AIM AND SCOPE OF THE PRESENT INVESTIGATION

The study of embryology at physico-chemical and molecular level is a comparatively recent discipline of science. Within its brief history of about two decades we have seen an amazing degree of advancement as evident from the numerous publications and development of sophisticated techniques in this field. Organisms from different levels of the phylogenetic scale have been used as experimental subject, comparative aspects of study are shedding more and more light on the mechanisms and causal analysis of development, newer patterns or processes are coming to light.

Newer fields are going to be opened in molecular embryology with the discovery of newer experimental tools and subjects. Sea-urchin is the most oftrepeated and exhaustively treated material; this is evident from a number of excellent reviews by Gustafson, T.(1); Monroy (1a) and Lallier (1b) and Gross (1c).

In two issues of the series "Advances in Morphogenesis" H. Denis (2) and C. Kafiani (3) presented masterly reviews on Xenopus (an amphibian) and fish respectively. The phylum mollusc has also been able to draw a sufficient amount of interest which is evident from the exhaustive reviews of Collier (4,5). Besides these a number of other invertebrates like Urechis, Nereis, Artemia or brine Shrimp, Ascaris and some insects like Beetle, Acheta domestica, Milk weed bug, Ascidia and snails (like Ilyanassa, Spisula, Limnea etc.) have also drawn the attention of a good number of investigators.
Among the other vertebrates the case of chick and mouse embryo has been investigated at molecular and biochemical level.

The molecular embryology of invertebrates has been treated in the review of Brahmachary (6). From the classical embryological point of view, the development of Limnaea was maximally investigated by Raven and Beenakkers (7), its biochemical aspects have mainly been worked out by Morril, J.B. (1961-65) and Brahmachary et al.

The present investigation mainly involves the protein and nucleic acid synthesis and as in most other fields of molecular embryology, efforts have been made to correlate the results with the regulation of differentiation at the gene level i.e., sequential expression of genetic code as well as control exerted by the factors at the translational level. It is a well established fact that the relation of nucleic acids and protein synthesis to the phases of embryonic development merit attention because these syntheses are the results and the operational criteria of gene transcription.

The interplay of these molecules is responsible for the initiation of cytoplasmic diversity and its final result i.e. cellular differentiation. The segregation of cytoplasmic substances along a radial (cortico-central) direction also plays an important role in the distribution of ecto- and endoplasmic in the cells of Limnaea eggs and thus resulting into cytoplasmic diversity.
Besides the work carried out on normal egg by isotopic tracer techniques, biochemical enucleation by the use of inhibitors like actinomycin and other chemical agents and preparation of homogenized i.e., disorganized egg systems have also been attempted. Another feature of this enquiry is the time mapping of the molecular processes. This includes changes in the rates of RNA synthesis with morphogenesis. Keen interest was taken specially to note the changes in the rate of biosynthesis of ribonucleic acid with the advancement of morphogenesis. The stability and catabolism of different classes of RNA at different developmental stages have also been studied, specially by the application of pulse and pulse chase techniques of different duration.

The investigation of heavy RNA, the study of their rate of genesis and decay and their intermediate catabolic product may shed light on synthesis of ribosomal class of RNA.

Methylation of nucleic acids, specially heavy or ribosomal and soluble RNA is a modern topic. By the use of $^{14}\text{C}$-methylmethionine, the nature of methylation and fate of the different classes of RNA have been studied.

The second section of this investigation embraces the nature of metabolism of S-containing compounds during early mitotic cycles and in the course of later embryogenesis. Rapkine (1931) was the pioneer in the field of metabolism of sulphur-containing substances while he was working out the problems of chemistry of mitosis. Later, Mazia and Dan discovered a number of important facts regarding the role of
Sulfur containing compounds specially - SH (Sulfhydryl) group in mitosis which has been concisely incorporated in the work of Mazia (11, 11a, 11b). As a result of their research, significant contributions have been made to our knowledge of the role of Sulfur in the biogenesis of S-containing proteins, the role of inorganic sulfate in the biosynthesis of heteropolysacharides; biosynthesis of S-containing nucleotides and so on. In this thesis we have also described the attempts to characterize the compound or compounds in which inorganic sulfur may be incorporated.
General Description of the Molluscan Development with special reference to Limnaea Sp.

*Limnaea sp.* is a gastropod belonging to phylum Mollusc. This organism is an oviparous freshwater mollusc; the four common species are *Limnaea stagnalis, Limnaea limosa, Limnaea auricularis* and *Limnaea peregra*, the first being the commonest Indian pond snail and mostly used for our experimental investigation while the last one is the offspring of isolated self-fertilizing individuals. The classical and descriptive embryology of *Limnaea stagnalis* has been exhaustively investigated and reviewed by C.P. Raven (7).

