An Ultrastructural Study of the Early Embryogenesis of the Zebrafish, Brachydanio Rerio

Eleanore Paunovich

Loyola University Chicago

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AN ULTRASTRUCTURAL STUDY OF THE EARLY EMBRYOGENESIS OF THE ZEBRAFISH, BRACHYDANIO RERIO

by

Eleonore Paunovich

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

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1977
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Words cannot fully acknowledge the deep sense of appreciation extended to my parents, Mihailo and Elisabeth Paunovich. Their moral and financial support of my education to this point has made my learning experience a rewarding one.
VITA

The author, Eleonore Paunovich, is the daughter of Mihailo and Elisabeth Paunovich. She was born July 17, 1952, in Gary, Indiana.

Her elementary education was obtained at Franklin School in Gary, Indiana, and her secondary education at Lew Wallace High School in Gary, where she graduated in June, 1970.

In September, 1970, she entered Indiana University Northwest, and in June, 1974, received the degree of Bachelor of Arts in Biology. While attending Indiana University Northwest, she was selected as a Hoosier Scholar by the Indiana Scholarship Commission. She served as president of the Beta Iota Omega Chapter of the American Institute of Biological Sciences, and was a member of the executive council of the IUN Student Government in 1973. In 1974 she served as chairperson of the IUN Health Fair.

In September, 1975, she was granted an assistantship in biology at Loyola University of Chicago. In June, 1977, she was awarded the Master of Science in Biology. She will be continuing studies at the Indiana University School of Dentistry.
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INTRODUCTION

The biogenesis of organelles involves a basic aspect of life processes: the transition from one level of organization, the molecular, to a higher level, the supramolecular. The mechanisms by which cells make molecules are much better understood than the processes by which the molecules are assembled into organelles. The problem of organelle biogenesis can be examined from two different but complementary viewpoints: conceptually, in terms of the basic principles involved, and operationally, in terms of the methods by which biogenesis is studied in the lab.

Organelle biogenesis gains a clearer perspective when viewed through an embryonic system. The cell differentiation that occurs in embryonic development results from a complex pattern of interactions between the nucleus and cytoplasm and between different groups of cells. When an egg is fertilized, a precise program of events is set in motion, a program that depends on factors, many of which are only beginning to be appreciated. An important
part of the story is the formation of different messenger RNA's in different cells as different genes are freed from the repression that keeps them inactive. Interesting cytological changes are associated with this gene activation.

A preliminary ultrastructural study of the early embryogenesis of a teleost, *Pimphales Notatus* (Rosner, 1972), has shown that developing zygotes exhibit considerable differences of membrane organization between early and later cleavage stages. During the blastula stage (5 hours), only the plasma membrane and nuclear membrane were visible. Conspicuous by their absence were membranes of common cellular organelles, such as mitochondria, Golgi apparatus, and even endoplasmic reticulum. The only discernible structures identifiable at this early stage have been tentatively identified as yolk transport channels, which are formed by the junction of plasma membranes of three cells in close juxtaposition. In contrast, a later developmental stage well along into somite differentiation (48 hours) illustrated considerable diversity of cytoplasmic membranes, with mitochondria and a well-developed rough endoplasmic reticulum readily apparent.
It thus appears that at some time between 5 and 48 hours, the biosynthesis of organelles has occurred. The ultimate aim of this structural study is to establish a timetable of organelle biogenesis in the early embryogenesis of a teleost. Such a timetable, presenting a picture of the membranes present, may be correlated with the present understanding of functional events during early embryogenesis. Such an association may build upon our concept of biological membrane functions through the establishment of: (1) compartmentalization within the cells during early embryogenesis, offering the containment of biomolecules required at definite cellular locations during the process of differentiation; and (2) possible cell-cell contacts which are critical to the determination process.
LITERATURE REVIEW

The idea of cell membrane existed for many years almost entirely at a conceptual level; physiological experiments suggested a barrier to the exchange of material between the cell and its surroundings. It was called a membrane because the barrier was thought to be a thin layer completely enclosing the cell contents. The membrane appeared to have definite mechanical and physical properties, and, even in the absence of any direct analyses, suggestions were made as to its structure and organization. The introduction of the electron microscope revealed the presence of many membrane systems present within the cell as well as the outer plasma membrane.

The term 'cell membrane' is used in at least three different senses—the anatomical, biochemical, and physiological. At the present there is intense activity aimed to bring these three concepts together so as to explain membrane structure and function in molecular terms. Chemical analysis of the components of cell membranes has now been made possible using chromatographic techniques.
showing the various lipid and protein components (Lanaz, 1974). Physical studies of the isolated components have begun to reveal the basic physical chemistry involved in cell membrane organization and the ways in which the components can mutually interact and affect each other (Wallach and Zahler, 1966). Finally, the introduction of model membrane systems has led to a more detailed understanding of the possible ways in which molecules can be selectively transported through the membrane.

