1968

Avian gastrulation: a fine-structural approach

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AVIAN GASTRULATION--A FINE-STRUCTURAL
APPROACH.

Iowa State University, Ph.D., 1968
Zoology

University Microfilms, Inc., Ann Arbor, Michigan
AVIAN GASTRULATION--A FINE-STRUCTURAL APPROACH

by

Nels Hamilton Granholm

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Zoology

Approved:

Signature was redacted for privacy.

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Ames, Iowa
1968
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The migration of cells is an important part of an animal's early embryology. After sperm and egg unite, the single-celled, fertilized egg (zygote) undergoes a series of cell divisions producing a multicellular embryo, a blastula. In many animals the blastula resembles a hollow sphere much like a tennis ball, with the embryo's cells or blastomeres providing the sphere's coat. However, in those animals whose egg has a yolk mass unequally distributed at one pole of the egg, the blastula is not a hollow sphere but rather a layered disc-like structure, the blastodisc. Once blastula formation has occurred, the tiny blastodisc is now ready to undergo a major transformation resulting in an embryo that begins to acquire characteristics of the adult. The process is gastrulation.

Gastrulation is the development of an ordered, triploblastic (having the three primary germ layers from which all definitive tissues are derived) embryo from a physically undifferentiated hollow sphere, or the formation of gastrula from blastula. This process is one of the earliest and most profound changes during animal development and is accomplished by the migrations of cells and tissues. More specifically, gastrulation is effected by the migration of certain presumptive organ-forming areas from the surface of the late blastula into the interior of the developing embryo.
Various patterns of cell movements observed during gastrulation in animal embryos have been given descriptive names. Some of these names include invagination, epiboly, emboly, immigration, concrescence, cell proliferation, polyinvagination, delamination, ingression, and others. Collectively these various types of cell migrations may be called gastrulation movements or more generally, morphogenetic cell movements, because they result in the production (generation) of new forms and structural arrangements. That morphogenetic cell movements are important in early development can hardly be doubted. They are largely responsible for the transformation of a one or two-layered undifferentiated blastula into a three-layered gastrula which demonstrates the adult's basic body plan. The surfaces of blastulae can be stained with "vital" dyes, and these stained areas may be followed as they migrate into the developing embryo's interior. The facts that patterns of migrations and internal destinations of blastula surface areas are regular and predictable suggest that morphogenetic cell movements are not random migrations of cells and tissues but highly ordered, directed movements. Yet the mechanism of these movements, and how these migrations are directed are not well known.

The avian blastula is a tiny two-layered disc-like structure resting on the surface of the yolk mass. The blastodisc or embryo of a freshly laid egg is about two
millimeters in diameter and consists of approximately 60,000
cells or blastomeres. In cross section the embryo is com-
posed of two undifferentiated epithelial layers separated
by a cavity.Apparently the bottom layer (hypoblast) is
"peeled off" or delaminated from the upper layer (epiblast)
while the egg is within the hen's oviduct. The space or
blastocoel between epiblast and hypoblast may be similar in
formation to the central cavity in spherically-shaped
blastulae. The hypoblast is equivalent to one of the three
primary germ layers--the entoderm, because this tissue gives
rise to the intestine and its various derivatives. The
epiblast contains the other two primary germ layers--ectoderm
and mesoderm.

Gastrulation movements in the avian embryo take two
basic forms. There is the migration of the area opaca
around the yolk (epiboly), and there is also the build up
of mesodermal cells beneath the primitive streak (invagina-
tion). This latter gastrulation movement provides the
embryo with its third primary germ layer--the mesoderm. The
understanding of those morphogenetic cell movements respon-
sible for the origin and production of the mesoblast, i.e.,
the way in which presumptive mesoderm is derived from the
epiblast, is the main emphasis of this study.

Avian gastrulation is mediated if not accomplished by
the primitive streak. The nature of the avian primitive
streak has long presented a problem for embryologists. It
functions either as an elongate blastopore (much like that of the amphibian embryo), as an active site of cell proliferation (blastema), or as a combination of both. Whether mesoblast cells originate as epiblast cells well removed from the neighborhood of the primitive streak which have migrated toward and through it, or epiblast cells of immediate origin (cell proliferation "in situ"), there is a net movement of epiblast cells out of the primitive streak. Initially, streak cells move into the mesoblast; secondarily, they migrate antero-laterally between epiblast and hypoblast layers. Thus, regardless of their origin, primitive streak cells migrate through the streak and into the mesoblast. It is the migration of the primitive streak cells and how this migration is accomplished that is the main question. Asked another way the question may state, "What is the mechanism of avian gastrulation?"

Avian gastrulation, like that of other animals, seems to involve some fundamental properties of cells. Some of these properties may be common to the mechanisms of all morphogenetic cell movements. These include motility, or the ability to move; selective adhesion, or the ability to make specific cell contacts at particular sites; cell-to-cell contact, or the ability to make and break cell attachments; and contractility, or the ability of a moving cell to produce contractile pseudopodia which may pull the cell in the direction of the adhesion. Another fundamental cell
property involved in the migration process may be the ability of a cell to regulate its shape.

The present study which deals with ultrastructural aspects of primitive streak cells in both the White Leghorn chicken (Gallus domesticus) and the Adélie penguin (Pygoscelis adeliae), contributes to an understanding of avian gastrulation. New information gained from this study includes a description of the different shapes of epiblast cells, the kinds of cell contacts and cell-to-cell attachments between invaginating epiblast cells, and the phenomena of pinocytotic vacuoles and cytoplasmic microtubules. This study presents a clear picture of primitive streak cell ultrastructure, and it discusses a mechanism of avian gastrulation in light of new information gained from the electron microscope.
Avian gastrulation has provided a stimulating and often controversial subject for nearly one hundred years. There are two major reasons why the literature is replete with information on avian embryology. There is the notion that existing phylogenetic schemes may be verified or disproven on the basis of an animal's embryology. And in years past, comparative embryologists attempted to find a basic or fundamental unit of animal gastrulation much as one tries to find the lowest common denominator in mathematics.

The infolding of one specific region of the blastula is basic to the gastrulation process and was given the name "invagination" (Haeckel, 1874, see translation, 1910, p. 123). The separation of cells and tissues was thought to be a fundamental process; hence the origin of the term delamination (Lankester, 1875). We now know that gastrulation is more than a simple invagination or delamination. But to define gastrulation in other than general terms is as foolish today as it was in the nineteenth century.

There are two periods during early bird development that may be thought of as periods of gastrulation. The first is the formation of the hypoblast or primary entoderm. The hypoblast, like the mesoblast, is apparently derived from the overlying epiblast. And a large portion of hypoblast development takes place in the hen's oviduct
prior to egg laying. The second period of avian gastrulation is the primitive streak stage, i.e., stages 2-4, 6-19 hours incubation (Hamburger and Hamilton, 1951). This review concentrates on the second period of avian gastrulation— the primitive streak stage.

Origin and Formation of the Primitive Streak

The concrescence theory of primitive streak formation was the major and most popular theory during the first part of the twentieth century. According to the concrescence theory, the primitive streak arises from the convergence of the blastoderm's margins toward the midline, i.e., the point where the blastodermal margins come or grow together. As early as 1876 Rauber (according to Romanoff, 1960) felt that the forerunner of the primitive streak is a crescent-shaped area in the postero-medial region of the area pellucida. The primitive streak is then derived from the convergence of the crescent's "tails" as the crescent's middle region moves in an anterior direction along the midline.

Pasteels (1937a) states that two types of cell movements occur in the area pellucida (embryo forming area of the blastoderm). One of these involves simultaneous movements of epiblast and hypoblast cells in a double vortex arrangement; the vortex on the right side of the presumptive primitive streak moves in a clockwise direction when viewing the streak from posterior to anterior, while that
vortex on the left produces cells moving in a counterclockwise direction. The second type of cell movement, which Pasteels termed "truly morphogenetic" is the movement of epiblast cells of the area pellucida from lateral positions toward the midline. Thus, Pasteels (1937a) suggested that the primitive streak develops from the invagination of epiblast cells which were originally located in the area pellucida some distance from the primitive streak. Wetzel (1929), stated that the primitive streak arises from the proliferation of cells "in situ". From the work of Pasteels (1937a, 1937b) and Wetzel (1929), one can discern the quiet beginnings of a recent embryological controversy on the origin and nature of the primitive streak. Is the primitive streak a "blastopore" or a "blastema"?

The immediate precursor of the primitive streak and thus of the avian embryo is a slight thickening in the blastoderm that occurs along the midline in the posterior region of the area pellucida. The streak first appears as an aggregate of densely packed cells in that same region. One critical point in the blastema versus blastopore controversy is the origin of these densely packed cells responsible for the observed linear thickening.

**Primitive streak as a "blastema"**

The word "blastema" indicates a structure having the potential to generate new cells. A healing wound producing
many cells during a regeneration process is a blastema; tissue giving rise to new offspring through the process of asexual reproduction may also be termed a blastema. The blastema theory of primitive streak origin and function considers the streak to be an active site of cell production. The formation and maintenance of the primitive streak depend upon the production of cells "in situ" in the "growth and movement center"--an area of high cell proliferation in the postero-medial part of the area pellucida (Spratt, 1963). Spratt and Haas (1965) have interpreted their experimental results as evidence for the blastema theory of primitive streak behavior. Using the techniques of vital staining and particle marking, the authors have marked virtually all areas of the chick blastoderm which are thought to migrate toward the streak and move through it. They conclude there is no concrete evidence for the involution of upper surface areas (epiblast cells) into a subsurface position, and they feel that the groove and folds of the primitive streak are the physical results of a rapid axial elongation of the streak while the blastoderm as a whole elongates more slowly. In regard to the role of the primitive streak Spratt and Haas (1965) state, "Our attempts to demonstrate unequivocally the movements of marked uppermost layer cells of either the early or definitive streak into middle or lowest layers of the streak have failed. It seems particularly improbable that any significant and systematic involution or invagination of upper surface areas could have escaped detection. The possibility of an
Ingression of single, scattered upon surface cells cannot be entirely excluded by the observations, but if it does occur, it would appear not to be an important mechanism for addition of cells to the middle or lowest layers."

**Primitive streak as a blastopore**

The word blastopore literally connotes a hole or pore through which embryonic cells migrate. Amphibian gastrulation is accomplished in part by the movement of presumptive entodermal and mesodermal cells from the blastula's surface through a pore into the embryo's interior. The avian primitive streak is thought by many to function as an elongated blastopore through which presumptive mesodermal and some entodermal cells invaginate.

Marking epiblast cells with vital dyes and following their movements in embryos of different ages is a technique which demonstrates movement of epiblast cells toward the primitive streak (Wetzel, 1929; Pasteels, 1937a; Malan, 1953; and Spratt, 1946). These movements of surface cells toward the primitive streak are termed "pregastrulation movements". Movements of cells from the streak to the interior of embryos have also been described (Pasteels, 1937a; Malan, 1953; and Hunt, 1937). Recently, a very thorough and well-documented study of Rosenquist (1966) on the transplantation of isotopically labeled grafts to unlabeled blastoderms has substantially reconfirmed the notion that the primitive streak functions as an elongate
blastopore. Rosenquist (1966) states,

"The experiments here reported show that there is a movement of epiblast toward the streak, invagination at the streak, and subsequent migration to both homolateral and heterolateral mesoderm, movements that are not compatible with the concept that the streak is a blastema but are compatible with the traditional view that the streak is a type of blastopore," and "Besides confirming in unequivocal fashion the invaginating movements that had been ascertained with vital dye staining and carbon marking methods by previous investigators, this method has permitted the movements of large areas of epiblast to be traced into the streak, where they descend ventrally and are distributed bilaterally in the entoderm and mesoderm."

