STUDIES ON CERTAIN ASPECTS OF EMBRYOGENESIS AND SEXUAL TRANSFORMATION IN THE ROTIFER ASPLANCHNA

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dedicated to my parents

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ASPLANCHNA BRIGHTWELLI

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Supervisor's Note

The thesis entitled " Studies on certain aspects of embryogenesis and sexual transformation in the rotifer Asplanchna" is a piece of original work of Shri N.V.Gopinath.

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CHAPTER I

CHAPTER I

INTRODUCTION

1.1 General Introduction

Rotifers constitute an interesting group of organisms. Yet they have attracted comparatively less attention of biologists. In recent years significant attempts have been made inorder to study certain phenomena typical to the group namely, cyclomorphosis, sexual mode of reproduction, and constancy of nuclear number in the adults. Owing to these peculiarities the rotifers could be important 'Biological tools' for investigations in the field of developmental biology. Indeed, computer oriented studies of the pattern of growth and symmetry are in progress in the laboratories of competent workers (Levinthol, 1971). It should be important at this stage to be able to describe certain aspects of the biology of this group.

Rotifers normally reproduce parthenogenetically although sexual reproduction intervenes in the life cycle for short periods. The transition between asexual and sexual reproduction is controlled by the environment. Laboratory studies with the genus Asplanchna have shown that it is possible to identify specific molecules as inducers, which act on embryos in utero to modify their development and determine the sexuality of the progenies.

The present study is an attempt to analyse the developmental phases and the nature of influence of the inducer molecules, on the developing embryos in the transition of asexual and sexual reproductive cycles in Asplanchna brightwelli sensu latissimo de Beauchamp, 1951.

1.2 Review of literature

Rotifers were first described by Leuwenhoek (Dobel,1960). Brightwell (1848 a, b) described Asplanchna brightwelli and gave relevent explanation on the structure of the males of these rotifers. The earlier studies (Linnaeus, 1758; Muller 1786; Cuvier, 1798; Lamarck, 1801; Dutrochet, 1812,1813 etc. cited by Hyman 1951) were mainly concerned with the identification and listing of these under certain phyla. Ehernberg (1838) separated these animals under the name Rotatoria, although the name Rotifera given by Dutrochet (1812) was already in common use.

Most of the later accounts on rotifers deal with the

morphological descriptions and in a few cases on the cyclomorphosis. The widely adopted taxonomic publication of Hudson & Gosse (1886-1889) was followed by leading workers in this field especially Beauchamp (1909), Wesenberg-Lund (1922-1927) and Harring & Myers (1922-1928) and several others. Edmondson (1963) made unique contribution by giving a chart of classification of rotifers up to the generic level. As a result of cyclomorphosis detectable variations often tend to confuse attempts for identification. A recent report of Sudzuki (1964) on the systematics of Japanese rotifers has been subjected to criticism (Gilbert, 1968). Some of the earlier works in the present worker's laboratory have dealt with the cyclomorphosis of certain rotifers and their taxonomic evaluation (Nayar, 1965, a,b,c, 1966-1968).

Cyclomorphosis in rotifers has been studied intensively for many years and has been reviewed by Wesenberg-Lund (1930) and Buchner et al (1957). But the causes and the significance of the phenomenon in nature are still subjects of dispute. Recently this subject has been extensively reviewed by Hutchinson (1967).

Among rotifers, cyclomorphosis is pronounced only in certain genera like Asplanchna, Brachionus and Keratella. Beauchamp (1951) reported Asplanchna as a difficult and variable genus. Systematics of the genus Asplanchna are still not well defined. The pronounced cyclomorphic effects in these organisms have resulted in the addition of many species under this genus. Edmondson (1963) has given an account of seven species following the catalogue of Wiszniewski (1954), leaving apart two species A. amphora and

A. ebbesborni as the polymorphic forms of A. sieboldi. The species differentiation characteristics within this genus were mainly based on the hump morphology (Rousselet, 1901) or on the number of nuclei in the gastric glandand the vitellarium (Sudzuki, 1964) or on the nature of the tooth in the inner margin of the scapus of the mastax (Daday, 1888). Powers (1912), Mitchell (1913 a ; b) and Mitchell & Powers (1914) found that the saccate A. brightwelli hatched from the resting eggs gave rise to large humped and companulate forms when dietary changes were made. Thus, the hump formation (Powers, 1912; Birky,1964) and the changes in the nuclear number of the vitellarium and the gastric glands (Birky & Field, 1966) have been experimentally proved to be the effects manifest in polymorphism owing to dietary change of the organism. Similarly, the tooth formation in the scapus is also reported to be the effect of polymorphism in Asplanchna. Hence Gilbert (1968) has also included A. intermedia tentatively under the species A. brightwelli. Although de Beauchamp (1951) has erected a supra species A. brightwelli sensu latissimo comprising A. sieboldi, A. ebbesborni, A. amphora, A.leydigi, and A. intermedia, further studies are required to determine the exact status of these species.

In Keratella and Brachionus, cyclomorphosis was reported to be effecting the spine length. Lauterborn (1901, 1904) Kratschmar, (1908) and Hartman (1920) found that terminal spine in Keratella cochlearis and K.aculeata grew shorter in sucessive generations from winter to summer, and later disappeared. Experiments conducted by Kratschmar (1913), Kolisko (1938) and Ruttner-Kolisko (1946, 1949) on Keratella quadrata and K. volga have shown that the spine length reduced rapidly in successive generations on transferring

the organisms to laboratory cultures. No conclusion could be drawn from their observations as to how the changes were effected.

In Brachionus, Gilbert (1966, 1967) and Gilbert & Waage (1967) reported the effect of 'asplanchna substance' inducing the formation of postero-lateral spine in the laboratory cultures and in the natural environments. Gilbert (1966) considered it as a predator-prey relationship between Asplanchna and Brachionus.

Anatomical studies of rotifers were started by Zelinka (1891) and followed up by a series of workers, in Epiphanes (Hydatina) (Beauchamp, 1907 , 1909; Murray, 1910; Martini, 1912); in Asplanchna (Tannreuther, 1919, 1920; Nachtway 1925); in Brachionus (Stoszberg, 1932); in Pedalia (Remane, 1932); etc. A widely accepted account of embryology has been given by Nachtway, (1925) on A. priodonta. Later de Beauchamp (1956) described the development of Ploesoma and Pray (1965) that of Monostyla. A recent report on the experimental studies on the embryology of A. girodi is that of Lechner (1966).

Rotifers show the phenomenon of eutely, where each organ shows a constant and species-specific number of nuclei. Martini (1912) reported the nuclear number in Hydatina senta to range from 900-1000 with each organ having a constant number of nuclei. On the contrary Shull (1918) recorded inconstancy of nuclei in the same organism. However, later reports of Tannreuther (1919, 1920), Nachtway (1925) and Hyman (1951) stated that mitosis ceases half-way during the development and further orientation of the cell leads to an adult with constant number

of nuclei in each organ. But studies of Birky & Field (1966) have proved the variation in the nuclear number of certain organs (vitellarium and gastric glands) of Asplanchna. Birky & Power (1968) have shown the nuclear number of other organs, including that of the body wall, to be constant in their position and number. This view is supported by the observations of Levinthol (1971, personal communication) in A. sieboldi, in which the brain is found to have 182 nuclei. Hence, it is presumed that the nuclear number in rotifers remains constant in each organ with the exception of vitellarium and gastric glands.

Rotifers are exclusively dioecious and exhibit marked sexual dimorphism (Hyman, 1951). Males were first reported by Brightwell (1848 a) in Asplanchna. Males in rotifer order seisonecea have the same grade of organization as females and are abundant at all times (Remane, 1929). Bdelloids are devoid of males. Pronounced sexual dimorphism is reported in Ploima. Wesenberg-Lund (1920-'23) has explained the male structure and organization in rotifers like Epiphanes, Collotheca, and Rhinoglena. Rousselet (1897) has referred the males of Asplanchna as "perambulatory bags of spermatozoa". Whitney (1917, 1918, 1919), Shull (1913) and Shull & Ladoff (1916) have worked on organs of the males and their development in Hydatina senta, Kahn (1921) Tauson (1927) and Mitchell (1913) in Asplanchna and Solberg & Dougherty (1959) in Brachionus. The structure of the sperm and its development was studied by Tauson (1927) in detail in Asplanchna. Latest study is that of Koehler (1965) on

spermatozoa of A. sieboldi.

The general structure of the rotifers has been given by Lange (1913), Remane (1929), Hyman (1951), Birky (1967b), and others. The life cycle of a rotifer was first worked out by Cohn (1856) in Brachionus urceolaris, and later by Maupas (1890a,b, 1891) in Hydatina senta. Observation of the mictic and amictic females, which differ markedly in reproductive parameters, was reported by Miller (1931) and de Beauchamp (1935). Birky (1964, '65, '67a) made a thorough study of the physiology and life cycle of Asplanchna brightwelli and explained the sexual and asexual cycles in detail. He has supported the views of earlier workers (van Enlanger & Lauterborn, 1897; Tauson,1924; Storch, 1923, 1924 etc.) that the amictic maturation division releases one polar body and forms diploid female progeny, while the mictic generation produces two polar bodies and form haploid male progeny. But the reports on the chromosome studies, carried out so far, are however, confusing. Whitney (1924, 1929) reported 26 haploid chromosomes in A. intermedia, while Tauson (1924 , 1927b) claimed a haploid number of 12 chromosomes in the same species. Koechler (1965) made detailed study of the types of spermatozoa in A. sieboldi, but he did not mention the chromosome number, except stating that the absolute chromosome number has not been determined with accuracy. Early literature suggests an interesting complication that males are apparently haploid organisms whereas females are diploid. Hence, the haploidy in males remains inconclusive.

Later studies on the life cycle of rotifers have shown that the mictic female production was caused due to the effect

of certain environmental factors. The genera Asplanchna and Brachionus have been used extensively in studies on the environmental control of mictic female production. These studies have been reviewed by Gilbert (1963, 1968), & Pourriot (1965).

The major influencing factors are reported to be starvation (Nausebaum, 1897; Mitchell, 1913b), changes in culture medium (Shull, 1911, 1912, 1915, 1916, 1918,1925; Kahn, 1921); temperature (Maupas, 1891; Moro, 1915); pH (Tauson, 1925 ,1926, 1927a,b; Luntz, 1929); photoperiodism (Laderman & Guttman, 1963); dietary change (Whitney, 1916, 1929), & population density (Wesenberg-Lund, 1930; Hertal, 1942; and Gilbert, 1963). However, Tannreuther (1919) and Whitney (1929) reported that the dietary inclusion of Chlamydomonas resulted in the production of mictic progeny in A. amphora. Buchner & Kirchle (1965) and Kirchle & Buchner (1966) repeated the experiment by adding Chlorella or Euglena to the cultures of paramecia fed A. sieboldi and concluded with successful results in mictic production. Gilbert (1967) extracted certain plant lipids from spinach leaves and found them to have profound influence on the reproductive cycle of Asplanchna. Gilbert & Thompson (1968) isolated and identified the plant lipid content as alpha tocopherol (Vitamin E) which acted as the inducing factor for the mictic female production in Asplanchna.

Recent reports of Birky and his co-workers (Birky, 1968, 1969; Birky & Power, 1969), based on their studies on polymorphism in rotifers indicate that the effects of tocopherols are not confined to the changes in the reproductive cycle alone, but that

they also induce certain polymorphic characters like the bodywall outgrowth (BWO) in Asplanchna. The mechanism by which tocopherol induces the mictic production in Asplanchna, however, is not understood.

The function of vitamin E compounds in invertebrates is very poorly known. Vishcever, et. al. (1938, 1939) reported that Vit. E enhances vigour and fecundity in cladoceran Daphnia magnum. Further, vit. E is suspected to stimulate egg-sac formation (Jakobi, 1957) in copepodes, and spermatogenetic activity in cricket Achata (Meikle, et. al., 1965). Asplanchna is the first reported organism in which vit. E has been found to initiate either a transition from parthenogenetic to sexual reproduction or a marked change in body morphology (Gilbert& Thompson, 1968).

CHAPTER II

CHAPTER II

MATERIALS AND METHODS

2.1 Test Organism

Two monogonont rotifers, namely Asplanchna brightwelli (Fig. I.1) sensu latissimo and Asplanchna sieboldi (Fig. X|.3) were used in the present study. A. brightwelli was used for the study of embryogenesis and sexual transformation and A. sieboldi for comparison of the observations on A. brightwelli with the variations known in the BWO response of A. seiboldi (Birky, 1968).

A. brightwelli was collected from a temporary pond in Pilani (Rajasthan) and the cultures were maintained in the laboratory throughout the work (1968-1972). On two occasions, the stock cultures were lost; due to water contamination in June, 1969 and due to temperature rise in the BOD incubator in January, 1971. Both the times fresh cultures were developed from resting eggs stored in Peace solution at 4°C. For all experimental purposes the laboratory bred animals were used. Specimens of A. seiboldi were obtained from Dartmouth College, Hanover, U.S.A., through the courtesy of Dr. John J. Gilbert and the cultures were maintained in the same manner as for A. brightwelli (Table 2.1). Both the species were identified by Dr. Gilbert.

2.2 Culture methods

Rotifer cultures have been maintained in laboratories, by different investigators, employing different rearing methods (Nathan & Landerman, 1959; Dougherty 1963; Birky 1964; Lansing 1964; Gilbert 1967, 1968). All the species of Asplanchna except A. priodonta have been successfully reared in the laboratory by certain workers. In the present work a few of these known rearing methods have been employed with modifications wherever needed to suit local conditions, in order to maintain laboratory cultures (Table 2.2, Graph 2.1).

Table 2.1

Species	Place of collection	Source	Type of specimen	Date of collection
i. Asplanchna brightwelli sensu lattissimo de Beauchamp, 1951	Pilani, India	Temporary pond	Female	23.8.1968
ii. Asplanchna sieboldi Leydig, 1854	Dartmouth College, Hanover, U.S.A.	Lab. culture, Deptt. of Biol. Sciences	Females & resting eggs	3.4.1970

Table 2.1 Source of stocks of Asplanchna in the present investigation.

Culture medium	No. of Amictic Female mothers	Ave.size adult female in μ	Average (96 hrs) offspring females	Remarks
1.Artificial pond water (Lansing, 1964)	10	600-850	5.3	
2.Baked lettuce (Birky, 1964)	12	800-1000	6.33	
3.Gilbert's media (GM)(Gilbert, 1967, '68)	9	800-1200	7.33	
4.Gilbert media (GM) with Hay Infusion	10	800-1000	7.6	1% Hay Infusion in Gilbert's media
5.Gilbert's media with Glucose	8	900-150-	6.75	1% Glucose(Analar) Gilbert's media
6.Malted Horlicks in Hay Infusion	10	500-800	8.4	0.05 gm Horlicks* in 98 ml distilled water & 2 ml of Hay Infusion

Table 2.2 Fecundity of Asplanchna brightwelli in different media.