Biological membranes constitute extremely complex mixtures of lipids, proteins, ions, etc. In spite of the greater complexity of membranes it seems clear to investigators that the general principles of lipid-protein composition are agreed upon. There will be modifying factors due to special structural features of biological membranes. The lipid may be assymetrically distributed between the two leaflets of the bilayer in the membrane (Bretscher, 1973). Different regions of the bilayer may be in different physical states, either rigid or fluid, and have different physical states, either rigid or fluid, and have different lipid composition (Singer, 1974). Divalent cations complexed to the lipids may alter their properties (Chapman, 1974).
At the present time the general ideas on the structure of biological membranes are perhaps less clear than before 1960; the reason is that, while at the same time there was but one model generally accepted for membrane structure, now we have a variety of different models. The Danielli-Davson model (1935) has satisfied the requirements for the properties of natural membranes for three decades.

In the later 60's some completely new hypotheses have been presented which reject the classical model: Bensen (1966), Green and Perdue (1966), Lenard and Sing (1966) and Wallach and Zahler (1966). They have been proposed to account for hydrophobic bonding of membrane proteins with the non-polar lipid chains. Lipoprotein subunits are envisaged to determine the membrane continuum in some of these models.

In a recent version of the Danielli hypotheses (Vandenheuvel, 1971) lipids form a bilayer with the polar heads turned toward the outside; proteins of two kinds cover the outer surfaces: a mononuclear layer, consisting of juxtaposed molecular protein units, and 'superstructural globular protein units.
Experimental work directed to prove or disprove the various models has resulted in refinements of the previous versions or even in the proposal of new models. Recently the conflicting experimental data have led to the tendency for a moderate compromise, so that some allowances have been made by both sides.

There is little doubt that a lipid bilayer is a common feature of all membranes; what must be clarified is whether there is protein penetration into the bilayer, and, if there is penetration, to what extent does it contribute to membrane structure and how it subverts the classical model. One party still supports the classical Danielli model (Hendler, 1971; and Vandenheuvel, 1971), but the possibility that proteins penetrate in part into the bilayer is not absolutely rejected; other investigators believe that protein subunits are immersed into a discontinuous lipid bilayer (Sjostrand, 1969; Glaser, Simpkins, Singer, Sheetz, and Chan, 1970).

During the past twenty years, much of developmental biology has been reinterpreted and re-examined in terms of gene activities which vary according to time, space, and cell type within a developing organism. These approaches have identified many of the mechanisms by which cells
synthesize large amounts of the different products that ultimately serve to identify the cells themselves (Rut­
tler, Wessels, and Grobstein, 1964). Long before embry­
onic cells can synthesize these products, however, they clearly differ from one another. Cells of the early ani­
mal embryo begin large-scale synthesis of their differenti­
tiated product or products only after a series of highly ordered morphogenetic movements and complex interactions. Although these movements and interactions must also be the results of maternal or embryonic gene products, no specific molecular hypothesis has ever been suggested which could account for these phenomena.

Tyler (1946) and Weiss (1947) independently suggested that cell surfaces contain complementary molecules which interact with each other specifically in a lock-and-key fashion. Molecules of this type could have at least two functions, according to Weiss. First, they could be deter­
minates in intercellular recognition if both members of each matching pair of molecules were bound to the external surfaces of particular cell types. In this way, a cell undergoing morphogenesis could determine the nature of its immediate environment and either move on, or if the sig­
nals were proper, remain and respond in a particular
fashion. Second, according to Weiss, cells might communicate by releasing soluble effectors which would bind their complementary receptors on the outsides of other cells. It was further suggested that growth control and organ size might be regulated in this way.

There are three systems in which macromolecules show a high degree of specific affinity for one another and which may be reasonable candidates for cell surface complementary molecules: antigens and antibodies, lectins and oligosacharides, and enzymes and substrates. The first two of these systems are unlikely to provide a general mechanism of recognition and communication of the types postulated by Weiss because one member of each pair is probably not a universal cell component. The known lectins generally originate from plants or invertebrates, whereas the immunoglobulins, at least in their usual form, are almost certainly located on only a small number of cell types. Enzymes and their substrates have the requisite specificity for one another and, in addition to simple binding, can interact in such a way that the substrate becomes altered.

Roth (1973) presents a model applicable only to the early events of cell interactions and not to those later
specializations which must be occurring by different mechanisms. For example, ultrastructural adhesive sites (Fawcett, 1959), low resistance coupling between cells (Lowenstein, 1973) and direct cell communication through apparent cytoplasmic continuity (Subak-Sharpe, Burk, Pitts and Pitts, 1972) are phenomena expressed by those initial encounters between cell and environment.

In the past several years the characterization of cell junctions in normal tissues has increased rapidly. Cell junctions are a class of short-range interactions between cells which require direct physical contact. They generally consist of a specialization of the plasma membrane as well as the cell surface or intercellular matrix. Junctional structures can be separated into five major groups, each with significant structural pleomorphism. These junctions have been described in developing tissues, and their appearances can often be associated with significant morphogenetic events (McNutt and Weinstein, 1970; deHaan and Sacks, 1972; Hand and Gobel, 1972; Sheridan, 1973; Wade et al, 1973).