Whether or not the primitive streak arises by the convergence and involution of epiblast cells, (blastoporal theory), or by the high mitotic activity of the "growth and movement center", (blastema theory, Spratt, 1963; Spratt and Haas, 1965), it appears that cells in the primitive streak are cells in motion moving at first into the mesoblast and then laterally and in an anterior direction between epiblast and hypoblast away from the unsegmented mesoblast.

Gastrulation Movements in Representative Animals

Early embryos of a number of different animals have been investigated in an attempt to discover fundamental aspects of gastrulation movements. The studies have been fruitful in clarifying some of the basic factors relevant to a generalized mechanism of gastrulation. Information gained from these studies pertinent to either avian gastrulation or to a generalized scheme of animal gastrulation
will now be discussed.

**Amphibian gastrulation**

Amphibian gastrulation has received much research attention and rightfully so. It is largely due to the efforts of Johannes Holtfreter that we know so much about morphogenetic cell movements. Holtfreter (1943a) felt the overall control in amphibian gastrulation was vested in a "surface coat", an extracellular substance which keeps the blastoporal cells tied together at their external ends. By uniting blastoporal cells, the "surface coat" allows a concerted invagination to take place. The existence of Holtfreter's "surface coat" seems doubtful, because electron microscope studies do not reveal its presence (Baker, 1965; Perry and Waddington, 1966). However the apparent behavior of a "surface coat", i.e., that of holding blastoporal cells tightly together, must have a physical basis. Tight cell-to-cell junctions at the apical (exterior) ends of invaginating amphibian blastoporal cells could be, in part, the physical basis of Holtfreter's "surface coat".

The recognition that flask or bottle-shaped cells are involved in amphibian gastrulation may also be attributed to Holtfreter (1943a, 1943b). Where bottle-shaped cells appear, the surface of the embryo is indented. Thus flask-shaped cells are involved in the invagination process, but is their involvement active or passive? Perhaps they are
responsible for initiating gastrulation, or perhaps they are just the passive by-products of the gastrulation process.

Studies on the mechanism of amphibian gastrulation have been profitable in identifying at least two of the important factors involved in morphogenetic cell movements. These are (1) specialized cell-to-cell attachments between migrating cells, and (2) the involvement of flask or bottle-shaped cells during the invagination process.

Echinoderm gastrulation

Echinoderm gastrulation is simply an invagination of one cell layer at the vegetal pole. There is neither an active growth nor an ingresslon of cells during the process. Gastrulation may be thought of as occurring in two parts. The primary phase begins the invagination with the archenteron tip reaching about one-third of the distance to the animal pole; the second phase involves elongate cytoplasmic processes or filopodia. As filopodia are spun out from mesenchyme cells of the archenteron tip, they attach to the inside of the blastocoelic wall near the animal pole and provide a contractile force pulling the archenteron tip and blastocoelic wall of the animal pole together (Gustafson and Wolpert, 1963). From studies on sea urchin gastrulation the importance of filopodia or similar cytoplasmic extensions in the movement of embryonic cells is obvious. The ability of a cell to "spin out" or produce
filopodia which attach to specific objects then contract, pulling the cell in the direction of the contraction, appears to be one of the basic ways in which cells of early embryos migrate. Contractile cytoplasmic extensions may well be one of the basic mechanisms in morphogenetic cell movements.

Avian epiboly

The incorporation of yolk into the embryo's body proper by the spreading of the area opaca around the yolk mass is a later gastrulation phase termed avian epiboly. The ability of the area opaca to spread over the yolk's surface is due to the presence and activity of certain marginal cells (New, 1959). An ultrastructural examination of these marginal cells (Bellairs, 1963) shows that each cell produces a long cytoplasmic process which extends out onto the yolk's surface, just beneath the vitelline membrane. The process may be 500 microns in length, 1/4 micron in depth, and may send projections upward into the overlying vitelline membrane. Bellairs (1963) states, "Cell processes probably pull the blastoderm by concerted amoeboid activity". The blastodermal margin adheres tightly to the overlying vitelline membrane as witnessed by anyone trying to dissect the vitelline membrane from the blastoderm. The two also remain connected through the rather arduous fixation and embedding procedure. Avian epiboly re-emphasizes the impor-
tance of cytoplasmic extensions and their apparent "contrac-
tile" behavior in morphogenetic cell movements.

**Teleost epiboly**

Gastrulation in teleosts is accomplished by a primary invagination of mesodermal and entodermal material into the interior of the developing embryo and by an epiboly of blastodermal cells around the yolk mass. Blastula cells invaginate over the lip of the blastodisc at one restricted area. This area may be considered a blastopore, since it appears to be the place where cells of the entoderm and mesoderm pass into the embryo.

Three separate components participate in epiboly of the teleost *Fundulus heteroclitus*. These include a superficial enveloping layer, a layer of deep blastodermal cells, and a syncytial layer on the surface of the yolk—the periblast (Trinkaus, 1965).

Teleost and avian epiboly are quite similar. Just as marginal cells of the avian blastoderm adhere to the under-
surface of the vitelline membrane, the teleost blastoderm appears to require the periblast as the surface over which it moves. The vitelline membrane's undersurface and the periblast's surface are examples of substrata. Another similarity between the two embryos is the importance of marginal cells during epiboly. If the contact between enveloping layer cells and periblast is severed in the
teleost embryo, epiboly is arrested and the blastoderm retracts (Trinkaus, 1966).

During the late blastula stage, just prior to the onset of epiboly, deep blastomeres are seen to produce lobopodia which secondarily develop into fan-shaped or filopodial-like extensions which connect to other cells near them. Upon lobopodial contraction, deep blastomeres move in the direction of the lobopodial adhesions.

Tissue culture studies on dissociated teleost embryos demonstrate that blastodermal cells behave differently at various stages of their development. Dissociated gastrula cells flatten rapidly to a glass surface, whereas dissociated blastula cells remain spherical showing little desire to flatten.

Cinematographic and fine-structural studies of teleost embryos reveal that deep blastomeres of the late blastula and early gastrula produce specialized lobopodia which adhere to other cells and contract; deep blastomeres of the 64-cell embryo (which is a much younger stage) show only small and unspecialized lobopodia (Trinkaus and Lentz, 1967). There is a time sequence of blastomere behavior; teleost blastomeres of a given age behave differently than those of earlier or later ages.

Studies on teleost epiboly demonstrate that the integrity of the contact between enveloping layer and periblast
layer is essential for tissue migration to take place. With the periblast acting as a substratum, marginal cells of the enveloping layer initiate the epiboly, while deep blastomeres with their lobopodial specializations provide the necessary momentum to move the bulk of the tissue around the yolk.

Comparisons and Conclusions

The examination of morphogenetic cell movements during amphibian gastrulation, echinoderm invagination, avian epiboly, and teleost epiboly demonstrates that those factors responsible for cell movements in one embryo may also be related to movements of cells in other embryos. By comparing the cell properties responsible for morphogenetic cell movements of one developmental system with those responsible for cell movements in others, one can list the cell properties which may be common to most morphogenetic cell movements in developing embryos.

A comparison of epiboly in chick and teleost embryos points out the involvement of the substratum. The undersurface of the avian vitelline membrane (membrane which covers the embryo and separates the white albumen from the yellow yolk) and the periblast (syncytial layer which rests directly on the yolk in teleost embryos) act as substrata in chicks and teleosts respectively. Substrata function as surfaces over which cells may move. Thus the relationship between moving cell and substratum is critically
important in the control of cell migration.

The phenomenon of surface adhesiveness is undoubtedly significant in cell movements. Surface adhesiveness may be defined as the degree to which one cell is attracted to another. Treating embryos with chemicals called dissociation agents causes the blastomeres to be split apart by disrupting their intercellular bonds. If the treatment is reasonably gentle, the blastomeres remain viable and can be kept in tissue culture where their behavior can be observed. Dissociation and tissue culture of teleost blastomeres shows that the degree of surface adhesiveness of the blastomeres is directly related to their age (Trinkaus, 1966). Early blastula cells act quite differently in tissue culture than do late blastula and early gastrula blastomeres.

Mesenchymal filopodia in sea urchins (echinoderms), lobopodia seen between the deep blastomeres of teleosts, and elongate (500 microns) cell processes of chick marginal cells may show one basic way in which embryonic cells move. Cells in motion appear to have the potential to elaborate cytoplasmic extensions. These pseudopodia, lobopodia, or filopodia then adhere to a specific object, contract, and draw the cell toward the point of adhesion.

Fine-structural investigations on teleost blastomeres (Trinkaus and Lentz, 1967) and on amphibian blastoporal cells (Baker, 1965; Perry and Waddington, 1966) demonstrate that specialized cell-to-cell attachments are involved and
Indeed necessary in the migration of cells. The apical contact zone, a specialized epithelial cell contact found in the tail epidermal cells of metamorphosing ascidians by Cloney (1966), has also been observed in the cells of the amphibian neural tube (Baker and Schroeder, 1967). It could be that specialized cell-to-cell attachments are the physical manifestation of Holtfreter's "surface coat". Thus morphogenetic cell movements appear to involve a number of cell properties. These include contractility, motility, differential surface adhesiveness, specialized cell-to-cell attachments, and the capacity of cells to change or regulate their shapes.

Tissue Culture Studies

After considering some of the morphogenetic cell movements associated with gastrulation in representative animals, one is able to list some cell properties that may be common to the basic process of cell migration. "In vitro" tissue culture studies allow the investigation of the same problem but from a slightly different perspective.

For many years people have been studying the behavior of cells in tissue culture and during cell dissociation-reconstitution experiments to discover those fundamental mechanisms that may be involved in the control of cell movements. Some of the phenomena which have received much attention include contact guidance, ruffled membranes, contact inhibition, and differential adhesiveness.
Contact guidance, an expression introduced by Paul Weiss (1961) points out the importance and influence of the substratum in the control of cell movements. Ruffled membranes are an important aspect of cells in motion. Apparently, ruffled membranes are the cell's "organ of motility" (Abercrombie, 1961; Ambrose, 1961). The various cytoplasmic extensions seen between migrating cells may well be the ruffled membranes of cells in tissue culture. Contact inhibition, an expression used by Abercrombie and Heaysman (1953, 1954), explains the fact that a moving cell in culture often stops moving upon contact with another cell. Differential adhesion is a concept attributed to Steinberg (1963) to explain why dissociated cells of different tissues tend to sort out randomly within cell aggregates and seek out identical cells (those derived from the same germ layer). There seems to be a "recognition of self" and the key to this recognition lies in the inherent differences of surface adhesiveness among cells. The excellent work of Townes and Holtfreter (1955) also demonstrates that cells of mixed aggregates tend to segregate according to type (germ layer) and tend to remould the aggregate in such a way that the various germ layer derivatives re-establish their positions relative to one another just like the definitive tissue or tissues from which the aggregates were made up.

The ultrastructural examination of teleost blastomeres
(Trinkaus and Lentz, 1967) and of avian primary mesodermal cells (Trelstad et al., 1967) now allows a correlation between the behavior of cells "in vitro" with the normal behavior of migrating cells during early morphogenesis. Avian mesodermal cells migrating away from the primitive streak produce lobopodia and filopodia which make contact with the epiblast's basement membrane (basal lamina), the hypoblast's surface, and other mesodermal cells themselves. Lesseps (1963) states that the cytoplasmic extensions observed in dissociated cells of eight day White Leghorn chick embryos are very much like fibroblast undulations reported by Ambrose (1961). The observed cytoplasmic extensions of embryonic cells may be the basis of "ruffled membrane" activity.

