1936. Etudes sur la gastrulation des vertébrés méroblastiques. I. Téleostéens. Arch. de Biol. 47:205–308.

Radice, G. P. 1980. The spreading of epithelial cells during wound closure in *Xenopus* larvae. Devel. Biol. 76:26–46.

Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. J. Cell Biol. 96:1–27.

Trinkaus, J. P. 1949. The surface gel layer of *Fundulus* eggs in relation to epiboly. Proc. Nat. Acad. Sci. U.S.A. 35:218–225.

Trinkaus, J. P. 1951*a*. A study of the mechanism of epiboly in the egg of *Fundulus heteroclitus.* J. Exp. Zool. 118:269–320.

Trinkaus, J. P. 1951*b*. Mechanism of periblast epiboly in *Fundulus heteroclitus.* Anat. Rec. 111:551.

Trinkaus, J. P. 1967. Procurement, maintenance and use of *Fundulus* eggs. *In* F. H. Wilt and N. K. Wessells (eds.), *Methods in developmental biology,* pp. 113–122. T. Y. Crowell Co., New York.

Trinkaus, J. P. 1971. Role of the periblast in *Fundulus* epiboly (in Russian). Ontogenesis 2:401–405.

Trinkaus, J. P. 1973. Surface activity and locomotion of *Fundulus* deep cells during blastula and gastrula stages. Devel. Biol. 30:68–103.

Trinkaus, J. P. 1980. Formation of protrusions of the cell surface during tissue cell movement. *In* R. O. Hynes and C. F. Fox (eds.), *Tumor cell surfaces and malignancy.* Progress in Clinical and Biological Research 41:887–906.

Trinkaus, J. P. 1982. Some thoughts on directional cell movements during morphogenesis. *In* R. Bellairs, A. Curtis, and G. Dunn (eds.), *Cell behaviour,* pp. 471–498, Cambridge Univ. Press.

Trinkaus, J. P. 1984. *Cells Into Organs. The forces that shape the embryo,* Second Revised Edition, pp. 543. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

Trinkaus, J. P. and C. A. Erickson. 1983. Protrusive activity, mode and rate of locomotion, and pattern of adhesion of *Fundulus* deep cells during gastrulation. J. Exp. Zool. 228:41–70.

Trinkaus, J. P. and T. L. Lentz. 1967. Surface specializations of *Fundulus* cells and their relation to cell movements during gastrulation. J. Cell Biol. 32:139–153.

Van Bameke, Ch. 1872. Premiers effets de la fécondation sur les oeufs de poisson: sur l'origine de la signification du feuillet muqueux ou glandulaire chez les poissons osseux. C. R. de l'Acad. des Sci. Tome LXXIV:1056–1060.

Van Haarlem, R. 1979. Contact inhibition of overlapping: One of the factors involved in deep cell epiboly of *Nothobranchius korthausae.* Devel. Biol. 70:171–179.

Van Haarlem, R. 1983. Early ontogeny of the annual fish genus *Nothobranchius*: Cleavage plane orientation and epiboly. J. Morphol. 176:31–42.

Wilson, H. V. 1889. The embryology of the sea bass (*Serranus atrarius*). Bull. U.S. Fish Commission IX:209–277.

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.