The cells in most adult tissues are connected by permeable junctions. Commonly, most cells in a given tissue are interconnected. Junctional interconnection is
is particularly widespread during embryonic development. At the morula stage, all cells of the newt embryo are connected to each other (Ito and Hori, 1966), and even at later stages of development these extensive interconnections are present between differentiating groups of cells whose descendant populations are connected in the adult (Potter, 1966).

Previous studies on cell junctions in developing systems have provided important information on the nature of the junctions that are present, as well as the timing or the developmental stages at which they first appear. These observations, in turn, have been related to important morphogenetic events, as well as the establishment of cell-to-cell adhesion, permeability barriers, and low-resistance pathways.

Events at the level of the cell membrane and cell surface which surround the differentiation of a cell junction have been studied (Gilula, 1972). The development of the septate junction during sea urchin embryogenesis illustrates a complete series of assembly steps at the membrane level concomitant with the generation of the mature junction.
The causal morphological analysis of determination and differentiation processes in early developmental stages has been successfully carried out, especially in insects, sea urchins, amphibians and mammals. In all these investigations the final outcome of isolation and transplantation experiments has been evaluated by histological analysis of the differentiated tissues.

Differentiation depends on the differential synthesis of RNA's and proteins. Ribosomes are actively synthesized during amphibian oogenesis and stored in the oocyte (Brown and Dawid, 1968). In amphibian embryos, the synthesis of messenger-RNA is low during cleavage, increasing from the blastula stage onward. Shortly afterwards, a considerable synthesis of 4S-RNA was observed. The synthesis of embryonic ribosomal-RNA becomes accelerated in the gastrula stage (Brown and Littna, 1966; Gurdon and Woodland, 1968). Proteins which are newly formed during the early developmental stages are in part translated on so-called "maternal" messengers, which are synthesized and stored in the oocytes, and in part on messengers, which are newly synthesized in the cleaving egg after fertilization. When early embryos or their tissues are treated with Actinomycin D to inhibit the synthesis of messenger-RNA,
these latter proteins cannot be produced and a series of
differentiation processes cannot occur. The translation
products of 'maternal' messengers include histones and
microtubule proteins (Gross, Jacobs-Lorena, Baglioni and

The regulation of the synthesis of ribosomal RNA
(rRNA) represents one of the key events in the control of
cell growth. It is only following a doubling of the
amount of DNA and ribosomal RNA that cell division usually
takes place. The sea urchin embryo represents an interes­
ting case in which such a regulation can be studied.
Here the synthesis of rRNA has been uncoupled from that of
DNA during oogenesis and during the cleavage period, being
very active in the oocyte, silent in the unfertilized egg,
very slow in the cleavage and active again from the begin­
ning of gastrulation (Giudice and Mutolo, 1967; Sconzo et
al, 1972).

Some changes in protein synthesis correlated with
development also have been reported with the use of
Actinomycin D on several embryonic systems, such as that
of the teleost (Schwartz and Wild, 1973) and the sea
urchin. These experiments suggest that the genetic infor­
mation required for the specific stages of development are
precisely expressed under a program of genetic control. Activation of required genes does not necessarily depend on an immediate transcription event, but often depends on the preserved stable messages, including maternal ones in oocytes whose transcription was much earlier than their utilization for translation (Schwartz and Wild, 1973; Silverman, Huh and Sun, 1974). Therefore, it seems likely that some of the proteins translated in the present studies are coded by the stored messages which had already been synthesized at the time of or even before the fertilization of the embryos.

Synthesis of RNA in early embryos has been extensively studied during recent years in a number of laboratories and has been shown to begin shortly after fertilization (Kafiani, Tatarskaya and Kanopkayte, 1958; Wilt, 1963; Brown and Littna, 1964; Decroly, Cape and Brachet, 1964; Nemer and Infant, 1965). Early embryos of *Xenopus* (Brown and Gurdon, 1964; Brown and Littna, 1964, 1966) and of sea urchins (Wilt, 1963; Nemer and Infant, 1965) synthesize predominantly or exclusively polydisperse RNA through the gastrula stage; this RNA is referred to as DNA-like RNA (dRNA).
The occurrence of continuous dRNA synthesis in early embryogenesis is in current conflict with the periodicity of the "morphogenetic function" of cell nuclei found by Neyfakh (1961, 1964, 1965) in embryos of a number of animal species. In fact, for a certain time after fertilization of eggs with a regulatory type of development, the nuclei remain morphogenetically inactive. Microsurgical (Briggs and King, 1959), chemical (Neyfakh, 1965) or radiation-induced (Shapiro and Lander, 1960) enucleation of the egg does not visibly affect early development up to the late blastula stage. After the initial period of inactivity, a period of 'morphogenetic activity' of the nuclei begins which assures the process of gastrulation.