* Hindustan milk food manufacturers, India; Horlicks Ltd., Slough, England

Cultures were maintained in glass cavity blocks(10 ml capacity), each containing 5 ml culture medium and 15-25 organisms. The PH of the media was maintained at 7.5. The stock cultures were daily fed with Paramecium aurelia propagated on paddy extract (prepared with 50 gms. of dried straw of Oryza sativa in one liter of boiled water, adjusted

to pH 6.5 with calcium carbonate), containing Aerobacter aerogenes. Care was taken to keep the cultures free of contamination from other organisms, especially bacteria and other protozoans. For feeding, a paramecium concentrate was prepared by filtering the paramecium culture through a nylon fabric (5 μ) followed by centrifuging. The sediment so obtained was washed in the rotifer culture media and recovered by another centrifugation. The settled mass of paramecia was directly transferred to the culture in the cavity blocks. Hay infusion method was found suitable for the maintenance of healthy paramecia cultures over a period of eight to nine weeks. The baked lettuce method of Sonneborn (1950) and Birky (1964) was also tried and found effective.

For the culture of Asplanchna, Gilbert's medium (GM) slightly modified with addition of glucose was used in experiments on the succinic dehydrogenase activity in Asplanchna (Gopinath & Datta Gupta, 1971a). The organisums showed 10-25% increase in size in this medium. In malted horlicks, with hay infusion medium, the organisms showed an increased (12-15%) reproductive rate. Asplanchna has also been found to survive successfully even in salt solutions (Hopkins & Peace, 1937; Dryl, 1959; and Gilbert, 1963), although artificial pond water (Lansing, 1964) was found less suitable. It is clear from these observations that the composition of the culture media has a significant influence on the growth and reproductive rate of Asplanchna, even though the organism can grow in varied culture media.

Care was taken to maintain healthy cultures of Asplanchna by avoiding starvation as well as accumulation of paramecia in the culture blocks. Excess of paramecia in the Asplanchna culture was found

to cause an inhibitory effect on the growth of the organism. Hence, the concentration of paramecia in the rotifer medium was maintained around 10^3 per ml, and the feeding was done in every 24 hours.

2.3 Fixation

Asplanchna being a very delicate and illoricate organism with a saccular structure hitherto employed methods for fixation (vide infra) were found unsuitable. Birky (1967) has suggested the exposure of Asplanchna to o.1% Novocaine for about 15 minutes before fixation. Later he (1969) recommended 0.01% Osmic acid for fixation and 70% ethanol for dehydration inorder to get well expanded specimens. Sudden dehydration is known to result in osmotic imbalance causing displacement of internal organelles, breakage of muscle strands and nerves, and distortion of cellular structures. This procedure, however, is useful as a general method for the gross anatomical studies, although cellular clarity and orientation pattern appear to be disturbed and distorted.

A series of experiments, as given in table 2.3, were conducted to find suitable technics for relaxation of the organism and its fixation.

Fixatives	Conc. Used	Temp. °C	Fixation time	% well fixed	Remarks
1. Acetone (BDH)	Con.	Room temp. 28	10 min.	98%	Nuclei clear; Not used for histochemical analysis
2. Bouin's fluid aqueous	5%	60	1 hr.	75-80%	Good results with cytochemical studies
3. Formalin Commercial (BDH)	5%	60	6 hrs.	80-85%	Imparts yellow color to the organism
4. Formalin Neutral (BDH)	5%	60	6 hrs.	80-85%	Specimens become transparent and clear
5. Osmic acid	0.01%	28	3 min.	40-50%	Requires 70% alcohol treatment to relax the specimens
6. Procain hydrochloride and Formalin	5% 5%	28	5 min. 6 hrs.	70-80%	PHC narcotized and fixed in formalin

Table 2.3 Effects of certain fixatives used for Asplanchna

Fixation procedures

Acetone: Organisms were collected in a cavity block containing a small quantity of water to which equal volume of acetone (BDH) was added. Fixation was immediate and organisms retained their original extended shape with utmost

transparency. After 5-10 minutes fixation, the nuclei in the various organs of the animal appeared as yellowish spots under the stereomicroscope. However, acetone fixation interferes with histochemical studies, as it dissolves most of the cellular inclusions.

Formalin (5% at 60°C)

This is a modification of Edmondson's (1963) hot water method adapted with an ordinary fixation procedure and has the added advantage of relaxing and fixing the organisms at one stretch. Animals were collected in a little volume of water to which identical volume of 5% neutral formalin, heated to 60°C, was added and kept covered with glass plate until the admixture cooled down to room temperature. This method yielded about 80-85% well-fixed organisms in normal stretched condition. Aqueous Bouin's, and Carnoy's showed good results with the above procedure, especially for those materials which were sectioned for cytological studies.

Procaine hydrochloride(PHC)-Formalin method.

Organisms were taken in a little amount of water and an equal amount of 5% aqeous solution of PHC (Metropolitan Drugs) was added. PHC was found to effect the muscular contraction of the animals and in about 5 minutes time the rotifers settled down in an extended condition showing movement of the ciliary circlet of corona only. Addition of fixative (Formalin, Bouin's, or Carnoy's) at

this stage fixed and killed the organisms in their natural shape and structure.

Subsequently organisms were preserved in 5% formalin or 70% ethanol.

2.4 Embedding and Sectioning

Rotifers, being minute delicate organisms, have created spacial problems in embedding and sectioning. The conventional paraffin embedding method caused appreciable distortion even at low concentrations of the processing chemicals. Various methods recommended for the embedding of rotifers are parloidion embedding for sectioning of Asplanchna (Whitney, 1929), methacrylate embedding (Birky, 1967); double embedding method with butyl methacrylate and paraffin for sectioning Philodina (Meadow, 1965) and epon embedding method for electron microscopy of Asplanchna (Luft, 1961). For maximum cytological stability of delicate organisms Davenport (1971, personal communication) suggested primary infiltration of the material with 5% celloidin and subsequent precipitation of the celloidin with chloroform followed by the paraffin processing as usual. But the application of this method was found to interfere with cytological observations. During the present investigations, two embedding technics have been found to be particularly convenient. They are as under:

1. Paraffin embedding using waterwax (carbowax; polyethylene glycol) as an intermediary matrix (Gopinath & Datta Gupta, 1971b) and

ii Gelatin embedding for freeze microtomy (based on Baker, 1944).

Paraffin embedding using waterwax as an intermediary matrix

Fixed specimens were washed well and transferred to a cavity slide with a little amount of water, sufficient to immerse them. Flakes of carbowax 4000 (polyethylene glycol - E.Merck) were added in small amounts at short intervals until the medium was brought to a saturation in 4-6 hours at room temperature. The materials were retained in the saturated solution of waterwax for one hour. This mode of infiltration was found convenient as it involved no transfer of animals and thus could avoid the damage of the material. The waterwax infiltrated materials were then dehydrated using ethanol (75% to absolute), over a period of 2 hours. The specimens were then transferred to a mixture of absolute alcohol and methyl benzoate (1:1) for 5 minutes and then cleared in pure methyl benzoate for 10 minutes. Carbowax was removed completely by two or three changes in benzene. This was followed by the benzene-paraffin, melted paraffin sequence involving an infiltration time of 6 to 8 hours. The blocks were prepared in the usual way and sections cut with an ordinary rotary microtome.

Gelatine embedding method

Reagents : Gelatin 20%

20 gms of gelatine (Gold normal Pulver, Riedel) were dissolved in hot distilled water (60°C); filtered through Whatman No.1

while hot and stored in refrigerator. A small amount of thymol was added to protect it from bacterial and fungal growth.

Formal calcium:	Formaldehyde	40%	100 ml
(Bakers, 1946)	(BDH)		
	Distilled water		500 ml
	Calcium chloride 10%		100 ml
	made upto one litar with water; a few marble chips were added for neutralisation.		

Procedure

Fixed specimens (vide supra) were washed well in running tap water, until the fixatives were removed. A small block of gelatin, cut with a dissection knife, was allowed to liquify in a watch glass to which the specimens were transferred. Infiltration was carried out for 24 hours at 37°C. A pin puncture on the body wall of the organism enabled quick and better infiltration. Embedding was done in another portion of gelatine and the material was placed in a refrigerator for solidification. The solidified material was cut into blocks and hardened in cold formol-calcium (4°C) for 36 hours. The blocks were trimmed and sections were cut on a freezing microtome (E.Leitz Wetzlar, with CO_2 supply). The sections (3-5 μ thick) were floated in distilled water for mounting and observation; or stored in 5% formol-calcium. Wet mounts were made in glycerine or glycerine jelly.

Mounting

Wet mounts of the organisms were prepared using 10% glycerine and following the mounting methods of Myers (1936). They were then dehydrated in a desiccator. For the observations of the mastax, specimens were treated with KOH according to the method described by Myers (1937).

Wet preparations were sealed with the adhesive 'Gestetner' stencil correcting fluid. Dry mounts were made using canada balsam or Euparol as the mounting medium.

2.5 Autoradiographic method

Observations on the nucleic acid and protein syntheses in the embryonic developmental stages of Asplanchna, and the incorporation of the tocopherols in their sexual transformation were made employing H^3 labelled compounds listed in Table 2.4.

In vivo labelling of Nucleic acid and Protein

In vivo labelling of the adult Asplanchna and in utero labelling of the embryos were done by exposing the adult organisms to tritiated lysine, thymidine and uridine in Dryl's (1959) or Gilbert's (1963) solution for 1 to 5 hours, keeping the final concentration of the isotopes approximately 100 µC/ml in the medium.

Experiment		labelled compound	sp. activity	Code No. & suppliers
Nucleic acid synthesis	DNA	H^3-thymidine	9000 mC/mM	ICT 3 Babha Atomic Research Centre, Trombay.
	RNA	H^3-uridine	3600 mC/mM	ICT 20 ,, ,,
Protein synthesis		H^3-lysine	1250 mC/mM	ICT 2 ,, ,,
Tocopherol incorporation		H^3-5 methyl DL-alpha tocopherol	200 mC/mM	Worthington Biochemical Centre, NJ. USA., through the courtesy of Mr. V.K. Daniel

Table 2.4 Details of labelled compounds used.

In vitro labelling

Embryos and the vitellarium of Asplanchna were labelled in vitro, by adding drop of the desired isotope into the culture chamber (page 23) in which the exposed embryos were kept in the pseudocoelomic fluid (PCF). Labelling for one minute with 500 µC/ml concentration of the isotopes gave adequate grain density for autoradiography.

Tocopherol incorporation

In 1 ml of the rotifer culture medium labelled H^3-5methyl DL-alpha tocopherol (con. 10^{-6}M) was added. Experimental animals were transferred into the culture slide. A series of experiments were conducted to measure the incorporation of labelled tocopherol at different stages of mictic production. Incorporation of the labelled

tocopherol at different stages of mictic production. Incorporation of the labelled tocopherol was counted in a Packard Tri-Carb scintillation counter. Details of the procedure are given in chapter IV.

Autoradiography

The procedures of Caro (1964) was mainly followed for autoradiographic studies. After labelling, the animals were fixed in ice cold 3:1 methanol-acetic acid. Squash preparations were made on a subbed slide (purified gelatine 5 gms, hot distilled water 1 liter, chrom.-alum. 0.5 gms; stored in refrigerator) in a drop of 45% acetic acid and covered for 5-10 minutes and the cover slips were flipped off by frosting the slide with liquid nitrogen. Slides were washed in 3:1 alcohol acetic acid mixture and the unincorporated precursors were extracted in 5% TCA (5°C) for 15 minutes. Dehydration was done in 70% ethanol and the slides were air dried for autoradiography.

The prepared slides were then covered with strip film AR 10 (Kodak) or nuclear track emulsion NTB 3(Kodak), and kept in light-tight slide boxes at 4°C. Autoradiographs were developed after two to three weeks exposure periods.

Developing was done in Microdol X (Kodak) developer for 5-7 minutes at 25°C and the fixation was done with Kodak acid fixer. The slides were then washed in distilled water before drying.

0.25 w/v aqueous solution of Toluidine blue (pH.6) was used as the stain. Excess stain was washed away in 95% alcohol, and

the slides were mounted in Euparol.

2.6 In vitro method for embryo development

For the study of the embryogenesis, embryos at different stages of growth were treated with labelled compounds. Treatment of growing embryos thus involves surgical applications.

Embryological and other experimental studies on rotifers are mostly confined to the genus Asplanchna. But the saccate body with an osmotic gradient maintained in between the pseudocoel and the external medium render the surgical procedure particularly difficult as it results in the death of the female organism and consequently the embryos within. Besides, these organisms are incapable of cell generation to heal up the wound caused by surgical procedure. In addition there is further difficulty in surgical application owing to the movement of the organism which cannot be arrested successfully. It thus became necessary to device a suitable method for the study. Lechner (1966) has avoided the dissection mechanisms, by selectively inactivating the cells of the embryos using an ultra-violet source. Birky (1967b) made use of a culture chamber for the in vitro development of the embryos. In the present work Birky's culture chamber has been remodelled in a simple manner and employed for the in vitro study.

Procedure

Gravid females were collected in a micro cavity slide (1.5 mm slides with 1 cm dia. dipressions). Specimens were washed

well with Dryl's solution (1956) mixed with penicillin and streptomycin (500 mg/liter) in order to eliminate bacterial contamination. The solution was removed using a micro pipette. This was followed by several washing. The organisms were then covered with a drop (enough to cover them) of liquid paraffin (medicinal pure, BDH). With the aid of fine pointed stainless steel needles (00-entomological pins sharpened on oil stone) the body walls of the organisms were ripped open under a stereomicroscope, so that the embryos were immersed in the pseudocoelomic fluid (PCF) of the parent body. Unwanted tissues and other organelles were removed with the help of needles. The thin liquid paraffin layer made a sealed aseptic chamber and the development of embryos in the PCF was found to be almost regular and normal.

Embryos of Asplanchna under these conditions developed in the normal pattern for several hours. The post-mitotic embryos in which the organ differentiation has taken place developed into a fully grown animal, whereas the mitotic phase embryos developed abnormalities after about 6-8 hours of normal growth. Neonates of post-mitotic embryos developed in vitro were weak. A sudden transfer of these organisms to a normal rotifer culture medium resulted in their death. But they survived when brought gradually to the temperature and pressure conditions of the normal medium. With the aid of a micropipette, a drop of the rotifer culture fluid was added to the PCF. When the neonates were found moving in the medium, further dropwise additions were made at intervals. Animals were transferred to normal medium after nearly 15 minutes. The probable aspects affecting the mitotic embryos, forbidding them from deve-

lopment after a certain stage, are discussed in later chapters (chapter 3 & 4).