Unlike the majority of marine gastropods, fresh water species usually do not develop free living veliger larvae and possess some characteristic properties such as *direct development* i.e. the development proceeds directly within the egg capsule until the formation of a fully differentiated young snail.

The eggs of *Limnaea* are non-cleidoic and ripe egg cells are about 120μ in diameter. Like other molluscan eggs these are spirally cleaving and have a mosaic pattern. Fertilization of the eggs takes place during the metaphase of the first maturation division. In gastropods the fertilization may occur at any place of the egg surface.

The early development of the molluscan egg is mainly dependent on ooplasmic segregation but the basic causal principles await further clarification. Raven (12) has put great
emphasis on cortical information also. A certain degree of regulative differentiation has been found to occur in a number of mosaic embryos (in some gastropods also) but in this species the majority of the differentiation processes occur in areas which have been irreversibly determined already during very early stages. The important phases and determinations in the embryonic development of gastropoda take place as early as in oogenesis. The egg cortex is supposed to contain the highly stable imprints for the regulation of the cleavage processes (12).

The development of Limnaea can be followed from the very beginning i.e., from the division of zygote by microscopic observation. At each division the cleavage furrows cut deeply into the egg. The ooplasmic segregation begins in the uncleaved egg and continues as a more or less unequal distribution of substances throughout the egg during subsequent cleavages. Upto the second cleavage, the cytoplasmic substances are distributed about equally among the blastomeres. At the third cleavage namely the formation of 8 cells from the 4-cell stage a significant difference in the cell sizes is evident. 4 micromeres are formed at this stage. Henceforth unequal distribution of cell substances continues as a result of micromere formation and spiral cleavage.

The Limnaea eggs are surrounded completely by a jelly capsule. The fluid within the capsule is of importance for growth because it is rich in nutritive material which is taken up by the embryos in the course of development. In Limnaea at the 40 cell stage of development the superficial cells of the
Egg ingest "albumen" from the surrounding egg capsule fluid which is laid down in the ectoplasmic part of the cells in special albumen vacuoles.

The normal sequence of developmental stages of Molluscs (Limnaea).

The development of oocytes takes 2-3 months when maturation is completed. Like other pulmonate snails Limnaea is also a hermaphroditic species but usually copulation between two individuals takes place. During copulation spermatozoa are exchanged between both partners. Insemination occurs within the oviduct. In addition, during the formation of an egg batch insemination of the individual eggs occur one after the other in the sequence in which they are included in the egg mass. Later, meiotic and first cleavage divisions occur in the same sequence. Therefore, the first phases of development moves as a wave along the eggs from one end of an egg batch (13). At the metaphase stage of the first meiotic division further development is arrested. This repression is released by the insemination.

Fertilization takes place between the beginning of maturation and metaphase of first maturation/division. The just laid eggs are in the stage of early anaphase of first maturation division. The following stages of the development of Limnaea eggs could be easily followed by microscopic observation and the characteristic stages could be recognized by some particular features.
The uncleaved egg:

The eggs are laid in the form of egg batches containing from 20 to 80 or even more egg capsules. Three different egg membranes can be distinguished. The primary or vitelline membrane is excreted by the cell cortex of the cocyte and is a very thin and delicate membrane, nearly invisible in the light microscope but definitely seen with the aid of the electron microscope. The secondary membrane or chorion is formed by follicle cells. It envelopes a rather large capsular space which is filled with a nutritive liquid. The egg cell swims freely within the capsule liquid. The tertiary membrane or jelly coat is formed by the cells of the oviduct. It is a jelly skin which enwraps the free egg capsules to form a united egg batch(13). At least in the species studied here the eggs, just after being laid, look like distorted spheres. But soon they become perfect spheres with very smooth surface contours. The stage persists for 2-3 hrs (when the first cleavage takes place). This period depends partly on the ambient temperature and partly on the inherent quality of the egg mass, i.e. variation among different egg masses is evident. In the ordinary room temperature (20°C. approximately) the time taken from post-laying period to first polar body elimination, from first polar body rejection to second polar body extrusion and from second polar body extrusion to first cleavage (cytokinesis) of the zygote have been determined in our laboratory condition, as 70 min, 55-65 min and 70-75 min respectively.
Fig. 1 - Schedule of development of Limnea and Sipunculus.

**Beginning of Oogenesis**

- **Total Period of Oogenesis**: ~30 days

- **Fertilization starts with entry of the sperm**

**Mid-oogenesis**

- **2 days**: Marked RNA synthesis
- **Last 5 days**: Last 3 days

**Extrusion of the 1st. polar body**

**Extrusion of the 2nd. polar body**

**Last 3 days**

**Fertilization ends with meeting of the pronuclei**

**Oviposition** (i.e., laying of eggs)

**1st. cleavage**

(i.e., formation of 2-cells)

**1st. laying of eggs**

**End of Oogenesis**
These are only slightly different from Raven's data. At the telophase of the first maturation division the first polar body is extruded. After late anaphase of second maturation division the second polar body is extruded, sperm aster is formed just before the elimination of the first polar body. The polar bodies are however visible for a long time thereafter.