The central concept of organelle biogenesis is that of self-assembly. This means in essence that the macromolecules of which organelles are composed contain within them information such that when mixed under the 'appropriate' conditions, they will more or less spontaneously associate with one another in specific patterns to build up more complicated structures. Self-assembly is a fact, having been demonstrated in a variety of biological systems, through successful test-tube reconstitution of structures from their components.
The cell can synthesize the proper amounts of requisite components at the correct time, and also can control its internal milieu in terms of ionic concentrations, pH, and so forth. Assembly processes are markedly affected by the presence or absence of particular ions, such as \( \text{Ca}^{2+} \), by temperature and by other factors that influence the modes and rates of molecular interactions. There is no doubt that the cell can control many such factors in precise fashion, although there are large gaps in present understanding of the underlying mechanisms.

The mitochondria have an important role in cell metabolism (Ambramova, 1974) in their role as the "power-house" producing the bulk of the energy for different cell functions; the mitochondria supply cells with the substrates for many biosyntheses, regulate glycolysis, etc. They contain their own DNA, but, while they have some autonomy, the mitochondria are at the same time under the control of the regulatory systems of the cell.

Embryonic mitochondria do not differ from mitochondria isolated from tissues of adult animals either in fundamental structural properties or in functions (Carrey and Grenville, 1959; Karasaki, 1959; Berg, Taylor and Hymphreis, 1962; Williams, 1965).
It has been shown by the work of these authors that during embryonic development there is a considerable increase in the intensity of respiration of the embryos, but the mechanism of this intensification remains unknown. In principle, this increase in respiration intensity can be due to an increase either in the quantity of respiratory enzymes or in their functional load. Ambramova (1965) showed that during the embryonic development of teleosts there occur some changes in the mitochondria which are expressed in that, as development proceeds, the intensity of the respiration of isolated mitochondria increases (Ambramova, 1968).

Interest in the biogenesis of organelles has been placed primarily on the mitochondria. Because the organelles--mitochondria, endoplasmic reticulum, lysosomes, Golgi apparatus, and so on--are defined by membranes of various types, this structural study will attempt to illustrate their separation from the undifferentiated cell sap in which they reside during the course of early embryogenesis.
MATERIALS AND METHODS

Breeding of embryos

In order to condition for breeding, adult zebra fish, Brachydanio rerio, were fed daily with brine shrimp, Purina trout chow, and Tetramin (R), a vegetable conditioning food. When suitable breeding color was achieved, the fish were acclimated to $26^\circ \pm 3^\circ$ C in dechlorinated tap water under a 16-hour daily photo-period. Water was continuously reconditioned by charcoal and glass wool filtration and in-tank aeration. A porous nylon net was used to make a trough so that the transparent fertilized eggs, being dimersal, could drop to the bottom of the tank, from where they were collected. The trough was necessitated by the fact that the fish normally eat their own spawn. Breeding was best accomplished with a ratio of one female to four males. However, when fish were kept in schools, there was continuous spawning with 50-100 eggs being found daily. Eggs were laid during the two-hour photo-period following the onset of light in the morning.
Culture of embryos

After breeding, the transparent eggs were removed from the tank bottom to large finger bowls containing dechlorinated tap water maintained at 26°C ± 3°C. Eggs were transferred with a Pasteur pipette into small petri dishes for further development at 26°C ± 3°C incubation.

The developmental stages at various times have been established by Hisaoka and Battle (1958). Time intervals are given from time of fertilization, with embryos reared at 26°C. The developmental stages are presented in Table 1.

Preparation of embryos for ultrastructural analysis

To establish the exact time of membrane assembly and organization, eight zygotes per developmental stage were fixed in 2% gluteraldehyde in 0.2M Sorenson's buffer, pH 7.2, for a minimum of two hours.

After fixation, gluteraldehyde was removed by aspiration and the zygotes were gently dechorionated with glass needles. Following dechorionation, the zygotes were washed gently in 0.2M Sorenson's buffer, and then post-fixed with Palade's fixative for 90 minutes at room temperature. The original formula proposed by Palade in 1952 used a strong buffering solution with osmium tetroxide.
The buffer chosen was the acetate-Veronal buffer of Michae­lis, pH 7.2. Apparently Palade empirically found this to be superior to inorganic buffers, and there was the fur­thur theoretical consideration that bivalent ions of tis­sue would not form insoluble precipitates with it (Pease, 1964).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Age</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>Recently fertilized</td>
</tr>
<tr>
<td>2</td>
<td>25&quot;</td>
<td>1-called blastodisc</td>
</tr>
<tr>
<td>3</td>
<td>35&quot;</td>
<td>2-celled ovum</td>
</tr>
<tr>
<td>4</td>
<td>43&quot;</td>
<td>4-celled ovum</td>
</tr>
<tr>
<td>5</td>
<td>1'11&quot;</td>
<td>8-celled ovum</td>
</tr>
<tr>
<td>6</td>
<td>1'30&quot;</td>
<td>16-celled ovum</td>
</tr>
<tr>
<td>7</td>
<td>1'45&quot;</td>
<td>32-celled ovum</td>
</tr>
<tr>
<td>8</td>
<td>1'50&quot;</td>
<td>Late cleavage</td>
</tr>
<tr>
<td>9</td>
<td>2'11&quot;</td>
<td>Early high blastula</td>
</tr>
<tr>
<td>10</td>
<td>2'33&quot;</td>
<td>Late high blastula</td>
</tr>
<tr>
<td>11</td>
<td>3'32&quot;</td>
<td>Flat blastula</td>
</tr>
<tr>
<td>12</td>
<td>4' 7&quot;</td>
<td>Very late blastula</td>
</tr>
<tr>
<td>13</td>
<td>4'56&quot;</td>
<td>Early gastrula</td>
</tr>
<tr>
<td>14</td>
<td>5'48&quot;</td>
<td>Blastoderm enveloping 1/3 yolk sphere</td>
</tr>
<tr>
<td>15</td>
<td>5'45&quot;</td>
<td>Blastoderm enveloping 1/2 yolk sphere</td>
</tr>
<tr>
<td>16</td>
<td>7'42&quot;</td>
<td>Blastoderm enveloping 3/4 yolk sphere</td>
</tr>
<tr>
<td>17</td>
<td>10'</td>
<td>Close of blastopore</td>
</tr>
<tr>
<td>18</td>
<td>14'</td>
<td>Optic vesicle</td>
</tr>
</tbody>
</table>