CHAPTER III

CHAPTER III

EMBRYOGENESIS AND SYNTHESIS DURING DEVELOPMENT

3.1. Introduction

The present day knowledge of the embryonic development of rotifers is mainly based on the studies on Asplanchna, although the embryology of Asplanchna may not be typical of the rotifer group in general (Hyman, 1951). A detailed study of the structural pattern of development of the amictic female generation was done by Nachtway (1925). The other contributions were from Jennings(1896), Tannreuther (1919, 1920) and de Beauchamp (1956). The study of Lechner (1966) on the embryology of A. girodi, employing the U.V. irradiation methods, has proved the importance of rotifers as a 'biological tool' (Nathan & Landerman 1959) for the developmental studies. All these investigations are essentially studies on the structural pattern during development.

In the present investigation attempts have been made to study:

i. the developmental phases and

ii. the syntheses of nucleic acids and proteins during embryogenesis.

3.2 Developmental phases in Asplanchna

The amictic and the mictic females of Asplanchna do not differ anatomically except in respect of their ovocytes (Shull, 1921). Amictic females (Plate I) develop diploid eggs which produce one polar body during maturation, and the diploid eggs develop parthenogenetically into females. Mictic females (Plate XII, fig.2 and Plate XIII, fig.2) develop haploid eggs which produce two polar bodies during maturation, and the haploid eggs develop into males (Plate XII, fig.4 and Plate XIII, fig.4) if unfertilized or into diploid thick shelled resting eggs, if fertilized. These low temperature and desiccation resistant eggs may resume development after a diapause of variable periods and always hatch into amictic females (See life cycle,Plate X). Thus the sexual process results in the production of both new genotypes and inactive stages, capable of withstanding ecologically unfavourable conditions (Gilbert, 1967).

The present studies deal with the divisional pattern and cell generation time during embryogenesis.

Methodology

The transparent nature of the organism facilitated observation of various developmental processes in the living rotifer through an Olympus stereo microscope (mag. 40-120x). The movements of the organism were restricted by transferring them to a 1.5 mm cavity slide, in which a few pieces of glass-wool were placed before setting a cover slip. The time

taken for different developmental steps were recorded with the help of a stop watch. Time was measured upto the 5th divisional phase, although it was possible to measure further divisional phases with varying degree of accuracy. Three series of observations, with ten organisms in each, were made on the amictic and the mictic generations. The period between two successive cleavages, which included the G_1, S and G_2 periods, was considered as the cell generation time. The observations are recorded in Table 3.1(page 39).

Results & Discussion

The embryonic development in Asplanchna is completed in utero and it requires about 20 hours at 25°C.

The process of development can be divided into three phases as follows:

Asplanchna Development
- .... Gametogenetic phase
  - Pre-vitelline phase
  - Vitelline phase
- .... Mitotic phase
- .... Post-mitotic phase

Gametogenetic phase

The gametogenetic phase of development in Asplanchna is the maturation period of the ovum until the initiation of the first cleavage of the embryo. This requires about five hours at 25°C and is carried out in two distinct steps namely:

i. a pre-vitelline phase and

ii. a vitelline phase

The pre-vitelline phase comprises the maturation division of the ovum in the ovary (Plate II, fig.3), leaving one polar body in the amictic generation and two polar bodies in the mictic male production (Tauson, 1924; Storch, 1923, '24; Gilbert, 1967). Amictic oocyte undergoes a single mitotic division whereas the mictic one undergoes the meiosis. In Asplanchna only one ovum (Plate II, fig.3) develops at a time in the ovary. The matured oocyte moves to the base of the vitellarium, which nourishes the developing embryo (Plate II, fig.1).

In the vitelline phase, the developing oocyte is attached to the base of the vitellarium and it receives the cytoplasmic complements for further development (Plate II, fig.2 and 3). The fully developed oocyte of mictic and the amictic female embryo measures about 250-300 μ whereas that of the male measures 200-250 μ. The developing oocytes receive all the nourishment from the vitellarium, and this is considered to be the reason for the rapid life cycle of Asplanchna (Bentfeld, 1968).

Mitotic phase

The mature oocyte enters the second phase of development (mitotic phase), which requires about six hours, effecting about 10 rounds of mitotic divisions. The first cleavage divides the embryo into two unequal halves, the bigger one forming the vegetal pole and the other the animal pole (Plate II, fig. 4). This division is followed by detachment of the embryo from the vitellarium and its further development takes place in the uterus. After the detachment of one embryo, initiation of the development of another starts

in the vitellarium within a period of 12-15 minutes. During the development of male embryos, in certain cases, the embryo gets detached from the vitellarium after the second or the third cleavage. Irrespective of the considerable variation in the detachment time of the embryo from the vitellarium (Table 3.1), the cell generation time in the development of male and female embryos was observed to be the same; i.e. within a range of 10 to 12 minutes.

The second cleavage takes place in the animal pole of the embryo whereas the vegetal pole, designated as the D-quadrant (Hyman 1951) remains without division (Plate II, fig.3). This division forms three blastomeres in the animal pole (ABC) (Plate II, fig.5). The next cleavages divide the ABC blastomeres spirally in two tiers, and is followed by the division of the D-quadrant into four blastomeres forming a ten cell stage embryo (Plate IIA fig.6). Among the four D-quadrant blastomeres, the largest one at the vegetal pole forms the primordium of the sex cell (Plate III, fig.1). The succeeding two divisions are in the peripheral blastomeres (ABC-cells), forming a stereoblastula stage in which the D-quadrant daughter cells are enclosed by the other blastomeres (Plate III, fig.1). Primordial germ cell and other D-quadrant cells at this stage of development are easily distinguished due to their size difference. Equational division of the germ cell forms the anterior vitellarium (Plate III, fig.2) and the posterior ovary primordial cells (Plate III, fig.2). The division in the ovary cells is quicker than that of the vitellarium.

At this stage, the cells in the anterior region of the surface layer of the embryo proliferate to form primordial pharynx

(Plate III, fig.2). These cells form a blind tube (Plate III, fig.3) which later opens out as the mouth. At the same time, proliferation of the cells from the dorsal body wall of the embryo forms the double layered cell structure which later transforms as walls of the stomach (Plate III, fig.3). The embryo now has a bilaterally symmetrical organization, with formation of primordia of organelles (Plate III, fig.4). A group of cells from the dorsal embryonic body wall forms the primordia of gastric glands (Plate IV, fig.1) anteriorly on either side of the stomach (Plate IV, fig.1&2) Salaivary glands and mastax are also formed from the cells separated out from the dorsal body wall of the embryo. A mass of cells from the posterior part of the ventral bodywall proliferate and forms the nephridia (Plate IV, fig.1). Corona and epidermal musculature are formed as parts of the epidermal layer. Mitotic stage of development stops with the formation of these organ promordia almost at the tenth cleavage stage. The embryos of the mitotic phase show no growth, and the cell division of different analgen stops at different times.

Post-mitotic phase

During this post-mitotic phase of embryonic development cell division stops along with DNA synthesis (vide infra). Organ differentiation and orientation are completed during this period. Tissues become syncytial at this stage, along with the establishment of different physiological systems. Connections of the stomach and the pharynx are established along with the pseudocoel formation (Plate IV, fig.3 and 4). Growth of the embryos takes place only

during this phase of development. The pseudocoel gets filled with the coelomic fluid, and the embryos start synthesising RNA at this stage. This phase covers a period of 8-9 hours. The cuticle formation takes place at this stage, as a hypodermal secretion. Corona and ciliary circlet start moving when the embryos attain full development.

3.3 Syntheses of Nucleic acids and Proteins during embryogenesis

The embryonic development of Asplanchna exhibits many interesting features. Cell division ceases at an early stage in development. Further differentiation leads to the formation of an adult with a definite number of nuclei in each organ (Birky et al, 1967). It is possible that the number of cells in the adult rotifers may be constant, as has been reported by Martini (1912) in Hydatina senta(quoted by Hyman, 1951). But it is difficult to say with certainty, the extent to which the number of cells in the whole organism would be constant. Hyman (1951) and Birky (1967) have stated that an adult female contains less than 1,000 nuclei in most species. At the same time Birky & Field (1966) have reported variation in the nuclear number in the vitellarium and the gastric glands of A. siebold . Identical observations have been made in the present study on A. brightwelli (Chapter IV). It is interesting to note that many of the classifications of rotifers are mainly based on the nuclear number in the gastric glands and the vitellarium (Sudzuki, 1964), although the number of nuclei in these

organs has been reported to be variable. Studies of Birky (1968) and some of the observations in the present study show constant number of nuclei in the bodywall of Asplanchna brightwelli and A. sieboldi, even in different morphotypes, although their position varies. Recent reports of Levinthol (1971)(personal communication) indicate a fixed number of nuclei (182 nos) in the brain of the adult female A. sieboldi.

The primary intention of this investigation was to study the nature of syntheses of nucleic acids and proteins in the embryogenesis of the rotifer Asplanchna, and their role in maintaining cell constancy in adult.

Autoradiographic techniques employing tritiated Thymidine, Uridine, and Lysine were used for the follow up of the DNA, RNA, and protein syntheses during the different developmental phases of Asplanchna.

Methodology

Gravid females of A. brightwelli collected from the mass culture were washed well in the culture medium and then in Dryl's solution (Dryl, 1959) with penicillin and streptomycin (500 mg/liter of Dryl's solution) and labelled.

In vitro labelling was done by dissecting the animals in the culture chamber (Chapter II) and adding the isotope at a concentration of 500 µC/ml. Labelling was done for periods ranging from one to thirty minutes. After labelling the embryos were fixed by

the addition of 3:1 alcohol-acetic acid.

In vivo labelling was done by allowing the organisms to grow for three to four hours in a medium containing isotope (100 μC/ml).

Preparation of squashes

Treated and fixed materials were washed well in 3:1 alcohol-acetic acid and were placed on subbed slides with a drop of cold acetic acid. Slides were covered with silicon coated cover slips and squash preparations were made. The cover slips were flicked off by freezing in liquid nitrogen. Unincorporated precursors were removed by extracting with 5% trichloroacetic acid (TCA) for 15 minutes, at 5°C. The acid was removed by washing with distilled water and the slides were dried for autoradiography.

Developing

Autoradiographs were prepared mainly following the methods of Caro (1964). Both Kodak NTB 3 liquid emulsion at 43-44°C, and Kodak AR 10 stripping films were used for coating the slides. The dried slides were stored in light-proof boxes at 4°C for an exposure period of 15-21 days. The slides were developed for 5-7 minutes at 25°C in Kodak microdol X developer and after washing in cold water, fixed in Kodak acid fixer for 10 minutes. Staining was done with 0.25%

aqueous Toluidine blue. Excess stain was washed off with 75% alcohol and mounting was done in Euparal.

Results and Discussion

The maturation of oocytes in Asplanchna brightwelli takes about five hours at 25°C. During this time cytoplasm streams into the maturing oocyte from the vitellarium and the oocyte undergoes rapid increase in its volume. This process can be observed under microscope (mag. 40 x 6.5) when the animal is kept on a slide under a coverslip supported with glass wool inorder to avoid pressure on the organism.

Synthesis in vitelline phase

Autoradipgraphs of the embryos of the vitelline phase labelled in vitro for three minutes show the incorporation of H^3-thymidine in certain nuclei of the vitellarium (Plate V,fig.1) and also in the developing oocyte (Plate V, fig.5). But the embryos of the same phase labelled in vitro for three minutes do not indicate the incorporation of H^3-uridine (Plate VIII, fig.1). In vitro labelling with H^3-lysine at the vitelline phase shows the synthesis of protein, both in the vitellarium and in the developing oocyte (Plate IX, fig.1 and 2)synthesis of protein, both in the vitellarium and in the developing oocyte (Plate IX, fig. 1 and 2).

Continuous labelling with H^3-thymidine in vivo for three to four hours shows most of the nuclei in the vitellarium labelled (Plate V, fig. 3 and 4). Certain cytoplasmic regions of

the vitellarium and the oocyte also are found to have incorporated H^3-thymidine (Plate V, fig. 4). When similar experiments were performed using H^3-uridine, the vitellarium and the developing oocyte incorporated the labelled substance to appreciable extent (Plate VII, fig. 3 and 5). Protein synthesis shows no change in experiments employing H^3-lysine.

Selective incorporation of thymidine in the vitellarium nuclei indicates that the synthesis of DNA takes place only in certain nuclei at a time. This is further proved by the labelling of more nuclei during the *in vivo* treatment for three to four hours. Similarly, the labelling in the cytoplasm of the vitellarium and the oocyte clearly shows that the cytoplasmic DNA synthesis is also taking place in these organs. Absence of the cytoplasmic DNA labelling in the *in vitro* treatment indicates that the cytoplasmic DNA synthesis is taking place at a lower rate, failing to give the labelling on short-term treatment with H^3-thymidine. Absence of cytoplasmic DNA in the later mitotic stage embryos (*vide infra*) suggests that these DNA complements, found in the maturing oocyte, have possibly been received from the vitellarium

In vitro labelling shows no incorporation of H^3-uridine in the developing oocyte (Plate VIII, fig. 1), whereas more RNA synthesis takes place in the vitellarium (Plate VII, fig.1 and 2). Hence it is presumed that the oocytes of the vitelline phase are incapable of RNA synthesis, whereas all the nuclei of the vitellarium uniformly synthesise RNA. The cytoplasmic RNA synthesis is also found to take place in the vitellarium (Plate VII, fig.2). *In vivo* treatment with H^3-uridine has shown the oocyte

with RNA, labelled. This further leads to the conclusion that the oocytes are receiving the RNA from the vitellarium along with the transfer of the cytoplasmic materials. The transfer of RNA from the vitellarium to the developing oocyte is clearly visible from (Plate VII, fig.4). On the contrary, protein synthesis takes place both in the vitellarium and in the oocytes of the vitelline phase.

Synthesis in the mitotic phase

The embryos of the mitotic phase labelled in vitro show H^3-thymidine incorporation only in certain cells (Plate V, fig. 6 and Plate VI, fig. 1 and 2) whereas the RNA synthesis is found to be lacking during this phase (Plate VIII, fig. 2 and 3). Protein synthesis is pronounced in the mitotic phase embryos (Plate IX, fig.3 and 4).