Avian Gastrulation--Epiblast Cell Invagination

Balinsky and Walther (1961) discuss the ultrastructure of primitive streak cells and present a theory on the mechanism of avian gastrulation. The migration of avian epiblast cells through the primitive streak is accomplished by a number of steps; these include a change in epiblast cell shape caused by the translocation of epiblast cell cytoplasm to the interior (away from the epiblast's free surface), loss of the "regular" epithelial-like connections between epiblast cells in the middle of the primitive streak (the active invagination zone), a further exaggeration of
cytoplasmic displacement causing the epiblast cell to assume a flask or bottle shape having as attenuated neck region, and the eventual withdrawal of the attenuated neck to a position beneath the epiblast's free surface (Balinsky and Walther, 1961).

The singular, most important aspect of the mechanism of avian gastrulation proposed both by Balinsky and Walther (1961) and by myself in this thesis is the necessity for the epiblast cell to undergo a change in shape. Balinsky and Walther (1961) state, "In the contracting outermost segment of the cell, it is sometimes possible to see strips of denser cytoplasm arranged transversely to the cell's long axis. Perhaps these strips represent a cytoplasmic pulling mechanism."

The idea that gastrulating cells may change shape by the contraction of a dense layer was suggested by Lewis (1947) who attributed invagination in the newt, Amblystoma punctatum to the contraction of a gel layer at the distal (outer) ends of the entodermal cells. Working with blastoporal cells of the treefrog Hyla regilla, Baker (1965) discusses the presence of a cortical "contracting-expanding dense layer" and postulates that this layer may be involved in the observed change in shape of the blastoporal cells as they migrate through the amphibian blastopore.

Lately, cytoplasmic filaments have been reported in cells undergoing a shape change, e.g. epidermal tail cells
in metamorphosing ascidians (Cloney, 1966) and cells of the neural tube of the treefrog (Baker, 1967). Cytoplasmic microtubular elements have also been observed in cells which are changing in shape, e.g. blastoporal cells of the newt (Perry and Waddington, 1966) and presumptive lens ectodermal cells (Byers and Porter, 1964). The suggestion is that these cytoplasmic elements, either filaments or microtubules, in some way influence the cell's shape.

The presence of vacuoles formed by cytoplasmic processes of two adjoining epiblast cells seems to be a regular feature of avian primitive streak cells (Balinsky and Walther, 1961; Ruggeri, 1967a). The vacuoles have a digestive function and contain ingested albumen (Ruggeri, 1967a). He postulates that within the vacuoles, water of the albumen is separated from its solid component, the water then being released into the intercellular spaces.

Trelstad et al. (1967) have completed a comprehensive study on cell contacts in the early avian blastoderm; they discuss the kinds and numbers of cell-to-cell attachments found in the primitive streak embryo and attempt to relate these to various tissue culture phenomena such as contact inhibition. For instance the authors state in their introduction,

"The occurrence of focal tight junctions (Farquhar and Palade, 1963, 1965) in migrating tissue is particularly interesting because of the possibility that tight junctions, acting as pathways of low
electrical resistance between cells (Potter et al., 1966; Sheridan, 1966) may mediate such phenomena as contact inhibition (Abercrombie and Heaysman, 1954) and thereby control the direction of tissue movements."

It is significant that positive correlations can be made between the behavior of cells "in vitro" with those in the living embryo. In this way, factors controlling cell movement may be elucidated.

This literature review shows that the origin of the avian primitive streak is still in doubt. Indeed, the function of the primitive streak is also in doubt; it acts either as a blastema or a blastopore. Perhaps it acts as a combination of the two—a "blastemopore". Studies on gastrulation movements in amphibians, echinoderms (sea urchins), teleosts, and birds demonstrate that morphogenetic cell movements are the expression of some basic cellular properties—motility, contractility, differential adhesion, and perhaps changes in cell shape.

"In vitro" studies present a number of phenomena which may be important factors in the control of cell movements. These include contact guidance, contact inhibition, and ruffled membranes.

Ultrastructural examinations of migrating cells during gastrulation have presented additional information regarding such concepts as cell migration and cell-to-cell attachments; they also allow correlations to be made between cell behavior "in vitro" with that "in vivo".
MATERIALS AND METHODS

Eggs of the White Leghorn chicken (*Gallus domesticus*) and Adélie penguin (*Pygoscelis adeliae*) were employed in this study. Chick eggs were artificially incubated at 99-100 degrees F. (37.2-37.8 degrees C.) for 12-18 hours (stages 3 and 4; Hamburger and Hamilton, 1951), while eggs of the Adélie penguin were incubated naturally until they reached the intermediate primitive streak and definitive primitive streak stages, i.e. until they resembled 12-18 hour incubated chick embryos.

Entire blastoderms were cut off the yolk's surface and immediately placed in a variation of avian's solution of Spratt (1947); it consists of 9.0 grams of sodium chloride, 0.42 grams of potassium chloride, and 0.25 grams of calcium chloride in one liter of distilled water. Within this solution vitelline membranes were dissected from the embryo's surface, and much of the adherent yolk was removed by rinsing.

Blastoderms were then transferred by pipette to an ice cold 1.2 percent glutaraldehyde solution containing a phosphate buffer (Sabatini et al., 1963) for 30-60 minutes. Following the notion that highly-hydrated embryonic tissue is preserved better at alkaline pH values, the pH was elevated to 7.6. As blastoderms are transferred to the fixative, gentle manipulation of the blastoderms with glass
needles' keeps them from rolling up.

After the initial fixation blastoderms are rinsed in the phosphate buffer and postfixed in cold 2.0 percent osmium tetroxide buffered with the same phosphate buffer (Millonig, 1962) at pH 7.8 for 20 minutes. Following the postfixation the embryos were again rinsed in pure buffer and placed in 30 percent ethanol.

Further dissection of the embryos while they are in the lower alcohols facilitates orientation of the tissue during the embedding procedure. Glass needles were used to remove all yolky fragments and as much of the area opaca as possible, leaving behind only the area pellucida. The embryos were then dehydrated in a graded series of alcohols (ethanol), saturated with propylene oxide, and flat embedded in an epoxy resin. The embedments used were araldite (after the modification of Luft, 1961), maraglas (Spurlock et al., 1963), epon (Luft, 1961), and an epon-araldite mixture. Blocks were sectioned on a Reichert ultramicrotome, sections picked up on 200-mesh parlodion coated grids, stained in uranyl acetate and lead citrate (Venable and Coggeshall, 1965), and viewed with RCA-EMU2-a and RCA-EMU3-f electron microscopes. Thick sections taken at 0.5-1.0 micron were photographed using a Wild phase microscope.

Other fixation and embedding techniques for embryonic and definitive chick tissues are outlined by Wilt and Wessels (1967). A dual fixation method for early chick
tissue employing cacodylate buffered glutaraldehyde and collidine buffered osmium tetroxide (Trelstad et al., 1967) yields very good tissue preservation.

In general I was satisfied with the preservation obtained by fixing avian embryos in phosphate buffered glutaraldehyde and phosphate buffered osmium tetroxide. Of the embedments used, a hard epon mixture (2B:1A) gave the most satisfactory image but was extremely difficult to section with glass knives. An epon-araaldite mixture provided good preservation and was suitable for sectioning.
RESULTS

The avian primitive streak embryo (stages 3 and 4; Ham­
burger and Hamilton, 1951) is little more than a tiny disc
composed of two layers having a space between them. The most
striking feature of this embryo is an elongate groove or
depression called the primitive streak which establishes the
anterio-posterior axis of the future adult.

The general architecture of the primitive streak embryo
(Figure 1)* is that of a bilaminar blastoderm composed of
two well-defined epithelia--an upper epiblast and a lower
hypoblast. Between these epithelia there exists a loose
meshwork of stellate-shaped mesodermal cells. The anterior
extension of the primitive streak is termed Hensen's node,
and the anterio-posterior depression in the blastoderm is
called the primitive groove. The embryo proper is confined
to a spherical and later pear-shaped area which has a
slightly clearer appearance than surrounding tissue. This
clearer appearance, due to the intervening space between
epiblast and hypoblast epithelia, bestows the name area
pellucida to the area of embryonic development. The surround-
ing tissue which rests directly on the yolk's surface with
no intervening space takes on a more opaque appearance and is
termed the area opaca.

*For all figures, see the end of this chapter.
The epiblast consists of a single layer of low columnar epithelial cells peripherally, but this layer gently merges into a four or five layered pseudostratified epithelial area in the middle of the primitive streak. In this region (the middle of the primitive streak) the cells at the epiblast's free surface are no longer exclusively columnar in shape; some have become cuboidal, some pyramidal, and others bottle-shaped. It is this pseudostratified area in the middle of the primitive streak where the invagination of epiblast cells may occur and thus, where the mesoderm has its origin. In this area all three primary germ layers—ectoderm, entoderm, and mesoderm—are continuous with one another.

Light Microscopy

Blastoderms of the White Leghorn chicken-*Gallus domesticus* and Adélie penguin-*Pygoscelis adeliae* are shown in the light photomicrographs of Figure 2. The blastoderms demonstrate their bilaminar nature with very thin entodermal epithelia and relatively thicker ectodermal epithelia (epiblasts). One observes that the regular columnar epithelium of the epiblast is lost in the area of the primitive streak. The mesodermal mass has the appearance of being derived from cells of the epiblast which flow ventrally into the mesoblast and laterally between epiblast and hypoblast. When viewed with the light microscope, cells
of the avian blastoderm possess relatively large nuclei and many yolk spheres or platelets distributed throughout the cytoplasm. Of the cytoplasmic constituents of blastoderm cells little more than yolk platelets and nuclei can be resolved with 0.5-1.0 micron, thick sections viewed with phase optics of the light microscope.

Epiblast cells at their free surface are joined together tightly showing little or no intercellular space between apposing membranes (Figure 2D; Figure 2E). These tight junctions located between the apical ends of epiblast cells give way ventrally to looser cell-to-cell attachments, than to large intercellular spaces. Stellate-shaped mesodermal cells are arranged in a loose meshwork showing very pronounced intercellular spaces (Figure 2B; Figure 2D). Many mesodermal cells form long finger-like cytoplasmic projections (F, Figure 2D) called filopodia which may connect them to the base of the epiblast, the hypoblast surface, or to other mesodermal cells in their immediate environment. At times these cytoplasmic extensions may even connect the same mesodermal cell to both epiblast and hypoblast simultaneously.

Light photomicrographs of the avian primitive streak also reveal the presence of many flask or bottle-shaped cells (B, Figure 2E). Bottle-shaped cells seem to be most numerous in that region of the primitive streak where invagination of
epiblast cells appears to be taking place. One is able to observe varying degrees of "bottleness" within the primitive streak; some of the cells possess neck regions which are more attenuated or drawn out than others. Although the center of the primitive streak is marked by the base of the primitive groove, it appears that epiblast cell invagination occurs some distance to one side and the other from the exact center of the streak, and this area may be termed the "active invagination zone" (AIZ, Figure 2B).

Electron Microscopy

A section cut transversely through the primitive streak (see Figure 1 for orientation) sections cells of the blastoderm in longitudinal planes. Electron micrographs (Figures 3, 4, and 7) demonstrate what is observed when transverse sections are made through the exact middle of the primitive streak; one sees the very bottom of the primitive groove, borders or margins of the primitive groove sloping upward on both sides of the primitive groove, and those epiblast cells which have their apical ends or free surfaces at the base of the primitive groove. It is these epiblast cells that we want to examine critically, because they are the ones that may actively be leaving the epiblast to become part of the mesoblast during epiblast cell invagination.