(Hisaoka and Battle, 1958)
See Appendix A for the formula for Palade's fixative.

Following osmification, the fixative was removed by aspiration. Embryos were rinsed in several changes of distilled-deionized water, since excessive quantities of osmium tetroxide could react with the alcohol. Dehydration of the embryos was carried through a graded series of ethyl alcohol dilutions (see Appendix A for dehydration schedule).

The purpose of dehydration is to remove all the free water from the fixed and washed tissue and to replace it with a suitable organic solvent such as ethanol. Removal of free water is necessary if the tissue is to be embedded in water-insoluble resins. However, when ethanol is employed for dehydration, the use of a transitional solvent is necessary because most resins are not readily miscible with ethanol. Although it is possible to use ethanol with epoxy resins, the speed of diffusion is very slow, and the impregnation of the tissue is not uniform. Therefore, to remedy this difficulty, propylene oxide was employed. Propylene oxide was used as the transitional solvent between ethanol and resin, since it is a homogeneous compound having an epoxy radical completely miscible with water. It dissolves resins more readily than ethanol,
and because it has a relatively low viscosity, it infil­
trates tissues readily and reduces the viscosity of the
resin mixture.

Immediately following dehydration in alcohol, the
embryos were immersed twice in propylene oxide for 15
minutes. The second change of propylene oxide was removed,
and 1.0 ml. of fresh propylene oxide was added to an equal
amount of freshly prepared resin to initiate the embedding
process. The resin mixture formula can be found in
Appendix A.

After one hour, the propylene oxide/resin mixture
was removed by aspiration and replaced with a mixture of
3 parts resin to one part propylene oxide, with infiltra­
tion occurring overnight at room temperature. The follow­
ing morning, the embryos were placed in a pure resin mix­
ture which was allowed to infiltrate further overnight at
room temperature, since the final appearance of the tissue
is influenced by the duration and the temperature of the
infiltrative process (Pease, 1964).

Following the period of infiltration, BEEM (R) gela­
tin capsules were filled to 1-2 mm. of the top with resin
mixture. The tissue specimen was transferred to the
labeled gelatin capsule on a tip of bamboo stick to achieve the desired position. The gelatin capsules were then polymerized in a drying oven according to the schedule found in Appendix A.

Following polymerization, the gelatin capsules were removed with the tissue specimen remaining embedded in the plastic block. In order for the tissue to be sectioned in a microtome, it was necessary to rough-trim the specimen block with a sharp razor blade. Rough-trimming was carried out with the specimen block clamped to a specimen holder mounted on a specimen-trimming block. The shape one wished to produce was a trapezoid pyramid with vertical or sloping sides. The sectioning face itself was to have parallel top and bottom edges, with its final shape being trapezoid. This ensured even specimen contact with the knife's edge and formation of a relatively straight ribbon of sections.

Standard electron-microscopy sectioning was carried out on a Porter Blum MT--2 ultramicrotome, with sections being viewed with the Hitachi HU-11B electron microscope. Fertilization represents a period of time from the penetration of the male pronucleus into the micropyle and its fusion with the female pronucleus. Because of this time
factor as well as the variances in temperature control in the present investigation, it was necessary to identify developmental stages through thick sections at the light microscopic level, rather than using the developmental schedule of Hisaoka and Battle (1958).

Micrographs were taken to establish the timetable for the laying down of membranes. Since there are no complete atlases of teleost embryogenesis at the ultrastructural level, a collection of various journals and books was used in identifying various organelles and membranes (Rennert, 1972; Gilula, 1973; Lowenstein, 1974; Marthy, 1975).
RESULTS

The embryology of the zebra fish, *Brachydanio rerio*, has been described previously (Hisaoka and Battle, 1958; Manner, 1975). A brief summary, which emphasizes developmental activities at the earlier stages, will aid in the understanding of the description of the results that follow.