In vivo labelling for three to four hours shows that the developing oocytes are also labelled with H^3-uridine (Plate VII, fig. 3 and 5). Incorporation of H^3-uridine is observed in the cytoplasm of the vitellarium (Plate VII, fig.2). It becomes evident from the above that along with the transmission of cytoplasmic contents (vide supra) the developing oocyte is also supplied with RNA from the vitellarium (Plate VII, fig.4).

Synthesis in the post-mitotic phase

The post-mitotic phase embryos show no H^3-thymidine incorporation except in the vitellarium nuclei (Plate VI, fig.3 and (Plate V, fig. 1). Certain cells of the vitellarium are labelled (Plate V, fig.1). But the H^3-uridine incorporation becomes more

evident in almost all the nuclei with high intensity labelling in the vitellarium (Plate VIII, fig.4). Protein is found equally labelled in all tissues of the organism (Plate IX, fig. 5 and 6).

These observations further show that RNA synthesis is resumed in the post-mitotic phase, possibly after the DNA synthesis and mitosis have ceased. DNA synthesis in a differentiated embryo is found to take place only in the vitellarium.

To summarise the observations in the developmental phase of Asplanchna embryos, it can be said that:

1. DNA synthesis takes place in the maturing oocyte and in the vitellarium, during the vitelline phase. Different nuclei of the vitellarium synthesise DNA at different times. DNA synthesis stops at different analgen. In the fully grown embryo and in the adult, only the vitellarium nuclei synthesise DNA.

2. All the vitellarium nuclei synthesise RNA uniformly. The developing oocytes are supplied with the RNA complements by the vitellarium, and no RNA synthesis takes place in the embryos till the mitotic phase is completed. Post-mitotic embryos resume the RNA synthesis.

3. Protein synthesis takes place in the embryos at all stages of development and in the vitellarium.

Parent Organisms / Amictic Females	1st CLT		Vit. detachment time		2nd CGT		2nd CLT		3rd CGT		3rd CLT		4th CGT		4th CLT	
	mins.	sec.	mins.	sec.	mins.	sec.	mins.	sec.	mins.	sec.	mins.	sec.	mins.	sec	mins.	sec
e + 0	0	45	8	45	11	10	0	45								
E + e + 0	1	28	1	35	10	00	1	24	12	00	1	12	10	30	0	55
0	1	22			9	00	1	37	7	19	1	04	9	27	0	45
2e + 0			9	00			1	00								
E + e + 0	0	45	2	31	11	01	1	37	9	15	1	38	9	40	0	53
2E + 0	0	53	6	35	13	10	1	30	12	25	1	25				
0	1	10	6	10	9	50	1	15								
0	1	20	2	10	10	20	1	30	8	17	1	10	10	20	0	40
E + 0	0	55	1	50	11	20	1	10	10	05	1	08	9	10	0	50
E +2e + 0	1	10			10	40	1	40	11	30	1	50	11	05	0	52
Average	1	05	4	47	10	47	1	02	9	58	1	20	10	02	0	49

Table 3.1 The developmental phases of amictic females of A. brightwelli showing the cleavage and cell generation time upto the 5th mitotic phase division

CLT - Cleavage time; CGT - Cell generation time; E -Embryos at post-mitotic phase;

e - Embryos of mitotic phase; 0 - Developing oocyte attached to vitellarium

CHAPTER IV

CHAPTER IV

SEXUAL TRANSFORMATION IN ASPLANCHNA

4.1 Introduction

The transition from parthenogenetic (asexual) to sexual reproduction (biparental), observed in many species of the order Ploima (Gilbert, 1968), is an interesting problem of the biology of rotifers. The sexual periods are comparatively brief in occurence and are owing to the parthenogenetic production of the mictic females. This sexual transformation is the result of the influence of certain stimuli namely pH variation, oxygen content of the medium, phtoperiodicity, starvation, dietary change etc. (Mitchell, 1913); Tannreuther, 1919; Shull, 1927; Whitney, 1929; Birky, 1964; Buchner & Kichle, 1965, Kichle & Buchner, 1966; Gilbert, 1967). However, the influence of these stimuli in transformation of amictic females into mictic ones is poorly understood.

Gilbert & Thomson (1968) have shown that the inclusion of alpha tocopherol (vit.E) in the diet of rotifers Asplanchna brightwelli and Asplanchna sieboldi causes several changes in the development of their parthenogenetic offsprings, namely:

i. the offsprings develop regular body wall outgrowth (BWO) or the humps,

ii) the offsprings have a higher mean number of nuclei in the gastric glands and the vitellaria, which are exceptions to the phenomenon of eutely, a characteristic feature of most of the rotifers, (Birky & Field, 1966)

iii) some of the offsprings grow into maturity as mictic females whose oocytes undergo meiotic rather than mitotic maturation division,

4.2 Effect of Vit.E on BWO response and nuclear number

Studies carried out on the BWO and the increase in the nuclear number of the vitellarium and the gastric glands of A. sieboldi and A. brightwelli have shown identical results (Plate XI, fig.2 and Table 4.1). A. sieboldi (Plate XII, fig.3 and Plate XIII, fig.3) show pronounced BWO and higher nuclear number in the vitellarium and gastric glands than A. brightwelli (Plate XII,fig.1 and Plate XIII, fig.1). The nuclear map (specimens fixed in acetone and map drawn using camera lucida) have shown the displacement of certain hypodermal nuclei in the humped forms without causing any change in the nuclear number (Plate XI, fig.2). In both A. sieboldi and A. brightwelli few of the marginal nuclei (Plate XI, fig.2, $n_2n_3n_4n_5$) were selectively activated for the hump formation. Similar effects were observed in the median nuclei also which were induced to form the posterior and the dorsal humps (Plate XI, fig.2 $d_1d_3d_4d_5$). Although A. sieboldi showed flap like lateral outgrowth of the bodywall, nuclear position

and the number almost remained similar to that of A. brightwelli. The nuclear counts made in the gastric glands and vitellarium (through phase contrast microscope, mag. 40x12.5) show a higher nuclear number in A. sieboldi (Table 4.1).

Species	Morphotype	Vitellarium nuclei mean no.	Gastric gland nuclei mean no.
Asplanchna brightwelli	Saccate	38.4 (36-48)	9.2 (8-12)
	Humped	46.2 (42-56)	13.5 (11-15)
Asplanchna sieboldi	Saccate	56.6 (49-62)	13.2 (10-16)
	Humped	64.0 (58-69)	23.1 (21-28)

Table 4.1 Nuclear number in the vitellarium and gastric gland of A. sieboldi and A. brightwelli.

Birky (1968, 1969) through his extensive studies on polymorphism has demonstrated in A. sieboldi that in the case of BWO response, dietary vitamin E controls the morphogenetic growth in the syncytical embryonic hypodermis without altering the cell number. At the same time, it also controls the number of mitotic divisions in the embryonic gastric glands and vitellarium without affecting the morphogenesis. The mechanism by which vit.E operates in two different ways in two specific target tissues of the same organism still remains unknown.

In the present work further experiments were performed to study:

A) the effect of vit.E at various stages of amictic embryogenesis, and

B) the nature of inheritance of the mictic trait.

The BWO response being a morphotypic variation associated with the mictic development, was taken as a parameter to assess the mictic production. The organisms have been broadly classified into three groups, on the basis of the level of morphotypic variations in response to the dietary inclusions of vit.E. These groups are as under:

1. Amictic saccate females (AM) (Plate XII, fig.1 and (Plate XIII, fig. 1)
2. Partially humped transitional forms (T) (Plate XII, fig. 2) and (Plate XIII, fig.2 and
3. Mictic fully humped females (M) (Plate XII, fig.4 and (Plate XIII, fig.4)

4.3 Effect of vit.E at various stages of amictic embryogenesis

Methodology

Amictic females (AM), bearing developing embryos of different age groups, were separated from the mass culture, washed in the culture fluid and transferred to the mictic culture medium (Chapter II) containing DL alpha tocopherol (10^{-5}M). Gravid amictic females, with embryos at different stages of development, were taken in small cavity blocks. Five series of experiments were conducted with 8 to 10 specimens in each series. Number of organisms in each cavity block was limited to 3 or 4 for the convenience of observation. Appropriate control was maintained in the normal media without DL alpha tocopherol. The progenies of one generation (P_1) upto the

5th or 6th offsprings separated and examined. The groupings were made on the basis of EWO response. Observations are recorded in Table 4.2

Experiment series	Embryos in the AM female at the time of transfer to vit. E medium	Offsprings of the P_1 generation P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	P_{16}
1.	2E + 2e	AM	AM	T	T	M	T
2.	E + 2e	AM	AM	T	T	T	M
3.	e + o	T	T	T	T	M	
4.	0	T	T	T	M	M	
5.	New born	T	T	T	M	M	M
6.	Control 2E + e	AM	AM	AM	AM	AM	AM

Table 4.2 Effect of vit.E on the embryos of Asplanchna brightwelli amictic generation. E- embryos after the gastrulation and differentiation of cells., e- embryos of the mictic phase, 0- the developing oocyte attached to the vitellarium. P_{11} - P_{16} progenies of the same generation in succession.

4.4 The nature of inheritance of the mictic trait

Methodology

Uniform batch of newly born, unfed amictic females were separated from the gravid amictic females from the mass culture

and kept in cavity blocks with normal culture media. Neonates were collected after 2-3 hours, and kept in separate cavity slides containing mictic culture medium and DL alpha tocopherol (10^{-7}M). Control was maintained in the normal media without DL alpha tocopherol. The organisms were fed with Paramaecium aurelia. The cavity slides with the neonates were kept at 25°C in a humid chamber and regularly observed.

Five series (G_1 - G_5) of experiments were conducted with 8-10 specimens in each line and the observations continued upto tenth generation in certain cases. Treatment with vit.E in the medium continued from one to five generations (G_1 - G_5 series). The continuity of the inheritance in the succeeding generations was studied by transfering the mictic offsprings of each series back to the normal medium without tocopherol. The results are summarised in Table 4.2.

Experiment series	Generations 0	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}
1. G_1 a	AM	AM	T	M	M	T	AM				
2. G_1 b	AM	AM	T	M	M	AM					
3. G_2	AM	AM	T	M	M	M	T	AM			
4. G_3	AM	AM	T	M	M	M	M	M	T	AM	
5. G_4	AM	AM	T	M	M	M	M	M	T	AM	
6. G_5	AM	AM	T	M	M	M	M	M	M	T	AM
7. Control	AM	AM	AM	AM	AM	AM	AM	AM	AM	AM	AM

Table 4.3 Effect of vit.E in A. brightwelli treated with/alpha DL tocopherol for varying number of generations (in box) and then transferred to normal culture media. AM-amictic female, M-mictic female, T-transitional form.

Result and Discussion

Mictic female production and BWO response in Asplanchna are apparently controlled by alpha tocopherol. Experiment 4.3 shows that the embryos of A. brightwelli remain labile to differentiation into mictic or amictic females until they reach the post-mitotic stage (E) through gastrulation and cell differentiation. Developing oocytes (O) attached to the vitellarium and undifferentiated mitotic embryos (e) are easily induced to mictic production by the maternal diet of vit.E as evident from the observations in series 3 & 4 of Expt. 4.3(Table 4.2). On the contrary, the post-mitotic embryos in which the organ differentiation has occured show no visible effects due to incorporation of tocopherols (1 & 2 series of Table 4.2) Neonates of the amictic females completely transform their progeny into mictic through the transitional stage (T) (series 5 Table 4.2).

These observations lead to the following possible inference:

i. Vit.E directly induces the embryos to mictic production
ii. Vit.E initiates the action or production of certain specific molecules responsible for the mictic production.

However, it becomes evident from the above results that vit.E is not associated with the oogenesis of the amictic females in the production of the mictic progeny.

The inducer molecules are possibly macro-molecular, as they are incapable of inducing the post-mitotic embryos in which the differentiation of the cells have taken place. Presumably the body-wall thickening of the developing embryos may act as a barrier to

the entry of the macromolecules into the animal cells, as has been found in the development of sea urchin egg (Monroy, 1964). Similarly it is reported (Bentfeld, 1968) that the impregnation of the sperms into the pseudocoel of the females is possible only before the hardening of the body wall. This is emphasized by the in vitro development of the post-mitotic embryos (Chapter II) devoid of maternal nourishment. The synthetic processes are found to be carried out by the embryos at the later stages of development (Chapter III). These results lead to a possible conclusion that the inducing molecules are having the macromolecular structure and their action is not associated with the maternal oogenesis; and in utero it can act only upon undifferentiated embryos.

The P_1 progenies of the Expt. 4.3 are mostly transitional forms (T) eventhough they develop in vit.E containing medium. This delayed response may be owing to:

i. insufficiency of the vit.E ingested by the organism or

ii. delayed production of the inducer molecules.

The intake of vit.E by the organism is mostly through the paramecia and hence its passage from the stomach to the target tissues is yet to be worked out.

The females of F_1 generation on alpha tocopherol diet from birth to the first offspring (0-24 hours) of the F_2 generation, are in the transitional stage (T) (Expt.4.4 Table 4.3). When these F_1 progenies are removed from the mictic medium to the normal their F_3 progenies are mictic (M) with more prominent hump

formation although they received no additional complement of vit.E (G_1 series). Further generations (F_4,F_5) show gradual decline in the response, forming again the transitional stage and finally reaching the amictic stage (AM) through F_5 or F_6 generation. In G_2 - G_5 series of experiments of 4B, the organisms were treated with vit.E for a number of generations varying from 2 - 5. The observations made in these experiments reveal that the influence of vit.E continues over 3-4 generations even when the organisms are subsequently grown in the absence of the vit.E. This indicates that once the optimum quantity of tocopherol is taken up addition of further quantities of vit.E to the medium does not have any additional effect on the organism. On the contrary, when the quantity falls short of the optimum requirement, the transitional forms are produced which finally lead to the production of normal amictic females. The gradual decline in the response through the generations leads to the conclusion that the magnitude of the mictic response to vit.E is a function of the amount of the inducer molecules present in the animals in each generation. The long labile period in Asplanchna development, in contrast to that of Brachionus (Gilbert, 1963, 1968), is due to the in utero development in which the nourishment is received from the maternal synthesis through the uterine fluid (Chapter III). The mictic induction caused in the developing embryos of the early stages further indicates that the inducer molecules are present in the uterus of the mother influencing the development embryos. The transmission to the progeny is likely to be through the PCF of the offspring formed during the post-mitotic intra-uterine development. This transmission process gradually decreases in the subsequent generations as further addition of inducing molecules are not made available in

in the absence of vit.E.