Primitive streak cells (epiblast cells) as well as other cells of the early avian blastoderm are characterized
by a great abundance of cytoplasmic constituents. The most obvious cytoplasmic constituents are the yolky intermediates, lipid droplets, various inclusions, and an array of membranous organelles. One readily identifiable yolky body called a granular yolky intermediate (Gr, Figure 3) has a dense core surrounded by a less dense region; the diameter of the whole structure varies from 1-3 microns. Clearer 1-3 micron agranular spheres are also dispersed throughout the cytoplasm. These are lipid droplets. Sometimes they have a uniform appearance (L, Figure 3), but at other times they demonstrate a striped appearance (Figure 4) with alternating clear and darker stripes. This "washboard" effect may be due to the fragility of lipid droplets, an unequal degree of osmium fixation throughout the droplet, and a physical formation of waves in the droplets caused during the sectioning procedure. The "washboard" effect is the rule rather than the exception when working with particles or droplets with a high lipid content.

Yolk granules and lipid droplets have no definite arrangement in the cell but are found randomly dispersed throughout the cytoplasm. At times these inclusions occur in such high concentrations that they cause the nuclear membrane to be deformed and inpocketed by their presence. Other cytoplasmic inclusions and membranous organelles are present in cells of the early blastoderm; their various aspects will be discussed later.
Cells of the primitive streak have different shapes. The theory on the mechanism of epiblast cell invagination presented in this paper depends upon the change in cell shape as a necessary requisite for invagination. In the light photomicrographs (Figure 2C; Figure 2E) one observes that cells of the epiblast possess many shapes. A variety of cell shapes may also be observed at the fine-structural level (Figures 3 and 5). Of the epiblast cells bordering the primitive pit (Figure 3), the attenuated neck of one bottle-shaped epiblast cell is seen adjacent to a cuboidal-like cell on one side and a more columnar-like cell on its other side. Epiblast cells (Figure 4) show slightly different shape variations with the majority of the cells beneath the primitive groove in a drawn out state. An elegant attenuated neck of an epiblast cell (Bot, Figure 5) is bordered on one side by a cuboidal-like cell and on the other by a less attenuated, nucleus-containing neck region of a columnar-like epiblast cell. These micrographs and others demonstrate that the variations in cellular shape observed at the light microscope level can also be detected at the ultrastructural level.

Certain epiblast cells detach themselves from the epiblast surface and migrate into the mesodermal mass. Prior to their detachment these epiblast cells become bottle-shaped with long attenuated necks which remain attached to
the epiblast's surface. After the necks of these flask-shaped cells become stretched or drawn out beyond a certain critical limit, they detach themselves and sink beneath the epiblast's surface. Some of these attenuated necks of epiblast cells (W, Figures 3 and 4) are no longer connected to the epiblast's free surface but have withdrawn beneath it. Apparently, some epiblast cells are actively withdrawing from the epiblast and entering the mesoblast. Cells which have withdrawn from the epiblast's free surface usually have a bottle-like shape. Factors responsible for the origin of bottle or flask-shaped cells will be discussed later.

**Cell-to-cell attachment**

During avian gastrulation epiblast cells in migration must initially break their old epithelial attachments, continually establish and break cellular contacts during their migration, and establish permanent attachments at their final destinations. A study on the mechanism of epiblast cell invagination must include an investigation on the nature of epiblast cell-to-cell attachments.

Contacts between cells of avian blastoderms show some variation. The variations observed in the domestic chicken are also seen in the Adelie penguin. There exist both zonulae occludentia and zonulae adhaerens (Farquhar and Palade, 1963, 1965) as well as combinations of these two termed "junctional complexes". A specialized type of
junctional complex is termed an "apical-subapical contact zone" (Cloney, 1966).

Tight junctions or zonulae occludentes, characterized by a fusion of apposing membranes resulting in the occlusion or obliteration of the intercellular space, are encountered between epiblast cells at their apical-most ends (the epiblast's free surface). In the chick embryo of 12-19 hours incubation and in the primitive streak embryo of the penguin, tight junctions near the epiblast's free surface are "focal" in nature, i.e., they occur over a very short linear distance, 1000 A or less, and should be called focal or minute tight junctions (MTJ, Figure 6B). These minute tight junctions occur predominately between primitive streak cells at the epiblast's free surface, and are associated with another specific type of cell-to-cell attachment called a close junction (CJ, Figure 6B).

Close junctions, also known as intermediate junctions and zonulae adherentes (Farquhar and Palade, 1963, 1965), are characterized by an intercellular space of 100-200 A, and a strict parallelism of the apposing cell membranes over a substantial linear distance which may be up to a micron in length (Figures 6B and 7). Close junctions compose the majority of cell contacts in the early avian blastoderm; they are found at cell junction of the epiblast's surface, as connections between mesodermal cells, as contacts between
cells of the hypoblast, and also between cytoplasmic extensions (lobopodia and filopodia) of mesodermal cells and the basal borders of both epiblast and hypoblast. When minute tight junctions associate with close junctions (and this is the general rule between apical ends of epiblast cells in the middle of the primitive streak), their specialized association is termed a "junctional complex."

At the epiblast's free surface in the middle of the primitive streak there exists a "simple junctional complex" which consists of one or two minute tight junctions in association with a close junction over a linear distance of 0.25-1.0 micron (SJC, Figures 3 and 7). Another type of junctional complex observed in the early avian embryo has also been observed in ascidians (a group of Protochordates); this "apical-subapical contact zone" (Cloney, 1966) has been observed in contracting epidermal cells of resorbing tails during ascidian metamorphosis. The apical contact zone (ACZ) is approximately 1-2 microns long with the limiting membranes of adjacent cells forming a series of minute tight junctions which alternate with close attachments showing an intercellular space of 100-200 A. Deeper into the epiblast cell the adjoining membranes follow a strict parallel course with the overall junction becoming winding and toruous. The intercellular space remains constant at 150-200 A and no minute tight junctions are evident. This deeper continuation of the junctional complex characterized by parallel
membranes and the absence of minute tight junctions is called the subapical contact zone (SCZ, Cloney, 1966). The apical-subapical contact zone (ASCZ, Figure 8) seem to occur lateral to the exact middle of the primitive streak and at the apical-most ends of the epiblast cells. The various junctional complexes give rise ventrally to rather large intercellular gaps or spaces which may be as great as 1-2 microns. The light photomicrographs (Figure 2) show the general structure of cell contacts in the primitive streak. Apical ends of epiblast cells are tightly joined together, while their basal ends and cells beneath them demonstrate a marked relaxation of cell bonds and a great abundance of intercellular space.

**Apical vesicles**

A low power electron micrograph of the epiblast's free surface reveals a definite pattern of surface evaginations. These evaginations appear to be vacuolate in nature, or variable size, and lie across apical junctions of neighboring cells. Since the vacuoles seem to originate by fusion of cytoplasmic processes from two adjoining epiblast cells, and since they come to lie over cell junctions, they may be referred to as junctional vacuoles (JV, Figure 9).

Another series of apical vacuoles may arise on the surface of epiblast cells in between the junctional vacuoles and independent of them. These may be referred to as inter-
Junctional vacuoles (IV, Figures 6A and 9). Both junctional and interjunctional vacuoles may be pinocytotic in nature. Occasionally an extremely large vacuole is present at the apical-most end of penguin epiblast cells just beneath the primitive groove (V, Figure 7). Possible functions of the vacuoles during epiblast cell invagination are discussed later.

**Cytoplasmic extensions**

Cells of all three germ layers of the early avian embryo possess cytoplasmic extensions; they are very reminiscent of the pseudopodia of amoebae. Depending upon their diameter and length, cytoplasmic extensions may be termed lobopodia or filopodia. Those having a relatively large diameter and short length are termed lobopodia, while those of a small diameter and longer length may be called filopodia. Light photomicrographs of the avian primitive streak demonstrate numerous long, finger-like filopodia extending from cells in the middle of the primitive streak to other cells around them (F, Figure 2D). Filopodia appear to function in the migration process. An epiblast cell may show a large number of interdigitations by sending out many filopodia (Figures 4 and 10) which make contact with either the filopodia of other cells or with the main body of other cells. An epiblast cell may also establish contact with adjacent cells by simply moving close to the cell in question and
making contact over a large surface area without the help of filopodia. Cells in the epiblast demonstrate both these types of connections simultaneously. Cells in motion within the mesoblast tend to have the filopodial-like connection predominately.

**Extracellular material**

There are a number of extracellular factors that are relevant to a consideration of avian gastrulation. The epiblast's surface is bathed in a fluid rich in albumen. The surface vacuoles (JV and IV) mentioned above may be involved in the incorporation of that albumen into the cytoplasm of the epiblast cells.

A supracellular "surface coat" may be secreted by epiblast cells and be disposed on the epiblast's surface. A "surface coat" was thought to be responsible for the coordination of entodermal cell invagination in the amphibian blastoporal region (Holtfreter, 1943a). However, in the present study, there is no concrete evidence for the existence of a "surface coat".

A common extracellular constituent of an epithelium is the basement membrane. The basement membrane is an amorphous, fibrillar material closely conforming to the basal border of the epithelium. A basement membrane (basal lamina) consisting of a 250-1000 A amorphous layer has been reported beneath the epiblast in slightly older avian embryos by two
authors (Trelstad et al., 1967; Ruggeri, 1967a). It was not observed in this study, because the area just beneath the primitive pit is not a well-defined epithelium but an area of cell migration.

A variety of extracellular material is seen within the primitive groove (Figures 3, 4, and 7). Included in this material are fragments of complex yolk particles, yolk granules, cell fragments, solid albumen, and other osmium-positive particles. Although some of this material may be present in the primitive groove of the embryo "in vivo", it is difficult to determine which components are normally present there and which components are artifact due to the handling of the embryo during dissection, fixation, and embedding. However the consistent discovery of material in the primitive groove does suggest that epiblast cells are migrating from peripheral positions of the area pellucida toward and through the streak bringing with them some debris.

Material within the intercellular spaces between epiblast cells, although appearing amorphous, has both a liquid and solid component (Figures 4, 6B, and 7). The origin and function of these components during early avian development will be discussed later.
Organelles and inclusions of epiblast cells

Epiblast cells contain many cytoplasmic components both membranous and particulate in nature. Cells of the early avian embryo are "undifferentiated" in regard to cellular specialization. However an examination of their constituents reveals the presence of many specialized organelles. Many of these organelles we associate with those later developmental events in embryogenesis termed histological differentiation. Thus cells of the early avian blastoderm, although structurally unspecialized, appear to be actively involved in "pre-differentiation" preparation.

Some of the components of epiblast cells may be associated with protein and lipid metabolism. Small particles less than 200 A in diameter are present in the ground plasm; these resemble free ribosomes. Membranous components of the endoplasmic reticulum are scarce and difficult to find. There are many elaborate and rather well-defined Golgi bodies (Gol, Figure 11) in the cytoplasm of epiblast cells. Indeed, the consistent discovery of Golgi complexes within the apical portions of the cell suggests that a regular "Golgi zone" exists within most epiblast cells.

Cell components which may be utilized as energy sources are also present in the cytoplasm of epiblast cells. These include yolky intermediates, 150-500 A glycogen particles found either as individuals or together in apparent rosette configurations (G, Figure 12A), and lipid droplets of vari-
able size. At times mitochondria may be seen almost completely surrounding lipid droplets. Since the major enzymes associated with triglyceride metabolism have been isolated from mitochondria, it is likely that lipid droplets in the early avian embryo are being utilized as an energy source.

Cytoplasmic microtubules are present in primitive streak cells of the avian blastoderm. These microtubules are straight, of indefinite length, and possess an overall diameter of 200-250 Å; they have a center of lower density than the surrounding core which gives them a hollow appearance. Apparently cytoplasmic microtubules of primitive streak cells are capable of originating in the cytoplasm, growing out or originating from a given point. They may be arranged or disposed in the epiblast cell in two, basically different ways.