The centrolecithal zebra fish egg is surrounded by a chorion. Cleavage is of the meroblastic type, with the first cleavage occurring approximately 1/2 hour after fertilization; ensuing cleavages occur at approximately 20-minute intervals at 26°C, resulting in a cap of cells, the blastoderm. Marginal cells lose their identity liberating their nuclei into the cytoplasm. The cells of the blastoderm continue to reproduce mitotically, resulting in a mound of cells, the high blastula. The high blastula flattens as the peripheral cells begin to migrate over the surface of the periblast, initiating gastrulation. Organogenesis starts within the next seven hours. Embryonic development is complete and hatching occurs typically at 96 hours.
In the present investigation, electron micrographs over the course of early embryogenesis showed progressive diversification of organelles from the early zygotic stages (Figure 1) to the gastrula stage (Figure 11). Increasing diversity was especially noticeable at the late cleavage stage (1'50''), high blastula (3'32'') and early gastrula (4'56'').

1. Eight Cell Stage

Cells of the embryo at this stage exhibited a plasma membrane, nucleus, and yolk transport channels (Figure 1). No nucleoli were observed within the nucleus of the cell. Mitochondria, exhibiting the characteristic double membrane, displayed very little if any cristal structure. In general, the complexity of the cristal structure of mitochondria reflects the energy requirements for the specific functions carried out by the cell. In cells that are relatively inactive, the mitochondria have simple internal structure (Lehninger, 1965).

The cytoplasm was very granular, due to the presence of free ribosomes and polysomal aggregates. "Yolk transport" channels, which were formed by the junction of plasma membranes of three cells in close juxtaposition,
contained osmiophilic inclusions which may be lipid in nature.

2. Late Cleavage Stage

At this stage, embryonic cells were undergoing very rapid mitotic division, forming a cap of cells, the blas­to derm (Figure 2). The nucleus presented itself with its characteristic double membrane (Figure 3). The cytoplasm remained dense, with an abundance of free ribosomes and polysomal aggregates (Figure 4).

In this same figure, mitochondria were spherical, with few cristae. The internal membrane of the mitochon­dria reflect their energy-generating mechanisms known to reside in the cytochrome chains while the matrix is the site of most Kreb's cycle enzymes (Lehninger, 1965). Yolk channels were less numerous than in the eight cell stage.

3. Blastula

At this stage of development, cells exhibited a cytoplasm which was less dense than in the earlier stages, free ribosomes and polysomal aggregates were present (Figure 5).

In the juxtanuclear region of the cell division of a centriole was visible (Figure 5). The chemical
composition, mode of replication, and exact functions of the centrioles are still very poorly understood, but they seem to have an important role in the submicroscopic organization of the cytoplasmic matrix in relation to cell division (Fawcett, 1969).

The mitochondria were found largely in the proximal cytoplasm frequently underlying or lateral to the nuclei (Figure 6). Cristae of many mitochondria were becoming well defined, exhibiting a greater morphological variability than was evident in earlier embryos.

Agranular endoplasmic reticulum was observed beginning to be laid down (Figure 7). The smooth surfaced channels, for the most part, were unbranched and appeared to be an entangled mass of long tubules instead of a network of branching elements. A granular endoplasmic reticulum was still absent.

Large ovoid droplets enclosed within a single membrane (Figures 7 and 8) appeared at this developmental stage. The fact that mitochondria-like structures were contained within Figure 8 suggests the organelles may be cytosegrosomes, the precursors of autophagic vessels. Annulate lamellae were also visible at this stage (Figure 6) in the vicinity of the nucleus. Such structures are believed to be precursors of the Golgi apparatus.
4. Early Gastrula

Cells at this stage (Figure 9) exhibited mitochondria with more numerous cristae than observed at the earlier stages. The cristae of the mitochondria were tubo-vesicular in nature, with the thin folds forming a series of incomplete transverse septa, ending blindly in the interior. Enzymes involved in the respiratory chain are located mainly in the mitochondrial membranes (Lehninger, 1965) and there is reason to believe that they are engaged in particular spatial configurations that favor the sequential reactions of the chain. The energy released by substrate oxidations is transferred ultimately to adenosine diphosphate in oxidative phosphorylation. This results in the storage of energy in the form of adenosine triphosphate.

Golgi cisternae first appeared at this stage (Figure 10). Although the function of the Golgi apparatus is not completely defined, it is known that it plays an important role in the secretory and packing process for export.

An increased distribution of agranular endoplasmic reticulum was observed throughout the cytoplasm (Figure 11). The general concept is that smooth endoplasmic
reticulum increases in quantity and structural complexity during the course of differentiation.
**FIGURE 1**

*Brachydanio rerio*, Eight-cell stage. Exhibits plasma membrane, nucleus (N), yolk transport channels (YT), and mitochondria (M), which show very little internal structure. Cytoplasm contains free ribosomes and polysomal aggregates. x 9,000.
Brachydanio rerio, Late cleavage. Light microscopic profile of embryonic cells at this stage; blastoderm (BD), blastocoele (BC) and yolk (Y). x 450.
FIGURE 3