The process of inheritance of mictic trait in Asplanchna can in no way be different from the embryological induction of the postereo-lateral spine formation (Gilbert, 1966), caused due to the 'asplanchna factor' and resulting in the possible cyclomorphosis, in Brachionus calcyflorus Pallas (Nayar, 1965). A reduced rate of induction in the succeeding progenies is observed in many cyclomorphic forms of rotifers (Hutchinson, 1967). All these observations support the passive transmission hypothesis (Birky, 1969). In Asplanchna, it may be explained as a function of certain endogenous inducer molecules present in the animal in each generation. The transmission of mictic response from the parent to the progeny is in the form of vit.E itself or in the form of another molecule whose synthesis requires high levels of alpha tocopherols.

4.5 Note on the intake of alpha tocopherol and its inheritance in Asplanchna

A. brightwelli has been grown in media containing 5-methyl H^3-labelled alpha tocopherol. An attempt has been made to study the proportion of this labelled substance that has been transferred in the succeeding generations.

Asplanchna females were reared from birth to 24 hours in culture medium containing H^3 labelled alpha tocopherol (10^{-6}M). The procedure of the earlier experiment was followed (Expt. 4.4) 10 specimens from each generation were isolated

and fixed in alcohol. The proportion of the labelled compound in each generation was estimated by taking the disintegration counts in a liquid scintillation counter (Packard, Tri-Carb).

Table 4.4 and Graph 4.1 show the percentage of the tocopherol contants retained in succeeding generations. Plate XIV, fig.1 shows the digramatic representation of the level of reduction and retantion of tocopherol in successive generations.

Generation	Vit.E content per female in ng.	% retention of H^3 (tocopherol)
I	0.142	4.71
II	0.0152	0.482
III	0.003316	0.11
IV	0.000775	0.026
V	0.000181	0.007

Table: 4.4 Vit.E content and its percent retention (on the basis of H^3-incorporation) of tocopherol in Asplanchna brightwelli

Graph 4.1 illustrates the percentage inheritance of H^3 in each generation, in a decreasing order with a sudden drop in the second generation progeny. However, the decline in each generation keeps a ratio of 1:0.25:0.25:0.25 relationship. The diagram (Plate XIV, fig.1) illustrates the probable mechanism by which the degree of inheritance is reduced in the succeeding generations. It is further suggestive that vit.E molecules are transmitted from one generation to other, without undergoing any apparent change in itself.

CHAPTER V

CHAPTER V

DISCUSSION

5.1 General discussion

The present study deals with certain developmental aspects of the rotifer A. brightwelli . The course of development of this organism involves three distinguishable steps; namely, gametogenetic, mitotic and post-mitotic phases. These developmental phases in this organism have not been specifically distinguished by earlier workers (Birky, et al, 1967, Gilbert (1968) etc.), although they have stated that mitotic division ceases at certain stage of development. In the present work, the gametogenetic phase has been distinguished as the processes leading to maturation of oocytes. This phase involves a pre-titelline and a vitelline phase. Pre-vitelline phase includes the processes of gamete formation in the ovary through the maturation division. This step is followed by transfer of the oocyte to base of the vitellarium, which serves as the nurse cells for development of the embryo. The mitotic phase comprises the divisional stage of the embryo development starting from the first cleavage that takes place when the embryos are attached to the vitelline phase. Mitotic phase continues for about eleven (Six) hours of development. The cell division stops after the formation of the organ primordia. The

post-mitotic phase is characterised by the organ formation and differentiation. The growth of the embryo is observed only during this phase of development. In the present work attempts have been made to elaborate the phases of development by a study of syntheses of major biological compounds namely, nucleic acids and proteins.

Complete development from mature egg to birth requires about 20 hours at 25°C (Nachtway, 1925; Lechner, 1966). In the ovary, only one oocyte at a time undergoes rapid growth. After the oocyte has fully grown and separated from the vitellarium to undergo further development in the uterus, another one in the ovary is initiated to grow. The duration of the growth of oocyte at 25°C is about 5 hours.

Monroy & Tayler (1967) state that in sea urchin egg, much of the macromolecular material of the growing oocyte is manufactured in other cells of the body (nurse cells) and supplied to the oocyte as finished products. A similar process, however, is not common in intra-uterine development, in which the complete nourishment is mainly carried out by cells of the mother, till the birth of the offspring. Though the oocyte development is intra-uterine in Asplanchna, its pattern of development is comparable to the telotrophic vorticles of insects, wherein the oocyte draws nourishment from the nurse cells through the nutritive cords (Imms, 1963). However, this mode of development in Asplanchna represents a lower grade of organisation as a viviparous organism.

5.2 Synthesis of Nucleic acids and Protein during development.

In A. brightwelli synthesis of DNA ceases at a certain stage during uterine development (Birky et al, 1967), alongwith the cessation of mitosis. Synthesis of DNA is found to stop at different times in different analgen. All the cells of the developing embryo are not found to synthesise DNA at the same time even in early stages of development (Chapter III). This may be one of the main reasons for the phenomenon of eutely exhibited by these organisms. Nuclear constancy (eutely), on the basis of this observation, appears to be a control mechanism which shuts off mitosis in the tissues prior to orientation of organs. Experiments employing labelled compounds reveal that incorporation of H^3-thymidine in the nuclear DNA indicates replication of the genome. In adult rotifers and in embryos at the post-mitotic stages, incorporation of H^3-thymidine is observed only in the vitellarium. During the post-mitotic development, the cell membranes break down and disappear completely in the vitellarium as well as in most of the other tissues. The vitellarium thus becomes a syncytial organ, showing nuclear DNA synthesis of variable extent during this period. The variability in time and rate of DNA synthesis in the nuclei of the vitellarium and the absence of DNA synthesis in the oocyte indicate that the cytoplasm of the reproductive organs does not control the DNA synthesis during development. This is further supported by the reports of the absence of DNA synthesis in the nucleus of oocytes in higher dipterens (Sharma, 1968) which draw nourishment from external sources. The nuclei of the vitellarium at this stage of development are probably becoming polyploid. Polyploidy and polytenization in

yolk glands are known to occur in insects (Raven 1961; King, 1964).

The maturing oocyte of the vitelline phase and the vitellarium shows incorporation of labelled thymidine in the nucleus and the cytoplasm. Cytoplasmic DNA shows labelling when exposed for a long time, especially in the oocytes. The presence of cytoplasmic DNA has earlier been reported in oocytes of sea urchin (Monroy,1964), amphibians (Haggis, 1964) and echinoderms (Tyler & Tyler, 1966a). But the exact amount and properties of this DNA have not been understood properly. In the case of Asplanchna the oocytes receive complete cytoplasmic complements directly from the vitellarium (Chapter III). Hence it is more likely that the cytoplasmic DNA synthesis is carried out in the vitellarium itself. Shmerling (1965) and Baltus et al (1965) have reported that in sea urchin the cytoplasmic DNA acts as a primer for both DNA and RNA syntheses. Cytoplasmic DNA has been reported in another aschelminth, the nematode, Caenorhabditis briggsae (Nonnenmacher-Godet & Dougherty, 1964). The precise function and location of this DNA in Asplanchna has not been determined, although it can be presumed that rotifers also possess DNA in their mitochondria like sea urchin (Piko et al, 1967), amphibian eggs (Dawid, 1966) and nematodes (Nonnenmacher & Dougherty, 1964).

Generally in animals, the synthesis of RNA continues actively throughout the oocyte development, although it is less pronounced in later stages. Experiments with oocytes of star fishes and frogs (Ficq, 1964) have shown that high molecular RNA is synthesized in the nucleus during most of the growth period. By precursor labelling it is proved to be ribosomal RNA (Edstrom et al, 1961). Similar results have been obtained in the toad Xenopus laevis,

in which considerable amount of RNA synthesis takes place during oocyte development (Davidson et al., 1964). But at the onset of maturation divisions this synthesis ceases, although a slow rate of syntheses of sRNA and mRNA have been detected. No further synthesis of ribosomal RNA was found to take place until the gastrula stage, whereas synthesis of heterogenous DNA like RNA is found during cleavage stages (Brown & Littna, 1964a). These results indicate that until gastrulation, no new ribosomal RNA are synthesised in the embryos; i.e., the protein syntheses are carried out on the ribosomes. Those ribosomes come from the vitellatium alongwith the cytoplasmic contents during oogenesis.

A similar situation is noted in Asplanchna in which the developing embryos of the mitotic phase and the oocytes of the vitelline phase do not synthesise RNA. Cytoplasmic contents of the oocyte are received from the vitellarium (Chapter III) wherein RNA synthesis is active throughout the developmental period. Intense labelling of RNA in Asplanchna gives more evidence to its polyribosomal or masked structure as observed in sea urchin and amphibians (cited above). During the mitotic phase of development, no RNA synthesis takes place in the embryos, which begins only after the organ differentiation in the post-mitotic embryo has been completed (Chapter III). These facts indicate that during the divisional stages of embryo, the maternal RNA is responsible for the development of the embryos. The embryo becomes able to independantly synthesise RNA only at a later stage, after the cessation of DNA synthesis. In adult rotifers, the nuclei and the cytoplasm are found to intensively incorporate H^3 uridine, in the vitallarium. RNA

synthesis in the vitellarium are probably rRNA and mRNA and are transferred to the growing oocyte in the vitelline phase, alongwith other cytoplasmic contents. These results suggest that the oocyte nucleus is inactive and the machinery for the protein synthesis are supplied by the vitallarium. Such observations have also been made by King (1960) in Drosophila. According to him ' the RNA which initially accumulates in the ooplasm arises from the nurse cells nuclei, but the subsequent build up of ooplasmic RNA is independant of nurse cells and oocyte DNA. It becomes evident from these observations that most of the synthesis processes for the oocyte development go on in the maternal templates in Asplanchna.The use of maternal tissue for the transcription of RNA give more evidence to the polyloid nature vitellarium nuclei that enable rapid RNA synthesis.

Ribosomal RNA is apparently not formed until the time of gastrulation in amphibian eggs and it is found to be correlated with the appearance of nucleoli in the nuclei of sea urchin (Coeden & Lahman, 1963). Cohn (1966) also reports that RNA synthesis occurs at the time of gastrulation, the appropriate enzyme presumably being synthesised in the development of Asplanchna. DNA synthesis ceases in the post-mitotic phase alongwith the appearance of nucleoli in the cells and the resumption of RNA synthesis (vide supra). These observations are identical to the development of amphibian and sea urchin eggs referred to above. It may be stated that during the development of Asplanchna, the masked RNA, received from the vitellarium, are unmasked in the early development period, and new RNA are synthesised once the organ differentiation has taken place in the post-mitotic embryos. RNA synthesis in the embryos of Asplanchna are preceded by the cessation of the DNA synthesis with the appearance of nucleoli in the cells.

Maggio & Catalano (1963) have reported a significant increase in the protein synthesis until the balstula stage, in sea urchin egg. But in Asplanchna the protein synthesis during the embryonic development is continuous in the developing embryos and the vitellarium. The complete machinery for the synthesis of proteins in the embryos is supplied by the vitellarium (vide supra). Hence synthesis of protein in embryos of Asplanchna is controlled by the ribosomal RNA complements received from the vitellarium. This is compareble with the observations made by Maggio et al (1964), that sea urchin egg contains RNA capable of acting as template RNA for protein synthesis in the ribosomes of rat liver and in sea urchin embryos. The mechanism of protein synthesis in the case of Asplancha is still at an infant stage of study to elucidate synthesis during embryonic development. However, it is evident that the syntheses in the early embryonic development of Asplanchna upto the onset of gastrulation and organ differentiation are indeed controlled by the cytoplasmic complements (especially RNA & cytoplasmic DNA) received by the developing oocyte during its vitelline phase.

5.3 Inheritance of mictic trait

Inclusion of vit.E in the diet of Asplanchna brings forth various responses, like bodywall outgrowth (BWO), increase in the number of vitellarium nuclei and gastric glands and the mictic female production (Chapter IV).

BWO response has been studied earlier in A. sieboldi by Birky (1968). The nuclear map of the bodywall of A. sieboldi and A. brightwelli (Chapter IV) shows no change in the number of the

nucleus but only in the position of the nuclei. Further, BWO response is specifically localized on certain nuclei of the bodywall. In A. sieboldi Birky (1968) claims that exogenous tecopherol causes tachyauxetic (excess) growth in specific cells in the embryonic hypodermis. Muscular dystrophy in higher vertebrates like rat, chick and rabbit (West et al 1967; Harper 1967 etc.) has been reported to be due to deficiency of vit.E BWO in Asplanchna is due to hypervitaminosis and hence is not comparable to the effects of vitamin deficiency in vertebrates. However, specific action of vit.E on certain nuclei of hypodermis becomes a matter of interest, as the hypodermis is a syncytium where all nuclei have a common cytoplasmic complement.

Apart from the BWO, the other changes associated with vit.E effect are variation of the nuclear number in gastric glands and vitellarium. This variation further suggests the influence of vit.E on mitotic divisions of certain specific cells. Interestingly, the effect of vit.E on the bodywall causes its outgrowth and changes the morphology without changing the nuclear number. But in the gastric glands and the vitellarium, the same material causes change in the mitotic division without affecting the morphology. Thus the influence of vit.E at these target tissues of the same organism is manifested in two entirely different ways. The effect of vit.E on mitosis has not been reported in any other animal. However, Hess & Manzel (1965) have described an increase in the incidence of centriole in the proximal convulted tubules of kidney of rat reared in the vit.E deficient medium. It was suggested that vit.E may prevent-SH group (required for mitosis) oxidation, and hence vit.E deficiency may inhabit the mitosis. It is also possible that increased amount of

vit.E might protect the spindle from oxidation in the gastric glands and vitellarium of Asplanchna. But Gilbert & Thompson (1968) and Birky & Gilbert, (1971) have stated that the different antioxidants and chemicals allied to tocopherols show no or little effect on the responses of Asplanchna. Hence, it can be presumed that the effect of tocopherol in Asplanchna is very specific and unique to the organism. It will be a premature statement, if we make any further assumption about the details of the control mechanism of mitosis and BWO in Asplanchna, without a study at the molecular level.

The present observations reveal that oocytes in the ovary, maturing oocytes attached to the vitellarium and the embryos of the early mitotic phase are influenced by the inclusion of vit.E in the diet. On the contrary embryos in which organ differentiation has occured, are not affected by changes in the maternal diet, and always hatch into amictic females. This indicates that the embryos are labile after their detachment from the vitellarium till they enter the post-mitotic phase. Hence, it appears that mictic production is not associated with maternal nourishment of developing oocyte or oogenesis. Probably the inducing molecules are taken up from the uterine fluid in which embryos develop. This is in contrast to the oviparious development of other rotifers. where fate of eggs to develop mictic or amictic is determined at the time of their extrusion from the maternal body, as reported by Shull (1912) in Epiphanes senta, Buchner (1941) in Brachionus calyciflorous,& Keratella quadrata, and Rutter Kolisko (1964) in B. rubens.The long labile period in Asplanchna may be attributed to the intrauterine development of the embryos.