Transverse microtubules (Tmt) are found running parallel to the epiblast's free surface a short distance beneath it (Tmt, Figure 7). Sections taken transverse to the primitive streak show these microtubules in cross section. They have an overall diameter of 200-225 Å and are consistent in their orientation, i.e., they are almost always found just beneath the epiblast's free surface running parallel to it. These microtubules do not occur in bundles or in any close-knit organization. They occur only as solitary tubules.

Another basically different arrangement of cytoplasmic microtubules is encountered within epiblast cells which are
either undergoing invagination or are in the final pre-invagination stages. These microtubules are arranged perpendicular to those described above and are disposed in a longitudinal direction. Longitudinal microtubules (Lmt) possess a definite organization which is sometimes encountered within the flask-shaped attenuated necks of epiblast cells (area enclosed within rectangle, Figures 10 and 12). These microtubules are also of indefinite length and are usually associated in bundles. Tracing them in an apico-basal direction, they appear to end blindly in the broadened basal portion of the same cell. They tend to be concentrated in the apical portions of epiblast cells.

Primitive streak cells are undergoing mitosis, and it is possible that some of the longitudinally arranged microtubules may be spindle fiber remnants of the mitotic apparatus. However, the position of these bundles in the most apical portion of epiblast cells (Lmt, Figure 12B) with no cell directly above, suggests that these bundles are not the remnants of an old mitotic apparatus. The presence, role, and significance of microtubules during avian gastrulation are discussed later.
Figure 1. Primitive streak embryo
    Anterior portion of the area pellucida of the avian primitive streak embryo sectioned transversely. The blastoderm consists of two epithelial layers interspersed with mesodermal tissue.
Hensen's node
primitive streak
epiblast
edge of area pellucida
mesoblast
hypoblast
migrating mesodermal cells
Figure 2A. Definitive primitive streak embryo
Chick definitive primitive streak stage showing the three major tissue zones making up the early avian embryo. Cells of the epiblast (E) are continuous with those in the mesoblast (M). The hypoblast (H), a thin single-celled epithelial layer, runs beneath the mesoblast. Light micrograph. (x490)

Figure 2B. Definitive primitive streak embryo
Chick definitive primitive streak stage showing the active invagination zone (AIZ), where cells of the epiblast appear to be migrating into the mesoblast. Light micrograph. (x340)

Figure 2C. Intermediate primitive streak embryo
Penguin intermediate primitive streak stage showing that the primitive streak (PS) is not well-defined. Mesoblast cells beneath the primitive groove demonstrate characteristic lateral migrations. Light micrograph. (x640)

Figure 2D. Definitive primitive streak embryo
Chick definitive primitive streak stage showing a well-defined primitive groove and many pseudopodial connections called filopodia (F) between cells of the mesoblast. Light micrograph. (x680)

Figure 2E. Definitive primitive streak embryo
Chick definitive primitive streak stage showing three bottle-shaped epiblast cells with long, attenuated neck regions (B). Light micrograph. (x800)
Figure 3. Chick primitive groove

Transverse section through the primitive streak similar to that shown in Figure 1. Epiblast cells are sectioned in longitudinal planes. The base of the primitive groove (P) is shown in the upper right. Simple junctional complexes (SJC) characteristic of epiblast cells in the middle of the primitive streak, are present and give rise ventrally to larger intercellular spaces. Epiblast cells contain many granular yolkic intermediates (Gr) and lipid droplets (L). Three epiblast cells bordering the base of the primitive groove display different shapes. A bottle-shaped cell (Bot) is bordered on one side by a cuboidal-shaped cell (Cub) and on the other by a columnar-shaped cell (Col). A withdrawn attenuated neck of an epiblast cell (W) is also present. Electron micrograph. (x12,800)
Figure 4. Chick primitive groove

Transverse section through the primitive streak at the base of the primitive groove (P). Cells in the streak are sectioned in a longitudinal plane. Both granular yolky intermediates (Gr) and lipid droplets (L) showing the "washboard" effect are present. Numerous long cytoplasmic extensions or filopodia are seen to connect epiblast cells beneath the primitive groove. One epiblast cell (W) appears to have withdrawn from the epiblast's free surface. Osmium-positive yolky intermediates and cell fragments are present in the primitive groove. Solid material (S) within the intercellular spaces is also present.

Electron micrograph. (x10,900)
Figure 5. Chick epiblast
Longitudinal section through epiblast cells just lateral to the primitive groove at the epiblast's free surface (ES). A bottle-shaped epiblast cell (Bot) is bordered on one side by a cuboidal epiblast cell (Cub) and on the other by the nucleus-containing neck region of a columnar epiblast cell (Col). A series of minute cell contacts are present between the attenuated bottle cell (Bot) and those cells adjacent to it. Electron micrograph. (x11,200)

Figure 5. (inset) Attenuated neck region
Longitudinal section through the neck region of bottle-shaped epiblast cell (Bot, Figure 5) showing longitudinally oriented microtubules (mt). Electron micrograph. (x46,000)
Figure 6A. Penguin epiblast
Transverse section through the penguin primitive streak just lateral to the primitive pit. The epiblast's free surface is seen in the upper left as are many surfaces vacuoles. The general organization of the epiblast is seen. Cuboidal epiblast cells possess basal nuclei with prominent nucleoli. Cells are tightly joined at the epiblast's free surface but give rise ventrally to larger intercellular spaces. Electron micrograph. (x2,500)

Figure 6B. Chick cell-to-cell attachments
Apical portion of an epiblast cell showing the cell junctions characteristic of the apical-most end of epiblast cells within the primitive streak. Minute tight junctions (MTJ), and close junctions (CJ) are observed. A combination of one or two minute tight junctions in association with a close junction is referred to as a "simple junc­tional complex". A granular yolky intermédia­te (Gr), mitochondria (M), solid material of the intercellular space (S), and an intercellular vacuole (V) are also present. Electron micrograph. (x46,000)
Figure 7. Penguin primitive groove
Transverse section through the primitive streak at the base of the primitive groove (P). Epiblast cells are sectioned longitudinally. Transverse microtubules (Tmt) are present just beneath the epiblast's free surface. Simple junctional complexes (SJC) characteristic of the apical ends of epiblast cells in the streak center are present. The apical end of one epiblast cell contains a large vacuole (V). Lipid droplets, mitochondria, and solid components of the intercellular material (S) are also seen. Electron micrograph. (x24,800)
Figure 8. Chick epiblast

Longitudinal section through the chick epiblast just lateral to the primitive groove. An apical-subapical contact zone (ASCZ) which may be characteristic of peripheral epiblast cells is seen at the epiblast's free surface (ES). Electron micrograph. (x16,800)
Figure 9. Penguin epiblast
Transverse section taken just lateral to the primitive groove in the penguin primitive streak embryo. Junctional vacuoles (JV) and inter-junctional vacuoles (IV) are seen at the epiblast's free surface (ES). Intracellular vacuoles (V) appear to contain the same material that bathes the epiblast's surface. Lipid droplets (L) are also present. Columnar epiblast cells contain an abundance of Golgi and mitochondrial material just distal to their large basal nuclei (N). Cell membranes can be traced deep into the epiblast from junctional vacuoles (JV) at the epiblast's free surface. Electron micrograph. (x7,200)
Figure 10. Chick epiblast

Longitudinal section of epiblast cells just beneath the base of the primitive groove. One epiblast cell has a long attenuated neck (W) which appears to have withdrawn from the epiblast's surface. Large intercellular spaces exist between epiblast cells. There are numerous point contacts between cells as well as longer, more permanent cell-to-cell attachments. Epiblast cells in this region appear to be migrating into the mesoblast below. The attenuated neck region within the rectangle is shown at a higher magnification in Figure 12A. Electron micrograph. (x10,900)
Figure 11. Chick epiblast cell, Golgi zone
Longitudinal section of the apical portion of an epiblast cell at the epiblast's free surface (ES). Three well-defined Golgi complexes (Gol) as well as numerous mitochondria are located in the cytoplasm between the nucleus and the epiblast's free surface. In the upper right, there is a specialized cell-to-cell attachment termed a "simple junctional complex". Electron micrograph. (x28,000)
Figure 12A. Longitudinal microtubules, chick
Longitudinal section of the attenuated neck region of an epiblast cell (part of the region enclosed within the rectangle of Figure 10) showing large intercellular spaces (IS) and microtubular elements (Lmt) having a 200-250 Å diameter. Densely staining particles within the microtubular array appear to be glycogen granules (G) and free ribosomes. Solid components of the intercellular material (S) are also present. Electron micrograph. (x58,000)

Figure 12B. Longitudinal microtubules, penguin
Longitudinal section of a long attenuated neck of a penguin epiblast cell which is just beneath the epiblast's free surface. The large intercellular space (IS) narrows becoming a close junction as one moves ventrally. Longitudinal microtubules (Lmt), 200-250 Å in diameter, extend from basal portions of this cell to an apical vacuole (not pictured). Electron micrograph. (x58,000)
Figure 13. Avian primitive streak cells
Semidiagramatic representation of invaginating primitive streak cells. A sequence of epiblast cell shapes is shown. Peripheral epiblast cells are cuboidal or low columnar in shape (1). Columnar-like epiblast cells (2), elongate columnar cells (3), and flask-shaped epiblast cells (4) are shown. One epiblast cell (5) has withdrawn from the epiblast's free surface. Notice the specialized cell-to-cell attachments at the epiblast's free surface, the concentration of Golgi material in the cells, the parallel alignment of the cytoplasmic components within bottle-shaped cells, and the cytoplasmic microtubules within bottle-shaped epiblast cells.
DISCUSSION

The present study, which supports the notion that cells of the epiblast leave the primitive streak and join the mesoblast, develops a theory of avian gastrulation based largely on the discovery of new structures within epiblast cells. This study, however, sheds little light on the blastema-like or blastopore-like nature of the primitive streak. Of these two schools, comprehensive evidence presented by Nelsen (1953), Romanoff (1960), Balinsky (1965), and Rosenquist (1966) on the nature of the primitive streak strengthens the traditional view that the primitive streak functions as an elongate blastopore.

A brief synopsis of the hypothesis of avian gastrulation is presented first in the discussion. This is followed by a consideration of those results pertinent to the general hypothesis. The discussion is then concluded by a comprehensive description of the mechanism of avian gastrulation.

Synopsis of Hypothesis of Avian Gastrulation

The freshly-laid avian egg contains a flat, two-layered, disc-like embryo. After 6-8 hours incubation the primitive streak begins to form. Epiblast cells in the middle of the primitive streak probably undergo a change in shape by becoming flask- or bottle-shaped and lose their columnar-cuboidal epithelial appearance. As greater numbers of primitive streak cells become bottle-shaped, the primitive groove
becomes deeper and the primitive folds more pronounced. Due to the change in shape, cytoplasm within bottle-shaped epiblast cells may be redistributed to more basal (interior) portions of the cell. Streaming of cytoplasm toward the basal poles of epiblast cells causes further attenuation or stretching of the neck regions. When this attenuation reaches a certain critical limit, cell junctions at the apical (exterior) ends of epiblast cells are ruptured, and the attenuated necks sink below the epiblast's free surface.