*Brachydanio rerio*, Late cleavage. Cytoplasm remains dense, with nucleus (N) and mitochondria (M), which are spherical, with few cristae. x 15,000.
Brachydanio rerio, Late cleavage. Dense cytoplasm with abundance of free ribosomes and polysomal aggregates (PA). x 22,000.
Brachydanio rerio...which is the..."ot (CT) 1yIm...x 25,000...
Brachydanio rerio, Blastula. Cells exhibit cytoplasm, which is not as dense as in earlier stages, with centriole (CT) lying near nucleus (N) and mitochondria (M). x 25,000.
Brachydanio rerio, Blastula. Mitochondria (M) exhibiting increased cristal packing; first indication of annulate lamellae being laid down (L) in cytoplasm. x 15,000.
FIGURE 7

*Brachydanio rerio*, Blastula. Cell exhibits an agranular endoplasmic reticulum (AR), cytosegrosome (AV), as well as mitochondria (M). x 25,000.
**FIGURE 8**

*Brachydanio rerio*, Blastula. Cytoskeleosome (AV) with mitochondria-like structure contained within. Agranular endoplasmic reticulum (AR) laid down in this developmental stage. x 25,000.
FIGURE 9

*Brachydanio rerio*, Gastrula. Cytoplasmic organization displays tubo-vesicular mitochondria (M). x 15,000.
Brachydanio rerio, Gastrula. Cell at this stage has nucleus (N), tubo-vesicular mitochondria (M) and agranular endoplasmic reticulum (AR). Golgi cisternae (G) being laid down near nucleus. x 22,000.
FIGURE 11

*Brachydanio rerio*, Gastrula. Cell exhibits increased distribution of agranular endoplasmic reticulum. Mitochondria (M) have acquired their typical morphological state. x 22,000.
DISCUSSION

The observations presented here, based on profiles in electron micrographs during the early embryogenesis of Brachydanio rerio, indicate a change from simple to a complex and specialized organization. During this period, organelles increased in number and in shape and structure, paralleled functional changes in the models.

Differential synthesis of RNA and ribonucleoproteins within the nucleolus within the embryo (Figures 1 and 2) is quiescent in activity during this time. The nucleolus serves as a reservoir for RNA and ribonucleoproteins; without them there is no RNA or ribosomes manufactured in the cell. This structural observation would account for the low mRNA synthesis observed by Gurdon and Woodland (1968) in the amphibian system. An inference may be made, therefore, that the mRNA synthesis during the nuclear...
DISCUSSION

The observations presented here, based on profiles in electron micrographs during the early embryogenesis of Brachdanio rerio, indicate a change from a simple to a complex system of cytoplasmic ultrastructural organization. During this time, the diversity of organelles increased, with many of them becoming changed in shape and structure as development progressed. The early ultrastructural organization of this teleost paralleled functional changes observed in other embryonic models.

Differentiation depends on the differential synthesis of RNA's and proteins. The lack of a nucleolus within the nucleus of the developing teleost embryo (Figures 1-4) suggests that the nucleus is quiescent in activity during this time. The nucleolus serves as reservoir for RNA and ribonucleoproteins; without them there is no RNA or ribosomes manufactured in the cell. This structural observation would account for the low mRNA synthesis observed by Gurdon and Woodland (1968) in the amphibian system. An inference may be made, therefore,
that the proteins which are formed during the early developmental stages are in part translated on so-called 'maternal' messengers, which are synthesized and stored in the oocytes. With the activation of the nucleolus, mRNA could be produced which would then permit the translation of RNA into protein as observed by Gross, et al (1973). Since mRNA was observed to increase from the blastula stage onward in the amphibian embryo (Brown and Littna, 1966; Gurdon and Woodland, 1968), one would also expect the synthesis of proteins to increase. This agrees with the observation by Neyfakh (1965) that nuclei in cells of early embryos are inactive, with a period of 'morphogenetic activity' of nuclei beginning at blastula, which in turn assures the process of gastrulation.

An increase of such macromolecules at this stage would offer the cell the needed "building blocks" to construct its supramolecular structures. This inference was observed by the increase in ultrastructural cytoplasmic diversity in the blastula (Figures 5-8) and gastrula (Figure 9-11). The appearance of autophagic-like vesicles in the blastula stage (Figures 6-7), which may be involved in autodigestion, digest the maternal
messengers, accounting for the peak in mRNA synthesis observed at this stage (Gross et al, 1973). This structural study also indicated an increase in the packing of cristae in the mitochondria as differentiation progressed (Figures 1, 4, 5, 11). Similar observations have been reported in other developing systems. Berg and Long (1964) noted that mitochondria of the 16-cell sea urchin embryo initially exhibited very little if any cristal structure, which increased during early gastrulation.