That vit.E does not have any influence on the post-mitotic

embryos of A. brightwelli suggests that:-

The thick cell membrane of the post-mitotic embryo does not permit entry of the inducing molecule. Even if the inducing molecules have entered the system they remain inactive, as the mitotic divisions have ceased at an early stage of development. The former seems to be more probable in the light of earlier reports. Tannreuther (1919) described the development of the syncytial hypodermis and regarded cuticle as a uniform amorphous secretion formed from the hypodermal cells before birth. Koechler (1965, 1966) has reported in A. sieboldi that the embryonic integument of the late stages is similar to that of the adult except that the hypodermal cells are generally thicker. Further, Birky (1967b), Gilbert (1968) and Koechler (1965b) have explained that due to the hardening of the cuticle, a few hours after birth the psuedocoel of the females are not impregnated with sperms. All these evidences show that the formation of the cuticle in the embryonic developmental phase itself, may primarily forbid the entry of any inducer molecule into the system of the post-mitotic embryos.

The other possibility may be delay of the inducer molecules to reach the target tissues. The intake of vit.E is through paramecia on which rotifers are fed. From the saccular stomach they have to pass through the psuedocoel to the uterine chamber. Post-mitotic development in Asplanchnis of about six hours duration and if the inducer molecules fail to reach the target tissues well ahead, they fail to exert any influence. In other words, post-mitotic embryos are not affected by dietary inclusions as embryogenesis is possibly faster than the time taken by inducer molecules to reach the

target.

It may be recalled that a delayed action of the inducer molecules is positively suggested by the results of experiment 4.4, wherein organisms of the F_3 generation showed more mitotic traits than those of F_2.

It is evident from the experimental observations (Chapter IV) that vit.E does not change the physiology or morphology of the organism permanently. The inclusion of the dietary vit.E in the first generation brings out the low-humped intermediary (T) forms and later the mictic forms. By removing vit.E from the diet, nuclear number in the gastric glands and vitellarium, as well as the degree of development of humps decline gradually in subsequent generations rather than ending abruptly in a single step. Magnitude of BWO and its expression in subsequent generations are related to the initial concentrations of tocopherol. When the initial concentration is low, the degree of expression of both show a decline. This suggests that the magnitude of response is proportional to the endogenous inducer molecules present in the animal, in each generation. The exact nature of the inducing molecules and their effect in bringing forth the mictic production in Asplanchna is yet to be determined. The effect of vit.E in the F_3 generations is more pronounced than in the F_2, even though the F_3 generations did not receive any additional supply of vit.E (Expt.4.4). It is assumable that vit.E, rather than being the inducer molecule initiates the formation of certain active inducers which promote mictic production . The gradual decline of influence of vit.E in succeeding generations, reveals that the synthesis of active inducer molecules takes place only in the presence of vit.E.

Studies on inheritance of the mictic response employing 5-methyl H^3 - labelled DL- alpha tocopherol (Chapter IV Expt. 4.5) yielded valuable results. In contrast to the above inferences, it was observed that the labelled 5-methyl vit.E was transmitted from the mother to the progeny. The studies also proved the transmission of methyl labelled vit.E through 4 - 5 generations in a receding order. The first generation progeny received 0.142 ng/female and in the fifth generation it was reduced to 0.000181 ng/female. With the reduction of the labelled vit.E content a proportional decline in the mictic response was observed in the subsequent generations. This corroborates that the mictic trait, as expressed by the production of mictic females, is directly proportional to the quantity of vit.E present in the organism. Further studies have shown that labelled vit.E is directly passed on to the progeny causing a comparable decline in the mictic expression from generation to generation.

Gilbert & Birky (1971) found that only DL tocopherol influences the mictic production in Asplanchna, whereas the other forms of vit.E had negligible or no effect at all. This would suggest that DL- tocopherol is specific in its action in inducing mictic production. Moreover, no transformation of vit.E is suggested in studies with labelled DL- tocopherol due to hydrolytic reactions (Rosenberg, 1945). Experiments to extract and separate the vit.E contents in different generations did not yield any result. Lack of labelled compounds and technical difficulties which could not be overcome arrested further studies. Though much more remains to be elucidated in the mode of inheritance in general and quantitative aspects of transmission in particular, what emerges out of the present studies is fascinating

that vit.E is transmitted in Asplanchna from generation to generation in all probability without any chemical change in transformation.

SUMMARY

SUMMARY

Certain aspects of development and sexual transformation in A. brightwelli and A. sieboldi have been studied. Three phases namely, gametogenetic, mitotic and post-mitotic phases of the course of development have been distinguished.

Embryonic development in Asplanchna in intrauterine. The maturing and immature oocytes are connected to the vitellarium by broad cytoplasmic bands. Developing embryos receive their cytoplasmic complements from the vitellarium. The mode of development of Asplanchna represents a lower grade of organization as a viviparous animal.

During the mitotic phase there is no increase in the size of the embryo. DNA synthesis stops at different times in different analgen. DNA synthesis in the vitellarium indicates polyplodization of its nuclei. Developing embryos

receive their nourishment from the vitellarium, including ribosomal RNA, and cytoplasmic DNA, alongwith cytoplasmic complements. Developing embryos of the mitotic phase do not synthesise RNA. Synthesis of RNA is resumed in the post-mitotic embryos concomittant with the cessation of DNA synthesis and appearance of nucleoli. Protein synthesis is a continuous process in the developing embryos and in the vitellarium. The machinery for protein synthesis is supplied by the vitellarium. In Asplanchna embryonic development upto organ differentiation, is controlled by the cytoplasmic complements received from the vitellarium (cytoplasmic DNA and RNA).

Incorporation of vit.E in the maternal diet influences the BWO expression. It also effects a variation in the nuclear number of gastric glands and vitellarium

Vit.E is specific in its mode of action. Incorporation of vit.E produces entirely different responses at different target tissues. Morphological variations are caused in the hypodermis, without affecting the nuclear divisions, whereas nuclear divisions are accelerated in the vitellarium and the gastric glands without causing any change in their respective morphology. In mictic females, vit.E alters the normal diploid maturation division. The effect of vit.E continues upto the fifth generation, and the extent of expression diminishes in succeeding generations. Effects

of vit.E do not make a permanent change in the morphology or physiology of Asplanchna . The level of response is proportional to the amount of vit.E molecules present in the organism in each generation.

Embryos of the mitotic phase are labile to the influence of vit.E. The mictic female production is not associated with the oogenesis of the organism. Studies carried out employing labelled tocopherol indicate the possibility that the molecules of vit.E undergo no change in the organism but are transmitted as such to one generation from the other with a reduction (70-90%) in its percentage.

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PLATE II

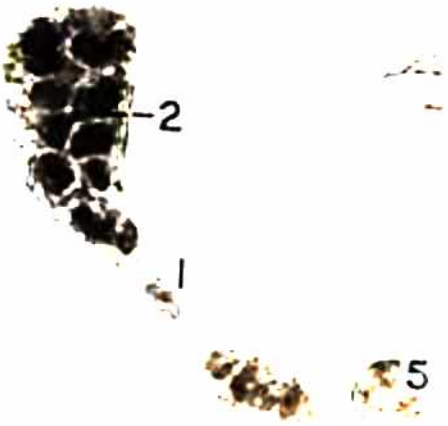
Fig.1.Vitellarium (1) with developing oocyte (4) in the vitelline phase. lobed nucleoli (2) , ovary (3) Acetone fixed x 800

Fig.2.Vitellarium (1), Gelatin section 5 u showing oocyte (5) x 1000

Fig.3.Developing embryo (4) of vitelline phase showing ovary (3) and oocytes (5). Gelatin section 5 u x 1400

Fig.4.First cleavage of embryo forming the vegetalpole (6) and animal pole (7) x 1600

1



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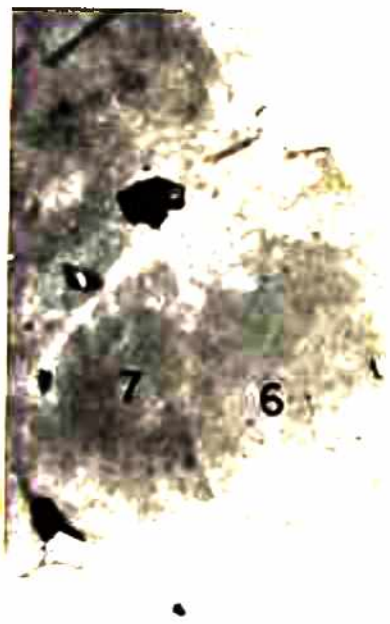

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PLATE - II

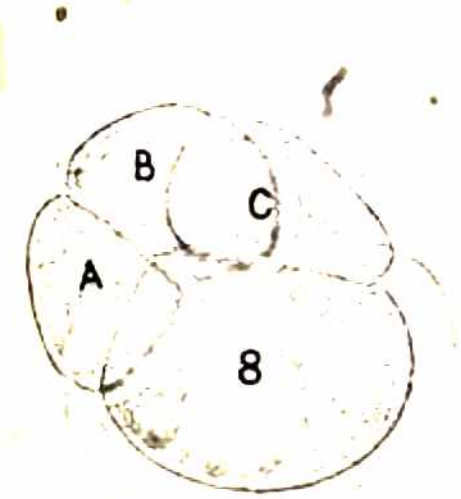
PLATE II A

Fig. 5. IInd cleavage of the embryos forming D-cell (8) and ABC blastomeres

Phase contrast photomicrograph x 1600

Fig. 6. IIIrd cleavage dividing ABC cells in two tiers forming 10 cell stage

Phase contrast photomicrograph x 1600



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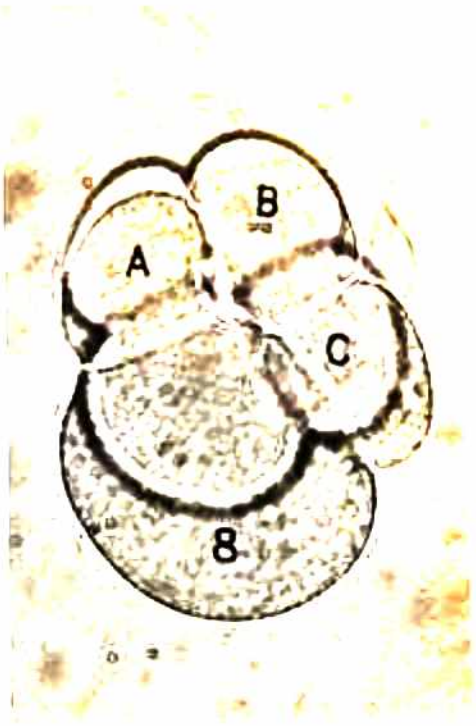

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PLATE - II A

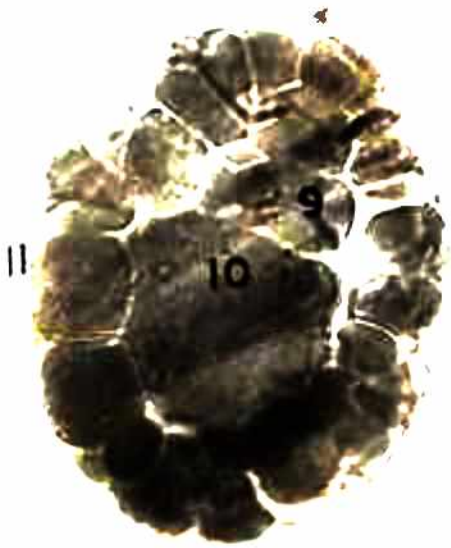
PLATE III

Fig. 1. Stereoblastula - with D-cell division forming the primordial sex cell (10) and other two cells (9). ABC blastomeres (11) divide and encircle the D-quadrant cells.
Gelatin section 5 u x 1600

Fig. 2. Sex primordial cell (10) divides forming the ovary primordium (12) and the vitelline primordium (13). Proliferation of bodywall forming primordium of the pharynx (14)
Gelatin section 5 u x 1600

Fig. 3. Sagittal section of primordial ovary (12) and pharyngial tube (14)
Gelatin section 5 u x 1500

Fig. 4. Pharyngial tube formation (15) and ovary primordial cells (12)
x 1200



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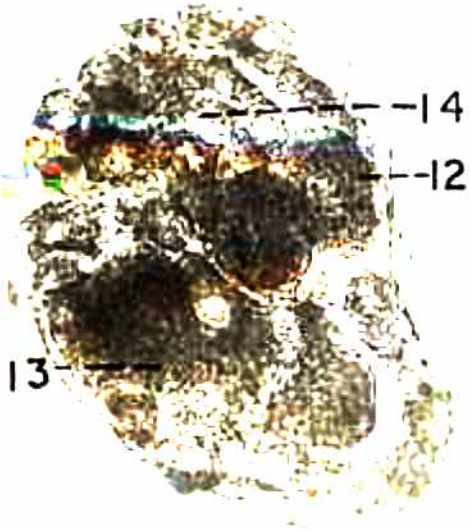

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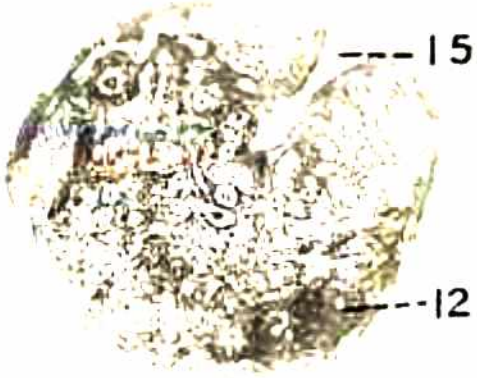

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PLATE -III

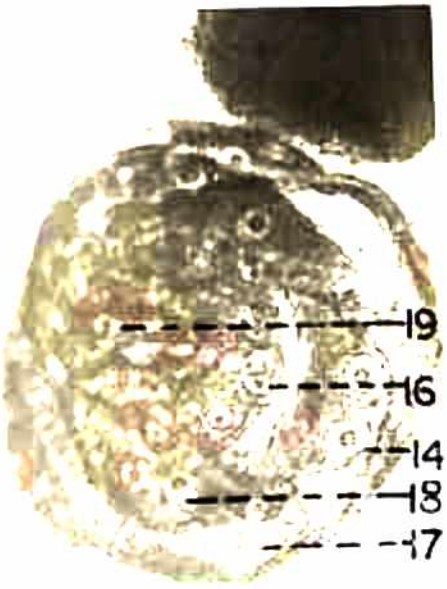
PLATE IV

Fig. 1. Frontal section - showing the bilateral symmetry of the organ primordia.pharynx (14) oesophagus (16) Nephridium (17) gastric gland cells (18) stomach cells (19) Gelatin section 5 u x 1000