During this period of avian gastrulation, migrating epiblast cells produce many cytoplasmic extensions which make contact with the cells around them. These pseudopodial-like extensions, termed lobopodia and filopodia, continually make and break contact with cells around them. They not only anchor epiblast cells as they undergo shape changes, but also may "pull" epiblast cells through the primitive streak into the mesoblast. Once primitive streak cells have broken from the epiblast's free surface, these "contractile" pseudopodia allow the invaginated epiblast cell to migrate into the mesoblast and eventually into the somite region. Cells which leave the epiblast's surface to join the mesoblast are replaced by epiblast cells which have migrated laterally to the primitive streak from peripheral positions of the area pellucida. Peripheral epiblast cells possess stronger cell-to-cell attachments than primitive streak cells. These tight attachments may be partially
responsible for the migration of peripheral epiblast cells to the streak.

Thus the postulated mechanism of avian gastrulation depends upon a change in shape of the epiblast cell which allows it to "slip" through the streak and into the mesoblast. Numerous epiblast pseudopodia and specialized cell-to-cell contacts aid the invagination and ensure that new epiblast cells are moved into position for their eventual invagination.

Differences Between Chick and Penguin Blastoderms

The early embryos of two rather different birds were used in this study because of the possibility that differences in the evolutionary history of these two birds could manifest themselves in their early embryology. By comparing the early embryology of these two birds one may gain more insight into the nature of the avian primitive streak.

The Adélie penguin (Pygoscelis adeliae) of the Order Sphenisciformes has a different evolutionary history than the White Leghorn chicken (Gallus domesticus) of the Order Galliformes. In fact, the evolutionary history of penguins is very much in doubt. One theory states that penguins once could fly but have now lost the ability. Another maintains that penguins are primitive birds and represent a "living 'missing-link' between modern birds and their flightless ancestors" (Euller, 1960). According to the embryological maxim "ontogeny recapitulates phylogeny", the embryonic
development of an animal may reveal its evolutionary history. Although the emphasis of this study is the mechanism of avian gastrulation, it is of interest to ask another basic question. Is there a difference between the early embryonic development in chicks and penguins?

One difference is readily seen. The embryo of the Adélie penguin must incubate from three to four days to reach the same stage of embryonic development as the 18 to 24 hour incubated chick embryo (Baker, 1968). The embryo is referred to as the definitive streak embryo. Comparing chick and penguin definitive streak embryos, one observes no striking difference between them. The arrangement of blastodermal layers, the general morphology of the cells, and even the cytoplasmic constituents within cells appear to be quite similar in these two birds.

There are two small variations observed in the epiblast cells of chick and penguin embryos. One difference deals with the kinds and concentrations of certain yolky intermediates. The other pertains to the presence of certain epiblast cell vacuoles in penguins but not readily seen in chicks.

The various yolky intermediates observed in chick blastodermal cells and an excellent account of their conversion to cytoplasm have been reported by Bellairs (1958, 1964). Blastodermal cells of the chick contain easily-recognizable
lipid droplets and yolky intermediates (Figures 3 and 4). Certain yolky intermediates are derived from granular yolk, i.e., type A drops (Bellairs, 1958). Although lipid droplets are present and recognizable as such in penguin embryos (L, Figure 9), the concentration of granular yolky intermediates is much lower in penguins than chicks.

Another minor difference between chick and penguin definitive streak embryos is the occurrence of relatively large vacuoles in the apical ends of those penguin epiblast cells which have just withdrawn their attenuated necks from the epiblast's free surface (V, Figure 7). At times these vacuoles are seen to completely fill apical ends of withdrawing epiblast cells and may even define the width of the neck. The origin of these huge vacuoles is uncertain. They may represent the product of coalescence of many smaller vacuoles which are forced into this apical-most region by a lateral compression over the length of the attenuated neck during the withdrawal of the epiblast cell from the epiblast's free surface. Balinsky and Walther (1961) suggest that some epiblast cell necks assume a mushroom or "T" shape just prior to their invagination. The horizontal part of the "T" lies flush on the epiblast's surface. It could be that during the ventral displacement of epiblast cells which accompanies invagination, the horizontal part of the "T" curves up and around and these cytoplasmic extensions contact each other and fuse. In this manner, a large vacuole may be incorporated
into the apical ends of invaginating penguin epiblast cells.

My findings relative to the differences between chick and penguin definitive streak embryos are slight and probably have little significance in terms of the main part of this thesis which is the mechanism of avian gastrulation. Since only one stage in the development of the penguin was studied, one does not have sufficient evidence to make comparisons between these two birds. It is interesting to note, however, that penguin epiblast cells do not contain the same granular yolky intermediates as do chick epiblast cells at a comparable stage. A structural and biochemical analysis of the components of penguin yolk could possibly reveal some information pertinent to the evolutionary history of penguins.

Primitive Streak Cells

Cells of the primitive streak embryo are homogeneous in regard to their cytoplasmic constituents. There is little cellular specialization with cells of all three germ layers having a similar appearance. Although morphologically undifferentiated, cells of the epiblast appear to be active, metabolizing cells which are making certain preparations for their future roles both immediate and distant.

The available evidence, both experimental and intuitive, suggests that epiblast cells are not passively moved through the primitive streak by some external force. Instead, they are able to direct their own movements. Gastrulating epi-
blast cells are capable of producing those internal components which may be necessary for the invagination process, i.e., cytoplasmic extensions (lobopodia and filopodia) and cytoplasmic microtubules. Epiblast cells also contain energy-yielding substrates which may allow the work of invagination to be carried out. Thus, this thesis suggests that epiblast cells themselves provide both the necessary internal structure or "invagination machinery" and the energy or driving force for avian gastrulation.

Since the displacement of epiblast cells through the primitive streak appears to be an energy-requiring process, it is logical to ask where the energy is being derived from. Energy-producing substrates in the form of glycogen and complex lipids are present in the cytoplasm. Large numbers of mitochondria are available, and their close association with lipid droplets suggests that triglycerides are being utilized as an energy source. The presence and disposition of numerous Golgi complexes in epiblast cells suggests (Fawcett, 1966) that they too may be involved in the lipid metabolism.

The ratio of nuclear volume to cytoplasmic volume is relatively high in cells of the early avian blastoderm. In fact, at times the nucleus may be seen to occupy more than half of the entire cell. Epiblast cells also have well-developed nucleoli. It is difficult to section through a nucleus without also sectioning through a prominent
nucleolus. Nucleoli are thought to be involved in the production of ribosomal RNA. The size of nuclei and prominence of nucleoli in epiblast cells tend to suggest that the nuclei are in an active synthetic state producing messenger RNA, ribosomal RNA, and probably soluble RNA as well. Since one may think of cellular differentiation as the elaboration of unique enzyme systems, then the active synthetic state of epiblast cell nuclei together with the presence of free ribosomes in the cytoplasm support the notion that epiblast cells are making preparations for differentiation.

Cytoplasmic Extensions

The invagination of epiblast cells and the subsequent antero-lateral migration of these newly-formed mesodermal cells between epiblast and hypoblast epithelia may depend on the presence and activity of cytoplasmic extensions. These extensions, also called pseudopodia, lobopodia, and filopodia, seem to be contractile in nature "pulling" the cell toward its destination.

Cells of very different origin and function have the ability to form pseudopodia. The most celebrated of these, of course, is the amoeba. Amoebae use their pseudopodia to ingest food and fluid as well as in amoeboid movement. Certain mammalian blood cells also have the potential to form cytoplasmic extensions. During an inflammatory process, leukocytes squeeze out of tiny capillaries (diapedesis),
produce pseudopodia, and migrate toward the site of infection. That the ability of cells to produce pseudopodia is almost universal is attested by the culture of various tissues. The cells of almost any tissue, whether ectodermal, endodermal, or mesodermal in embryonic origin, when explanted in tissue culture, will break away from the mass, produce pseudopodia, and actively migrate forming a zone of migration.

Embryonic cells involved in morphogenetic cell movements also possess pseudopodia. In some organisms, pseudopodia are obviously contractile, e.g., filopodia derived from primary mesenchymal cells of sea urchins (Gustafson and Wolpert, 1963) and processes of chick marginal cells seen during avian epiboly. Pseudopodia may also act as holding or anchoring devices, e.g., cytoplasmic extensions in the neck regions of amphibian blastoporal cells called "longitudinal flanges" presumably tie apical ends of the blastoporal cells together to form "a rather firm longitudinal bundle" (Perry and Waddington, 1966). Pseudopodia observed between epiblast cells in the early avian embryo (Figure 4) appear to be acting both as anchoring devices to hold apical ends of the cells together and as contractile elements to help pull epiblast cells through the streak. Since part of the general hypothesis of avian gastrulation assumes that pseudopodia are contractile and exert a "pulling force", then evidence to document their contractile
Certain pseudopodia have been shown to possess cytoplasmic filaments. Filopodia acting as contractile elements between the mesodermal cells of 18 hour and older chick embryos contain filaments about 50 \( \text{A} \) in diameter (Trelstad et al., 1967). Pseudopodial filaments may well be common components of pseudopodia. A dynamic demonstration of the role of contractile filaments can be seen in the tail epidermal cells of the ascidians Amaroucium constellatum and Distaplia occidentalis during the tail resorption phase of metamorphosis. During tail resorption, which occurs rapidly within six minutes, randomly arranged 50-70 \( \text{A} \) filaments align themselves parallel to the axis of contraction in the epidermal cell apices, and contract the apical portions of epidermal cells (and thus the entire tail) to 5.5 percent of their larval length (Cloney, 1966).

In a comprehensive review article dealing with cell structures which may be associated with amoeboid movement, Wohlfarth-Botterman (1964) found no evidence to suggest the presence of cytoplasmic filaments in the pseudopodia of amoeboid cells. However, with new fixation techniques Trelstad et al. (1967) and Fawcett (1966) show that filaments are present in cytoplasmic extensions. It is postulated that filaments such as skeletal muscle filaments are capable of sliding past one another producing an overall contraction.
Thus it is not unreasonable to suggest that epiblast cell pseudopodia may play an active contractile role in avian gastrulation.

Cell-to-Cell Attachments

A consideration of cell-to-cell attachments in gastrulating embryos is important because aspects of both morphogenetic cell movements and early morphogenesis may be influenced by cell contacts. Cells migrating as individuals such as invaginating epiblast cells must possess ways to disrupt old cell contacts, establish temporary contacts while in motion, and form more permanent attachments upon arrival at their definitive sites. On the other hand, in migrating tissue sheets contacts must be maintained between cells in order to coordinate the overall migration. During the numerous invaginations and evaginations which occur during an animal's early embryology, contacts between cells must be specialized to render their junctions less susceptible to disruption. The intercellular material, which is thought to be a mucopolysaccharide, probably regulates to some degree the uniform spacing between cells as do the cohesive forces between membranes of apposing cells. The cellular transmission of stimuli either in the form of chemical inducing agents or electrical impulses may be influenced by cell contacts. Thus the movement of cells and transmission of stimuli between cells are related to
the various cell-to-cell attachments.

During epiblast cell invagination a number of specialized cell-to-cell attachments are observed. "Simple junctional complexes" (which I have defined as cell contacts consisting of one or two minute tight junctions in association with a close junction over a linear distance of 0.25-1.0 micron) are observed between the apical-most ends of those epiblast cells concentrated toward the very middle of the primitive streak, i.e., within the "active invagination zone" (SJC, Figures 3 and 7). Another specialized epiblast cell contact is encountered between the apical ends of epiblast cells lateral to the middle of the primitive streak. This apical-subapical contact zone (ASCZ, Figure 8) is approximately 1-2 microns long with the plasmalemmas of adjacent cells forming a series of minute tight junctions with alternate close junction attachments which have an intercellular space of 100-200 Å. The "simple junctional complex" looks very much like a smaller, less-involved version of the apical-subapical contact zone.