The mitochondria show a simple morphological state during the very early stages of embryogenesis (Figures 1-4). Since the mitochondrial metabolic activities are directly related to the number and structural complexity of cristae (Linnane, Vitols, and Nowland, 1962; Lehninger, 1965), it is assumed that these organelles must be relatively quiescent in these early stages. Furthermore, since reversible ultrastructural changes associated with respiratory related transformations have been reported for the isolated liver mitochondria (Hackenbrock, 1968), it is possible that the structural changes in cristae reported in this study reflect such physiological variation in Brachdanio rerio.
The internal membrane of the mitochondria reflect the energy-generating mechanisms which are known to reside in the cytochrome chains (Lehninger, 1965), as well as the enzymes involved in the respiratory chain. It has been observed that the intensity of respiration of the embryos increases during embryonic development (Carey and Grenville, 1959; Karasaki, 1959; Berg, Taylor and Hymphreis, 1962; Williams, 1965). Ambramova (1965) observed such an increase in teleost embryos. This increase in respiration intensity could be due to the increase in quantity of respiratory enzymes, reflected by the increased packing of cristae. Such functional observations could be correlated with the structural observations in this study (Figures 1, 4, 5, 11).

If these inferences are valid, very rapid changes in activity could accompany the observed morphological alterations of the organelles during early embryogenesis. Under such physiological changes, metabolism may shift from anaerobic glycolysis to aerobic glycolysis, manufacturing pyruvate, a precursor of acetyl Co-A, which in turn would produce much larger quantities of cellular adenosine triphosphate to accelerate the biosynthetic
machinery of the cell and growth. Once the mitochondria become functional, the sites of protein synthesis and the packaging of macromolecules could increase, due to increased availability of ATP. Such inferences are gathered by the observation that once the mitochondria have acquired their characteristic morphological shape at high blastula (Figures 5-8), other organelle membranes are formed, such as the endoplasmic reticulum and Golgi cisternae at the gastrula stage (Figure 10).

The annulate lamellae, which were observed in a cluster of smooth-surfaced vesicles in the cytoplasm have been observed in developing systems as the precursors of the Golgi complex (Aizenshtadt and Detlaf, 1972). Many problems regarding the development and functional significance of the Golgi apparatus and its interrelationships with other membranous structures have not been fully solved.

With the progression of embryogenesis it was observed that the "yolk channels" observed in the early stages (Figure 1) decreased in number with developmental time. It should be noted that these "yolk channels" were the only cell-junction observed during the early embryogenesis of Brachydanio rerio. The existence of cell junctions was
not pursued in this study, because much higher magnifications were needed to detect these structures.

The present investigation serves as a baseline for future investigations attempting to relate structure and functional changes occurring during early embryogenesis of teleosts. Such correlates are extremely useful in environmental and teratogenic studies.
SUMMARY

Embryos of *Brachydanio rerio* were studied at the ultrastructural level to observe changes in the cytoplasmic organization of embryonic cells through gastrulation. Cytoplasmic organization proceeded from "simple" to complex system in terms of cellular ultrastructure. In the eight-cell stage, cells of the embryo exhibited a plasma membrane, nucleus, granular cytoplasm and mitochondria, which displayed very little if any cristal structure. Cells at later cleavage stages showed a further increase in cristal packing. At the blastula stage, mitochondria exhibited a greater morphological variation than was evident in the earlier stages. At this stage, the smooth endoplasmic reticulum, annulate lamellae and cytosegresomes were all laid down. With gastrulation, mitochondria were well defined in cristal structure, and the Golgi apparatus was present. Such morphological changes paralleled functional changes reported in other embryonic models.
REFERENCES


APPENDIX A
Palade's fixative was prepared as follows:

Stock buffer solution: (0.28 M, pH 7.2)

- Sodium Veronal: 2.88 g.
- Sodium acetate: 1.15 g.
- Distilled-deionized water to make 100 ml.

0.1 N HCl:

- Concentrated HCl (36%, 11.6M): 8.6 ml.
- Distilled-deionized water to make 1 liter

Stock OsO₄: (2)

- OsO₄ (aqueous, 4%): 10 ml.
- Distilled-deionized water: 10 ml.

Fixative: (1% OsO₄, buffered)

- Buffer: 5 ml.
- 0.1N HCl: 5 ml.
- Distilled-deionized water: 2.5 ml.
- 2% OsO₄: 12.5 ml.
Dehydration schedule:

<table>
<thead>
<tr>
<th></th>
<th>Change 1</th>
<th>Change 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% EtOH</td>
<td>15 mins.</td>
<td>15 mins.</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>15 mins.</td>
<td>15 mins.</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>15 mins.</td>
<td>15 mins.</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>20 mins.</td>
<td>20 mins.</td>
</tr>
</tbody>
</table>

Epon mixture:

Stock mixture A "DDSA"  
Epon 62 ml.  
DDSA 100 ml.

Stock mixture B "NMA"  
Epon 100 ml.  
NMA 89 ml.

Final mixture: 6 ml. (A) + 4 ml. (B) + 0.15 ml. DMP-30

Polymerization schedule:

(a) overnight at 35°C
(b) next day at 45°C
(c) next night at 60°C
The thesis submitted by Eleonore Pavnovich has been read and approved by the following committee:

Dr. A. J. Rotermund, Director
Associate Professor, Biology, Loyola

Dr. Harold Manner, Chairman
and Professor, Biology, Loyola

Dr. Jan Savitz
Associate Professor
Biology, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

May 9, 1977
Date

Albert J. Rotermund
Director's Signature