Fig. 2. Sagittal section, pharynx tube (15) stomach (20) vitellarium cells (13) Gelatin section 5 u x 1200

Fig. 3. Saggital section pharyngial tube reaching the stomach. vitellarium (1) pharynx (21) gastric gland cells (18) oesophagus (22) mouth (23) Gelatin section 5 u x 1400

Fig. 4. Section fully developed embryos. stomach and pharynx (21) forming the connection oesophagus (22) mouth (23) Gelatin section 5 u x 800



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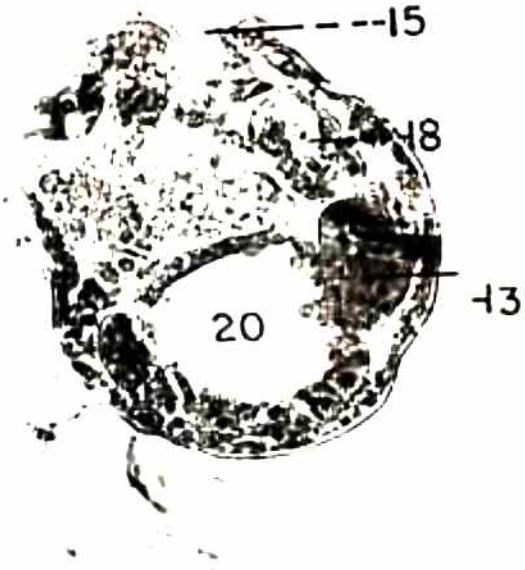

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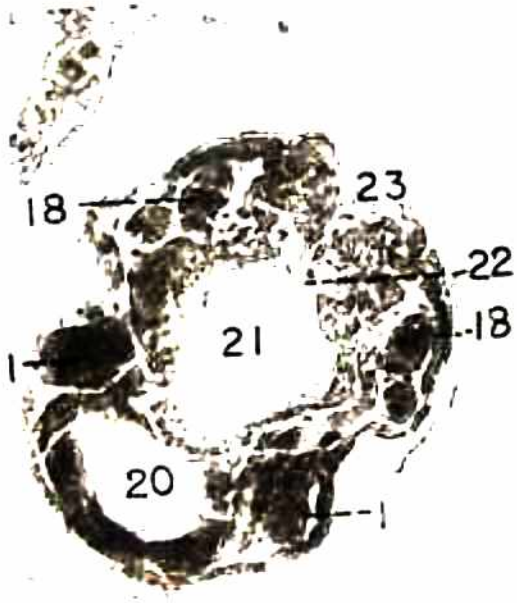

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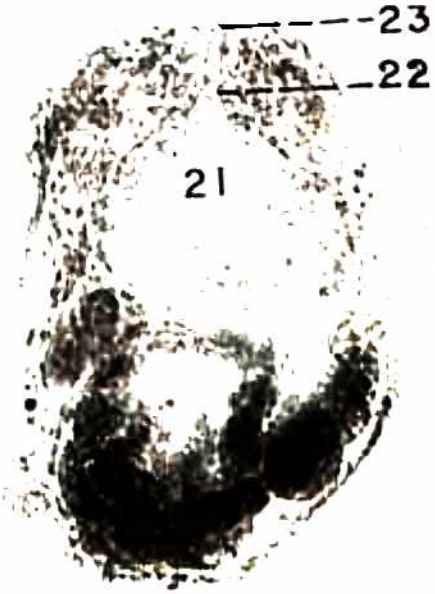

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PLATE -IV

PLATE V

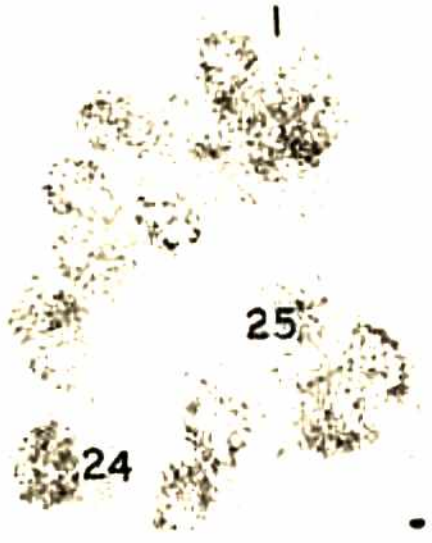
Fig. 1. Autoradiograph of post-mitotic embryo-H^3-thymidine incorporation in vitellarium (1). Only few vitellarium nuclear labelled (24) 3 minutes labelling 100 uc/ml thymidine x 600

Fig. 2. Autoradiograph of the vitellarium with 1 hr. labelling with H^3-thymidine almost all the nuclei are heavily labelled (24). cytoplasmic DNA spotted (25) x 1000

Fig. 3. Autoradiograph of vitellarium. Continuous 3 hrs. labelling with H^3-thymidine. All the nuclei labelled (24) alongwith cytoplasmic DNA (25) x 1400

Fig. 4. Autoradiograph- H^3 thydimine labelled developing oocyte (4) attached to vitellarium (1) continuous In vivo labelling for 3 hours.Cytoplasmic DNA labelled in the oocyte x 1600

1



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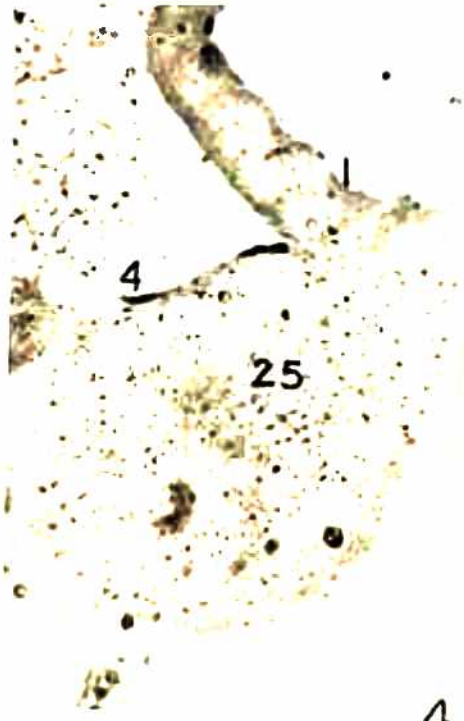

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PLATE -V

PLATE VA

Fig. 6. Autoradiograph of mitotic stage embryo (27)-In vitro 30 minutes labelling- only certain cells are labelled. x 800

Fig. 5. Autoradiograph thymidine labelled 3 minutes. The nuclei (26) of the oocyte (4) is heavily labelled x 800

5

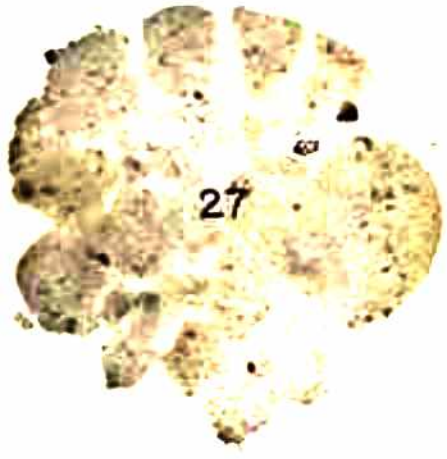

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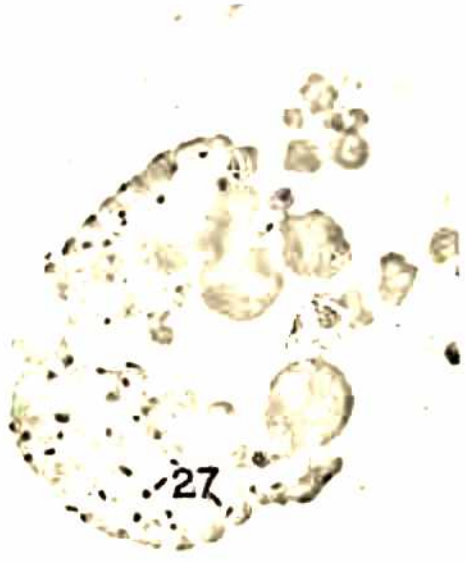
PLATE - V A

PLATE VI

Fig. 1. Autoradiograph 15 minutes labelled H^3-thymidine. mitotic stage embryos of the stereoblastula stage. D-cells are not labelled. ABC blastomers have incorporated labelled thymidine (27) x 800

Fig. 2. Autoradiograph mitotic phase 30 minutes labelling. Only one cell nucleus synthesise DNA (27). cytoplasmic DNA labelled in certain cells x 1400

Fig. 3. Autoradiograph post-mitotic embryo 15 minutes labelling certain vitellarium nuclei are labelled (24) x 800



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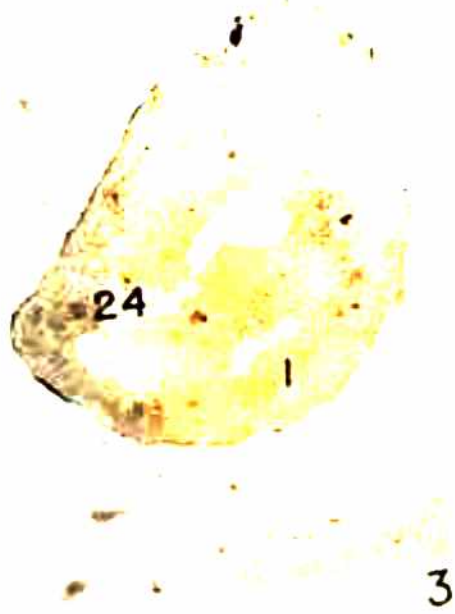

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PLATE -VI

PLATE VII

Fig. 1. Autoradiograph H^3-uridine 30 minutes labelling Heavy incorporation of uridine in the nuclei (28) of the vitellarium x 600

Fig. 2. Autoradiograph H^3-uridine 30 minutes labelling cytoplasmic RNA (29) and the nuclei heavily labelled (28) x 1600

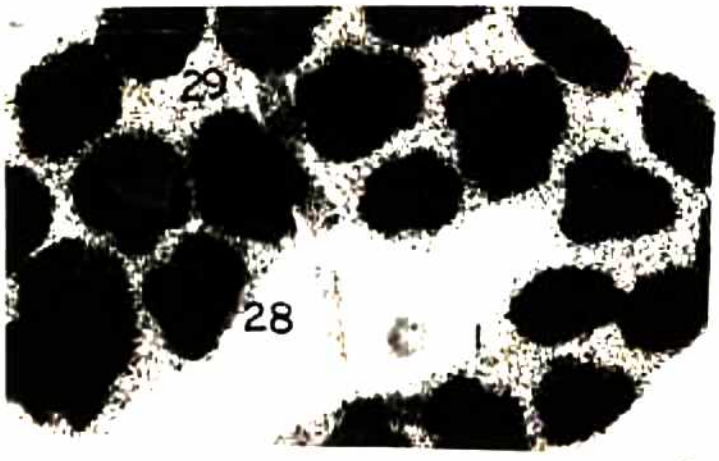
Fig. 3. Autoradiograph H^3-uridine 15 minutes labelling Developing embryo (4) in the vitelline phase is babelled, other mitotic stage embryos (30) have no incorporation of uridine x 400

Fig. 4. Autoradiograph Fig.3-enlarged. Heavily labelled RNA passed from the vittelarium to the oocyte (32) Note: RNA passing from the vittelarium to the oocyte (31) x 1200

Fig. 5. Autoradiograph H^3-uridine continuous 3 hrs. labelling- Vitellarium dark labelled (1) developing oocyte heavily labelled (4) RNA in oocytes received from thevittelarium gastric glands (33) also labelled. x 600

Fig. 6. Autoradiograph showing the RNA (32) in the developing oocyte continuous labelling (4) x 1600

1



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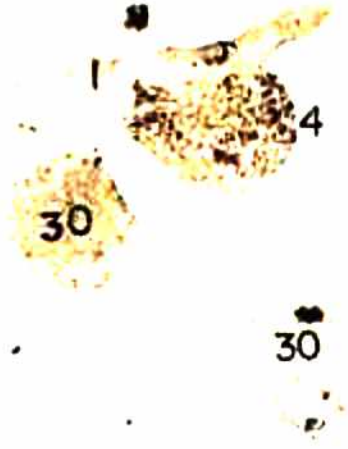

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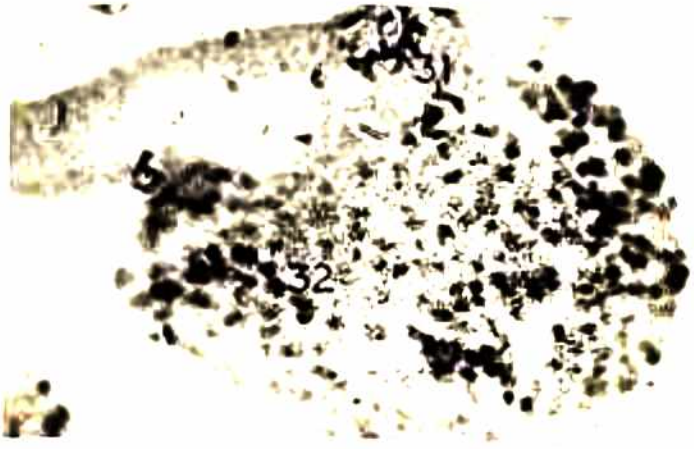

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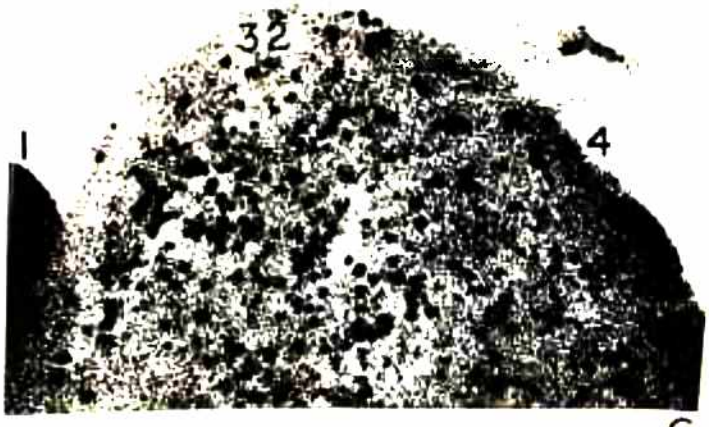