It is tempting to postulate that epiblast cells located to one side of the primitive streak, i.e., to one side of the "active invagination zone", are more tightly connected to each other than primitive streak cells because these peripheral cells possess apical-subapical contact zone junctions. As peripheral epiblast cells are displaced or
"pulled" medially toward the streak, their tightly adhering apical-subapical contact zone junctions may be gently disrupted and become transformed to the "simple junctional complex" observed at the apical-most end of those epiblast cells about to undergo invagination. It is interesting to note that some of the peripheral epiblast cells possess the apical-subapical contact zones. These same junctions are also found in the contracting epidermal cells of ascidian tails during metamorphosis (Cloney, 1966), where there appears to be an extraordinary stress on the cell-to-cell attachments. Thus as epiblast cells undergo invagination moving into the mesoblast, apical-subapical contact zone junctions may be partly responsible for replenishing the supply of primitive streak cells by keeping peripheral epiblast cells tied tightly together while a pulling force draws them medially toward the streak.

Another cell-to-cell attachment described as an epithelial cell specialization in other animals is observed in part in the early avian embryo. Of the three components of the "epithelial junctional complex" (zonula occludens, zonula adhaerens, and macula adhaerens; Farquhar and Palade, 1963, 1965), the first two, the tight and close junctions, are observed in the early avian blastoderm. The observation (Balinsky and Walther, 1961; Overton, 1962; and Trelstad et al., 1967) that desmosomes or maculae adhaerens are not present in the early stages of avian development is confirmed
by this study. Desmosomes have been reported in stage 12 embryos in the entoderm lateral to the primitive streak (Trelstad et al., 1967).

Balinsky and Walther (1961) reported that there are two major types of cell-to-cell attachments between primitive streak cells. They referred to these as (1) "simple contacts" with 113 Å intercellular spaces, and (2) "denser sections of cell boundaries". "Simple contacts" are the same as close junctions (zonulae adherentes, Farquhar and Palade, 1963, 1965), and they supply the majority of cell contacts in the early avian blastoderm. Close junctions are found as connections between individual mesodermal cells, between mesodermal cells and the basal borders of both epiblast and hypoblast epithelia, and between the filopodia of invaginating epiblast cells just beneath the primitive streak. "Denser sections of cell boundaries" (Balinsky and Walther, 1961) have been further clarified in this study, and are called "simple junctional complexes". Thus epiblast cells possess three major types of cell-to-cell attachments. These include apical-subapical contact zones, "simple junctional complexes" and close junctions.

Surface Evaginations

In a low power electron micrograph of the epiblast's free surface (Figures 6A and 9) regular patterns of surface evaginations are evident. Some of these vacuolate surface
Evaginations appear to be formed by cytoplasmic processes of two contiguous epiblast cells and are termed junctional vacuoles (JV, Figure 9). Other vacuoles arise between cell junctions and are independent of them. These have the name interjunctional vacuoles (IV). These vacuolate surface evaginations may represent a form of pinocytosis (Balinsky and Walther, 1961; Ruggeri, 1967a). It may be that fluid bathing the epiblast's surface is taken into the epiblast cells and allowed to mix with the ground plasm. Ruggeri (1967a) ascribes a digestive function to the vacuoles and suggests they contain ingested albumen. He further postulates that within the vacuoles water of the albumen is separated from its solid component; the water then is released into the intercellular spaces. The passage of water into the intercellular spaces between neighboring epiblast cells may have an effect on the intercellular material. The degree of adhesion between cells may be altered allowing junctions to loosen, and epiblast cells may slide past one another into the mesoblast.

Junctional and interjunctional vacuoles may be important to the normal functioning of epiblast cells. The possible pinocytotic origin of vacuoles suggests that they may be involved in the metabolism of the cell by providing nutrients in the form of albumen (Ruggeri, 1967a). Vacuoles may also be involved in hydrating the intercellular material which secondarily causes a loosening of cell-to-cell junctions.
Furthermore, they may be involved in sol-gel relationships within the cell thus contributing to the causes of cytoplasmic streaming.

Factors Responsible for the Change in Epiblast Cell Shape

The change in shape of avian epiblast cells from the typical cuboidal-columnar form to the flask or bottle configuration may be essential to the mechanism of avian gastrulation. As a result of this shape change, internal cellular components are translocated from apical (external) to basal (internal) portions of the cell by cytoplasmic streaming. This redistribution of cytoplasm and its various constituents further attenuates the neck region. The cycle continues until invagination is completed.

The change in shape can be accomplished by the cells themselves. They need not be assisted by some external force such as lateral compression by neighboring cells (Balinsky, 1965). The notion that gastrulating cells can actively change their shapes is further supported by the fact that amphibian blastoporal cells, even when dissected away from gastrulae, still maintain their bottle shapes (Holtfreter, 1943a). Thus the change in cell shape is a function of the cell itself, and the factors causing this shape change must be sought within the cell.

Any factor or force which causes a change in cell shape must ultimately exert its influence on the proteinaceous
"backbone" of the cell, the cytoplasmic matrix. The most direct way to change a cell's shape would be a localized reaction of the cytoplasmic matrix itself. Such a reaction could be the contraction of proteinaceous components within the cytoplasmic matrix. Indeed, this is what Baker (1965) suggests for the mechanism of shape change in amphibian blastoporal cells, i.e., a contraction of a subcortical "dense layer" which is presumably proteinaceous in nature. Another less direct but equally effective way to change a cell's shape would be the introduction or differentiation of certain cytoplasmic components which secondarily alter the cytoplasmic matrix. Such cytoplasmic components might be cytoplasmic filaments or microtubules. Both of these filamentous organelles have been described in cells undergoing shape changes (Baker, 1967; Perry and Waddington, 1966b). Cytoplasmic microtubules have also been isolated in avian epiblast cells by Ruggeri (1967b) and in this study. They may play a fundamental role in avian gastrulation.

Cytoplasmic microtubules are defined as straight tubules of indefinite length with an outside diameter of 200-300 Å and a center of low density giving them a hollow appearance (Fawcett, 1966). They are thought mainly to be involved in the elongation of cells (Byers and Porter, 1964; Perry and Waddington, 1966), the maintenance of cell shape (Taylor, 1966; Kessell, 1967), and the transport of fluid (Slatterback, 1963).
Cytoplasmic microtubules within epiblast cells may be responsible either directly or indirectly for the change in epiblast cell shape. They may act directly by means of an elongation mechanism thereby causing the epiblast cell to elongate in only one direction. They may mediate or "oversee" the translocation of cytoplasm which secondarily causes a change in cell shape. Or, in the last instance, cytoplasmic microtubules may have a more passive role in maintaining or stabilizing cell shape after that shape has been established by other factors. Although the manner in which cytoplasmic microtubules act within the cell is not known, their intimate involvement in the changing of cell shape will be demonstrated below.

Waddington (1940) was able to demonstrate a weak birefringence in the yolk-free necks of invaginating amphibian blastoporal cells, and he concluded that certain filamentous elements align themselves perpendicular to the egg's surface within the necks of blastoporal cells. Waddington (1940) felt that the alignment of filaments was due to a change in the protein constituents, and he named this change "fibrisation". He further postulated that "it is partly the result of the fibrisation of cell proteins that causes elongation of amphibian blastoporal cells".

A fine-structural examination of the neck regions of tree-frog blastoporal cells (Baker, 1965) failed to demonstrate the presence of filamentous elements. However the study did
demonstrate the presence of a subcortical dense layer. Baker (1965) feels that blastoporal cells change their shapes by an alternate contraction and elongation of this subcortical dense layer which is probably composed of a proteinaceous matrix with interspersed protein fibers.

A similar ultrastructural examination of the blastoporal cells in the newt, *Triturus alpestris* reveals a distinct zonation pattern within flask-shaped blastoporal cells and the presence of 300 A diameter cytoplasmic microtubules disposed mainly in the direction of cell elongation (Perry and Waddington, 1966). Although a subcortical zone of dense, granular material, very reminiscent of the "dense layer" of Baker (1965) is present, Perry and Waddington (1966) are of the opinion that the dense zone is "a passive accumulation of superfluous cortical material" and suggest that the elongation of blastoporal cells during amphibian gastrulation is caused by the elongation of cytoplasmic microtubules.

These cellular elements have also been identified in embryonic ectodermal cells undergoing shape changes during neurulation and lens differentiation, e.g., presumptive neural plate cells of the newt, *Triturus alpestris* by Waddington and Perry (1966) and presumptive lens cells of the chick by Byers and Porter (1964) respectively.

Waddington and Perry (1966) have shown that ultrastructural features of neural plate cells are similar to those of blastoporal cells in *Triturus alpestris*. Zona-
tion patterns within flask-shaped neural plate cells, i.e., a sub-cortical dense, granular layer, a more proximal vesicular zone, and an inner zone consisting of the main body of the cell, and the presence and arrangement of cytoplasmic microtubules are nearly identical in the neural and blastoporal cells. Neural plate cells undergo the same sort of shape transformations as do blastoporal cells; cells originally cuboidal in shape become prismatic or pyramidal, then elongate becoming columnar and eventually bottle-shaped. Thus the mechanism of neurulation may be quite similar to the mechanism of gastrulation, at least in the newt-Triturus alpestris.

The appearance of an organized array of cytoplasmic microtubules with the concomitant elongation of both neural plate and blastoporal cells strongly suggests that elongation is related to the microtubules. Although microtubules may be found in almost all cells of the newt, they occur only in high concentrations in those cells which are actively elongating (Perry and Waddington, 1966).

There is a close relationship between early stages of cell elongation and the occurrence of cytoplasmic microtubules in the presumptive lens ectodermal cells during embryonic development in the White Rock domestic fowl (Byers and Porter, 1964). During eye differentiation, cells of the head ectoderm are induced by the optic vessicle to form the lens placode, which in turn, rounds up and moves into the invagi­nated optic cup to become the definitive lens. The lens
placode arises through the elongation and palisading (aligning in rows) of head ectoderm cells. The appearance of microtubules about 230 Å in diameter in the cortices of elongating lens placode cells, their orientation parallel to the axis of elongation, and the time synchronization between the establishment of the parallel microtubular array with lens cell elongation (Byers and Porter, 1964), further implicate microtubules in the elongation process.

Cytoplasmic microtubules are thought generally to be involved in the alteration of cell shape, the maintenance of cell shape and the internal movement of cytoplasm. The reasons for postulating these functions are basically twofold. The first is the consistent discovery of cytoplasmic microtubules in many different cell types during a variety of physiological states, e.g., the cortices of certain plant cells engaged in wall formation (Ledbetter and Porter, 1963), primary mesenchyme cells of sea urchin embryos which undergo a complete reorganization of their cytoplasmic contents (Tilney and Gibbins, 1966), within developing grasshopper spermatozoa cytoplasmic microtubules involved in the elongation of the nucleus (Kessell, 1967), and in invaginating avian epiblast cells. Thus cytoplasmic microtubules appear to be common organelles, almost ubiquitous.

The second reason pertains to the similarity in structure between cytoplasmic microtubules and those microtubular elements found within other discrete cellular structures,
1.c., flagella, cilia, sperm tails (flagella), and spindle fibers of the mitotic apparatus. Even the substructure of these microtubular elements is strikingly similar. Phillips (1966) feels that cytoplasmic microtubules differ chemically from cilia and flagella, but suggests there are morphological and functional similarities. The investigation of suitable plant material by negative staining (Ledbetter and Porter, 1963) demonstrates that cytoplasmic microtubules have almost an identical substructure to that of spindle and flagellar fibers. Plant cytoplasmic microtubules possess 13 filamentous subunits arranged longitudinally with a center-to-center spacing of 55-60 A (Ledbetter and Porter, 1963). Barnicot (1966) showed that spindle fibers from cultures of newt hearts and human fibroblasts have an outside diameter of 250-270 A, and each is composed of a number of fibrils which have the appearance of rows of connected 35 A diameter granules with a center-to-center spacing of approximately 50 A. Finally Pease (1963) showed that the fibrils of rat sperm tails proved to be cylindrical units composed of 10 longitudinally oriented filaments with a width of 35-40 A and a center-to-center spacing of 55-60 A. Thus, from an ultrastructural standpoint, cytoplasmic microtubules are very much like the peripheral doublets in cilia, flagella, sperm tails, and spindle fibers of the mitotic apparatus.