Unlike other gastropods and lamellibranchs there is no actual fusion of the male and female pronuclei as established by Raven. The pronuclei apply themselves closely against each other and after the copulation of pronuclei has taken place, the cleavage spindle makes its appearance. Before cleavage a slight indentation is observed which causes the individual egg to take the shape of a dumbbell. The direction of cleavage planes and the mutual sizes of blastomeres are dependent on (a) the direction and place of cleavage spindles and (b) on local activity of the egg cortex directly influencing the course of the cleavage furrows (8, 9).

A schematic diagram of the schedule of development of *Limnaea sp.* at room temperature (at 20-25°C.) is presented in Fig. 1.

**The Morula:**

After the first cleavage each cleavage cycle persists for about one hour (at the room temperature) up to 8-cell stage when the macromeres and micromeres are well distinguished. Subsequently there remain a phase lag of the division of the
blastomeres because of the slower rate of division and greater
time required for segmentation of the micromeres in comparison
to macromeres. The reappearance of the nucleoli is evident from
16-cell stage onwards (10). Just half an hour after each division,
the whole egg mass assumes a 'compressed' stage so that the
two blastomeres are not clear and the egg mass almost seems to
be a single cell. In the next half hour, the cleavage furrows
develop and the dividing cells are very prominent. By repeated
division, the egg mass is converted to a ball of cells.

The Blastula:

After repeated divisions the advanced morula merges into
the blastula. This is easily distinguished from the gastrula
because of morphological changes.

The Gastrula:

The stage starts with an invagination preceded by a
repeated division of the micromeres. A pit-like structure
is seen at the vegetative pole. The regular arrangement of
the columnar epithelium and appearance of a blastopore is
found at this stage. At the end of gastrulation invagination
of the shell gland begins. In Limnaea, mesoderm formation
begins a considerable time before gastrulation.

The Trochophore:

In Limnaea sp. a more or less typical trochophore larva
is usually formed. It can swim freely by rotating within the
egg capsular fluid, with the aid of its trochs.
The typical trochophore larva is almost a sphere; the upper pole is provided with a tuft of long cilia and sensory cells - the so called apical sense organ. At a point on its equator is the mouth leading into the gut which passes via a little round stomach and a short intestine to open at an anus at the lower pole then running round the sphere just above the equator is the main ciliated girdle, - the prototroch or pre-oral band as it is variously called. The cilia give the little sphere a spinning rotation like a top; this sends it waltzing along so that it can continually draw into the mouth little particles of food from its environment by ciliary currents. In the earliest trochophore this movement is very slow but it gradually increases with the aging and is quite vigorous at the late trochophore stage when the peripheral part of the embryo is found to be densely populate by a large number of vaculated cells which can be stained deeply with neutral red.

The rudimentary shell gland in this stage is represented by a thickening of the ectoderm, which first invaginates (14).

The Veliger:

In most gastropods or snails the prototroch grows out into a pair of large lobes, one on either side; these extensions not only support a greater weight by increasing the ciliated area, but also enable the larva to direct its movements on a perfectly even keel. It now becomes known as veliger. In some veligers the lobes are drawn out into great arm like processes giving the
Some important stages of development of Limnaea sp.

A. Egg batches of L. Stagnalis. s, t, represent secondary and tertiary egg membranes (taken from Hess (13)).

B. 2-cell stage.

C. 8-16 cell stage.

D. Very early trophophore.

E. Very advanced trophophore.

F. Very advanced veliger.

G. Pre-hatching shell.
animal a most remarkable appearance. In this stage the most important characteristic feature of the anatomy of gastropods mollusc is the twisting of the main part of the viscera through $180^\circ$ quite independent of the spiral twisting of the shell. In case of Limnaea, the true veliger is not found but larva passes through characteristic stages within the capsule. The whole intestinal tract shows further development and takes deep neutral red stain. The pigmented eyes become vesicular and the larval beating heart becomes more clearly visible. The shell begins to be visible around the dorsal and posterior side (14).

Some of the important stages are represented in photographs. (Fig. 2)

No true metamorphosis is found in case of the development of Limnaea. In Limnaea, there is direct development without free living larval stage within firm egg capsule from which the young snail finally hatches (leaving the egg capsule as folded empty hankarchief). The hatching or emergence of young, normally developed snails occurs at about the twentieth day after ovoposition.
References


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