Objects having the same structure may be thought to carry
out similar functions. The implication of the contractile, elongating, or sliding filament nature of the "motor apparatus" of cilia and flagella (Satir, 1965) may also be applied to cytoplasmic microtubules. The ability of spindle fibers to rapidly "polymerize" or to grow out from a point of origin almost spontaneously during metaphase, and their ability to either contract or elongate during anaphase of mitosis may also be postulated for cytoplasmic microtubules. Tilney and Gibbins (1966) have further shown that cytoplasmic microtubules are very much like spindle fibers of the mitotic apparatus. By treating sea urchin gastrulae with colchicine, cytoplasmic microtubules within the mesenchymae cells of the archenteron tip completely disappear, and the cells round up. Treatment of these cells with "heavy water" (deuterium oxide) causes a stabilization of microtubules as well as stabilization of mesenchymal cell shape. Colchicine and heavy water have the same effect on spindle fibers.

Thus their ubiquity and their structural as well as functional resemblance to cilia, flagella, and spindle fibers have fostered the idea that cytoplasmic microtubules are involved in the maintenance and alteration of cell shape and in the movement of cytoplasm within the cell. But how do they act? At this time there is no concrete evidence to either support or deny any theory of microtubular action. However there are a few theories, and some are more likely than others.
Cytoplasmic microtubules may provide the motile force for an inherent cytoplasmic movement. It is doubtful that cytoplasmic microtubules "transport" cytoplasm with them as they polymerize or grow out from a point of origin. And it is difficult to imagine microtubules providing a surface, substrate, or channel over which or through which cytoplasm may be directed. It is much less difficult to postulate a sliding-filament arrangement in which a translational force could be applied to propel cytoplasm. By the gradual translocation of cytoplasm along the microtubules, elongation of the cell could take place.

Isolated cells of amphibian gastrulae which had been undergoing elongation at the time of dissection, demonstrated wave-like peristaltic movements (Holtfreter, 1944). Commenting on this observation, Byers and Porter (1964) suggest that cytoplasmic microtubules may be acting as an "intracytoplasmic peristalsis" to cause translocation of the cytoplasm. The suggestion of an "intracytoplasmic peristalsis" within elongating amphibian cells has some merit, because cytoplasmic microtubules have been described in the elongating amphibian cells (Baker, 1967; Perry and Waddington, 1966; Waddington and Perry, 1966). Microtubular peristaltic waves could conceivably displace cytoplasm. However, one rarely finds any other than a straight microtubular configuration. But it is possible that peristalsis at the microtubular level could easily go undetected. The observation (Byers and Porter,
1964) that smaller organelles seem to redistribute themselves prior to the appearance of microtubules and cell elongation suggested that there may be some pre-elongation "organizing influence". Perhaps this "influence" is the commencement of a few microtubular peristaltic waves which are "firing up" to take part in the larger, more coordinated waves when entire bundles of microtubules "beat" together.

Cytoplasmic microtubules may cause a localization gelation of the cell's proteinaceous matrix, thereby causing cytoplasmic translocation. It may be that "polymerization" of microtubules causes the "unfolding" of numerous protein complexes allowing peptide chains to interact forming cross linkages. The interlocking of proteins causes a localized gelation in one region of the cell and forces cytoplasmic inclusions and organelles into less gelated regions. As greater numbers of microtubules arise the gelation zone becomes stronger forcing more cytoplasm into other regions. Soon the region of localized gelation begins to shrink due to the paucity of cytoplasm. The cycle of cytoplasmic translocation begins, and invagination is underway.

Comprehensive Description of the Mechanism of Avian Gastrulation

Classical embryological studies have shown that the freshly-laid avian egg consists of a flat, two-layered blastodisc. Formation of the primitive streak and invagination of epiblast cells takes place within a restricted region of the
blastodisc termed the area pellucida. The 6-8 hours incubation the posterior portion of the area pellucida becomes thickened due to the movement of peripheral epiblast cells toward the midline.

Data from the present study indicate that some of these peripheral epiblast cells undergo a predictable sequence of shape transformations as they migrate to and through the primitive streak. Figure 13 demonstrates the various epiblast cell shapes encountered during the gastrulation process. Peripheral epiblast cells are largely cuboidal or low columnar (1, Figure 13) in shape. Some of these peripheral epiblast cells become more columnar in shape (2, Figure 13), later highly columnar (3, Figure 13), and finally bottle-shaped with long, attenuated necks (4, Figure 13). Eventually the attenuated necks of bottle-shaped cells "slip" beneath the epiblast's free surface (5, Figure 13), and the cells are free to migrate into the mesoblast.

The actual change in epiblast cell shape seems to be initiated by the presence of cytoplasmic microtubules. At 6-8 hours incubation some of the epiblast cells in the middle of the primitive streak probably begin the invagination process by producing cytoplasmic microtubules in their apical regions. The cytoplasmic microtubules start the "cytoplasmic translocation cycle" which is nearly irreversible once it begins. When the cytoplasm is redistributed to basal portions of the epiblast cell, this displacement
causes a further stretching or attenuation of neck regions. They in turn become smaller in diameter, forcing more cytoplasm out of their localized region and into the basal portions of the cell to start the cycle anew.

Microtubules are capable of initiating cytoplasmic streaming. They act either to cause localized regions of gelation in the apical portion of the cell, or they are able to propel the cytoplasm in an apicobasal direction by concerted peristaltic waves. However they accomplish this job, the important thing is that they may get the cytoplasm "rolling", and in this way, initiate invagination.

When primitive streak cells withdraw beneath the epiblast's free surface, other cells move into the "active invagination zone" to take their place. Epiblast cells just to one side of the streak are more closely associated along their lateral borders as well as their apical zones than are those cells of the "active invagination zone". In fact, it appears that the apical-subapical contact zone (ASCZ) of certain peripheral epiblast cells gives rise to the less extensive form of cell-to-cell attachment characteristic of those cells about to undergo invagination in the streak—the "simple junctional complex". And this transformation from ASCZ to "simple junctional complex" occurs while the epiblast cell migrates from a peripheral position to the "active invagination zone". Junctional vacuoles (JV) which appear to be formed by cytoplasmic extensions of two neigh-
boring cells and interjunctional vacuoles (IV) may be involved in the transformation of cell-to-cell attachments by hydrating the intercellular material causing junctions to loosen. These pinocytotic vacuoles may also be involved in the nutrition of epiblast cells by providing albumen and in sol-gel changes within the cytoplasm during the change in shape of epiblast cells by introducing quantities of exogenous fluid.

Cytoplasmic extensions in the form of lobopodia and filopodia are involved in the invagination process. These pseudopodia probably simultaneously anchor epiblast cells while they are undergoing shape changes and "pull" basal portions of the cell to aid the attenuation process. After the attenuated necks of epiblast cells sink below the epiblast's free surface these "contractile" cytoplasmic extensions "pull" the cells into the mesoblast. Not all cells of the epiblast undergo invagination simultaneously. It is often the case that an elongated epiblast cell will be positioned next to a cuboidal-shaped cell. This cuboidal-shaped cell provides a relatively stationary object which epiblast pseudopodia attach to and which also may send out pseudopodia. The net result of the attenuation process and the interplay of pseudopodia is that the bottle-shaped cell is able to "slip" past the more stationary epiblast cell, and in this manner, withdraw from the epiblast into the mesoblast.
In summary, during avian gastrulation certain epiblast cells become long, attenuated flask-shaped cells. Due to this change in epiblast cell shape, the activity of certain pseudopodia, the ability of certain epiblast cells to maintain a stationary position relative to others, and the disruption of strong, intercellular attachments, invagination occurs. The primitive streak becomes well-defined with a deep primitive groove as more and more epiblast cells undergo invagination. Cells lost from the epiblast to the mesoblast are replaced by peripheral cells which are "pulled" to the streak center by tightly adhering cell-to-cell attachments.
SUMMARY AND CONCLUSIONS

A study on the mechanism of epiblast cell invagination during avian gastrulation has been carried out using the primitive streak embryos of the White Leghorn chicken (Gallus domesticus) and the Adélie penguin (Pygoscelis adeliae). The ultrastructure of primitive streak cells has been described.

The following conclusions were made:

1. Information gained from this study can neither support nor deny either the blastema-like or the blastopore-like nature of the avian primitive streak. On an ultrastructural basis however, primitive streak cells strongly resemble amphibian blastoporal cells.

2. Cells of the primitive streak possess specialized cell-to-cell attachments, lobopodial and filopodial cytoplasmic extensions (pseudopodia), and homogeneous cytoplasmic constituents.

3. Cytoplasmic constituents common to epiblast cells include intracellular vacuoles, Golgi complexes, mitochondria, glycogen particles, free ribosomes, numerous granular yolky intermediates, and lipid droplets.

4. On a comparative basis, only one difference was discovered between the definitive streak embryos of the chick and penguin in terms of cytoplasmic constituents. Epiblast cells of the penguin do not possess the same kinds and concentrations of granular yolky intermediates as the epiblast.
cells of the chick at a comparable stage.

5. Cells of the primitive streak in both the chick and penguin demonstrate a variety of cell shapes. Cells may be cuboidal, columnar, flask or bottle-shaped, and almost any combination of the above.

6. Cytoplasmic microtubules of 200-250 A in diameter are present in primitive streak cells of the epiblast. Bundles of longitudinally-oriented microtubules are found in the attenuated neck regions of some bottle-shaped epiblast cells.

7. The hypothesis of avian gastrulation presented in this study depends upon a change in epiblast cell shape to accomplish the invagination. It is the elaboration of cytoplasmic microtubules that may cause the translocation of cytoplasm from apical (exterior) to basal (interior) portions of the cell. Due to the translocation of cytoplasm the shape change is accomplished, and bottle-shaped primitive streak cells invaginate to become part of the mesoblast.
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ACKNOWLEDGEMENTS

I sincerely thank Dr. John R. Baker for his willingness to help, his generosity, his honesty, and most important, his friendship. Our trip to Antarctica will never be forgotten.

Thanks go to Dr. Oscar E. Tauber for his understanding and kindness, the financial aid he provided in the way of assistantships and research expenses, and his ability to never be too busy to do whatever he can.

Thanks to Dr. Martin J. Ulmer for criticizing my dissertation and making valuable suggestions. In a larger sense, thanks to Dr. Ulmer for providing an example of excellence in the academic realm and other realms as well.

Thanks to Dr. David R. Griffith for teaching me more physiology in one quarter than I learned in four years of undergraduate school.

Thanks go to Dr. Walter R. Hearn. His honest desire to really get to know an individual is truly refreshing.

Thanks to Dr. Walter J. Humphreys who helped me with many of the fine-structural aspects of this study.

One thousand and one thanks go to Delores Oliver for taking care of the thousand and one details that seem to go part and parcel with any graduate program.

My sincere thanks to Iowa State University for providing
the necessary physical things plus those certain intangibles which make a collection of books, buildings, and disciplines into a productive university.

My father, mother, and dear wife DeAnna share in this achievement with me.

Finally, this study was supported in part by funds from the United States Antarctic Research Program under NSF Grant GA-1135.