6

PLATE -VII

PLATE VIII

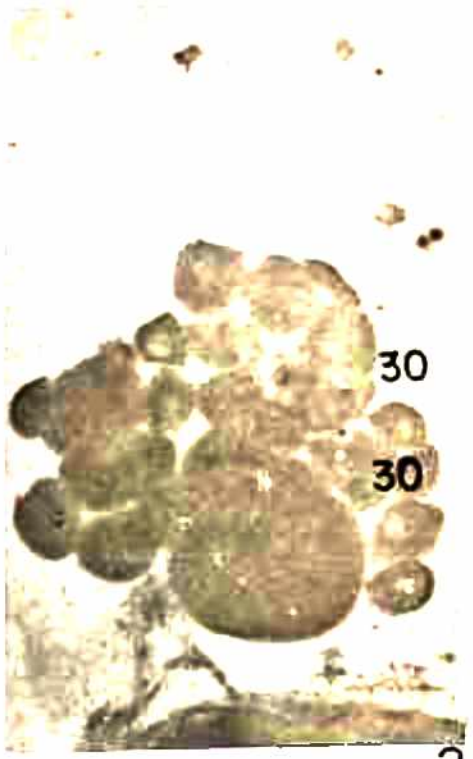
Fig. 1. Autoradiograph H^3-uridine 5 minutes labelling no incorporation of uridine in developing oocyte x 800

Fig. 2. Autoradiograph H^3-uridine 15 minutes labelling mitotic stage embryo without any incorporation of uridine RNA synthesis completely absent in these cells (30) x 800

Fig. 3. Autoradiograph H^3-uridine 15 minutes labelling embryos at different stages of development. No incorporation x 1000

Fig.4 . Autoradiograph H^3-uridine post-mitotic embryo almost all the nuclei synthesise RNA- in vitellarium (28) and gastric glands (33) rapid synthesis takes place x 600

1



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3

4

PLATE - VIII

PLATE IX

Fig. 1. Autoradiograph H^3-lysine vitellarium heavily labelled (34) x 600

Fig. 2. Autoradiograph H^3-lysine embryo in the divisional stage protein is labelled (35) x 800

Fig. 3. AutoradiographH^3-lysine protein heavily labelled x 600

Fig. 4. Mitotic embryos heavy labelling of protein x 600

1

2

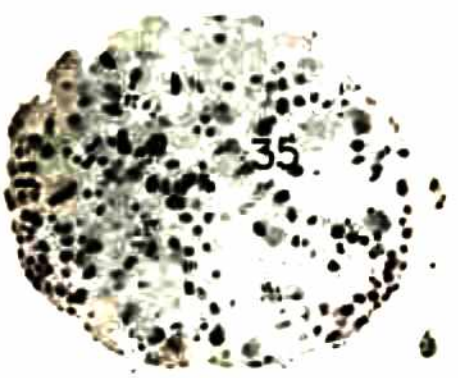

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PLATE - IX

PLATE IX A

Fig. 5. Post-mitotic embryos autoradiograph H^3-lysine heavy incorporation x 800

Fig. 6. Autoradiograph H^3-lysine fully developed embryo heavy incorporation of lysine x 800

5 6

PLATE-IXA

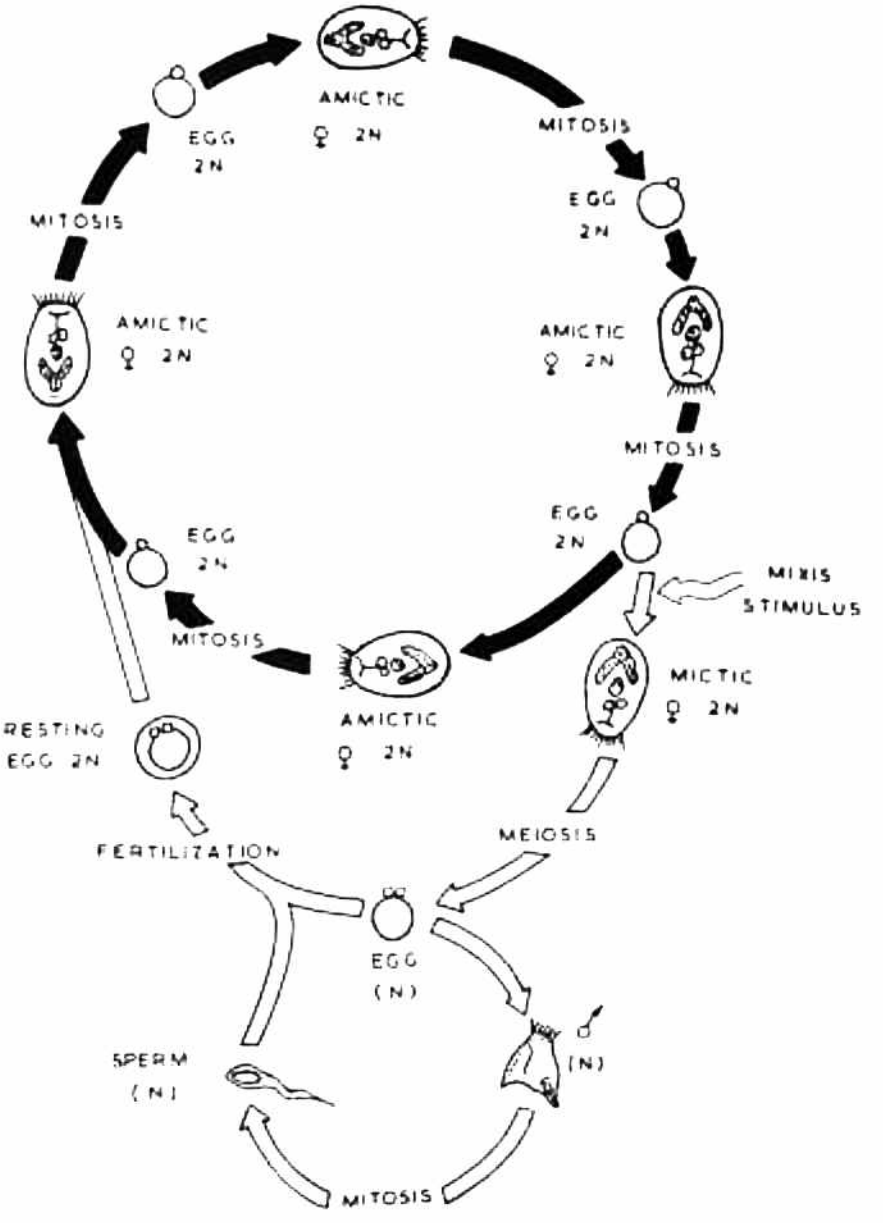
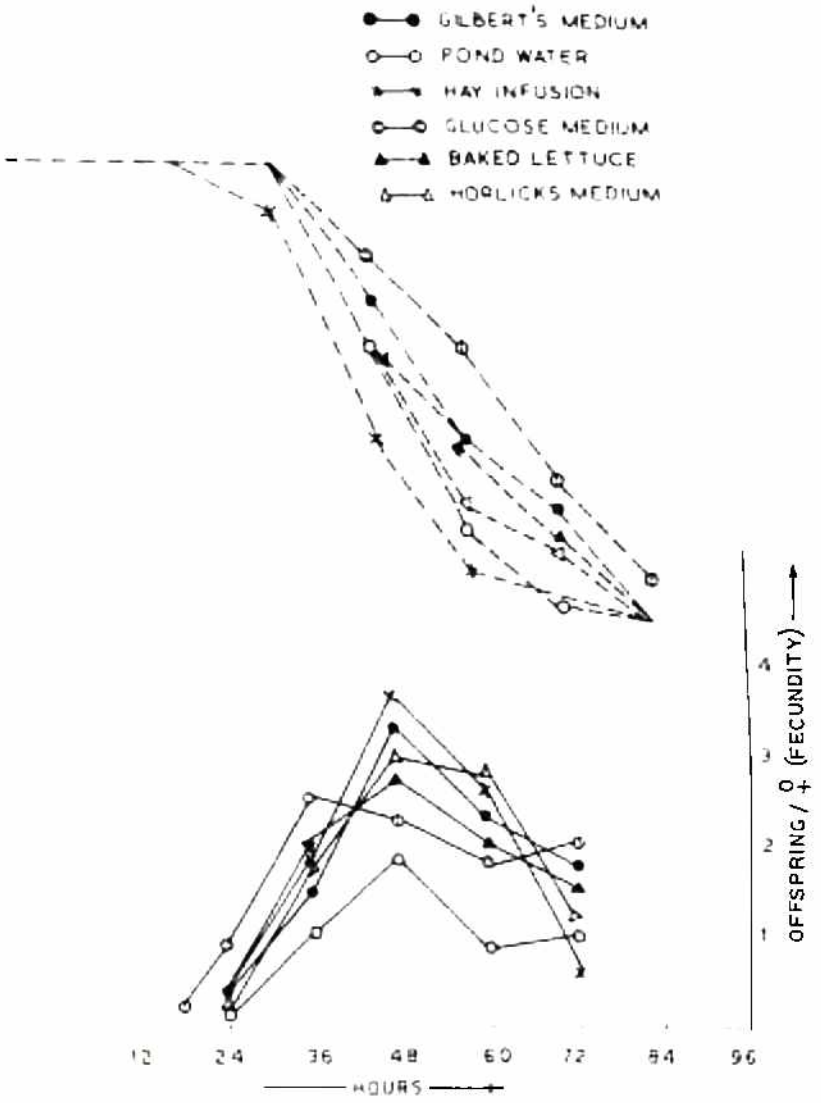


PLATE. X LIFE CYCLE OF ASPLANCHNA

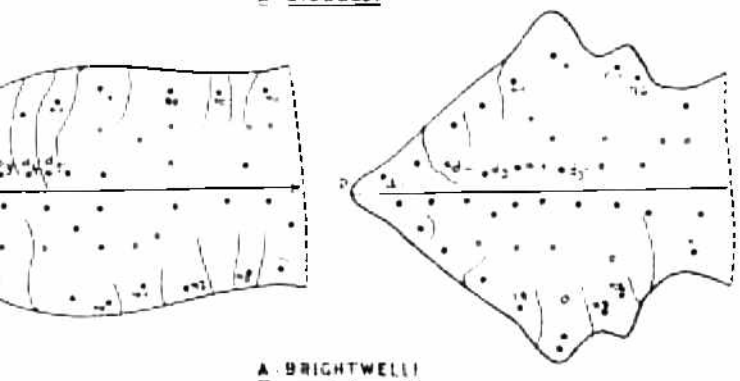
AMICTIC REPRODUCTIVE CYCLE

MICTIC REPRODUCTIVE CYCLE.



THE FECUNDITY (SOLID LINE) AND SURVIVAL % (DOTTED LINE) OF A BRIGHTWELL IN DIFFERENT CULTURE MEDIA

A. SIEBOLDI



A. BRIGHTWELLI

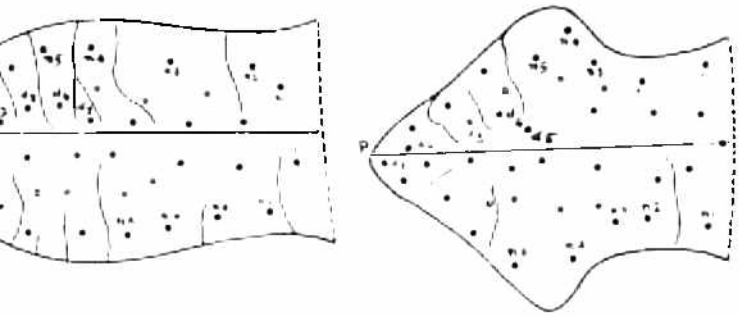


FIG 2

NUCLEAR MAPS OF HYPODERMIS OF AMICTIC (LEFT) AND MICTIC (RIGHT) FEMALES OF A SIEBOLDI AND A BRIGHTWELLI

○ - MEDIAN ◐ - SUB MEDIAN ● - MARGINAL

P L A T E XII

Fig. 1. Transitional low humped form Asplanchna brightwelli x 250

Fig. 2. Fully humped Asplanchna brightwelli x 250

Fig. 3. Fully humped Asplanchna sieboldi x 250

Fig. 4. Male Asplanchna brightwelli x 400

C- Corona; DH-Dorsal hump; Co-Copulatory organ; E-embryo
GR- Gastric glands remenents; LH- Lateral humps; M-Mastax
S-Stomach; T-Testis

1

2

3

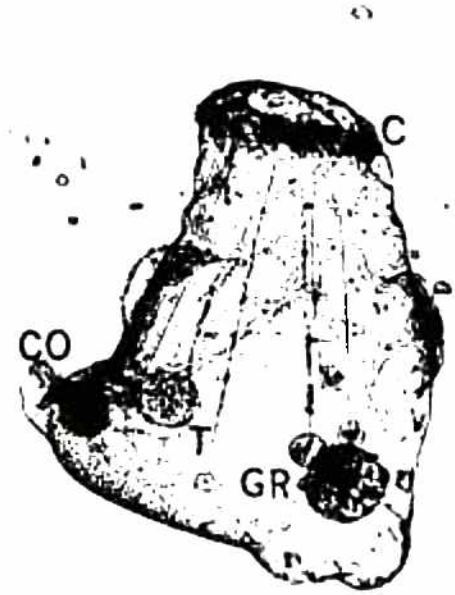


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PLATE-XII

PLATE XIII

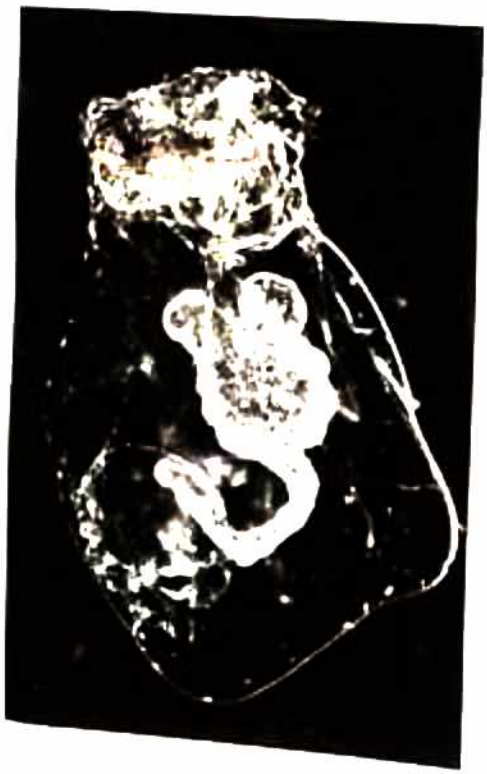
Phase contrast photographs

Fig. 1. *Asplanchna sieboldi* humped form x 300

Fig. 2. *Asplanchna brightwelli* humped form x 300

Fig. 3. *Asplanchna brightwelli* amictic female x 300

Fig. 4. *Asplanchna sieboldi* male x 400



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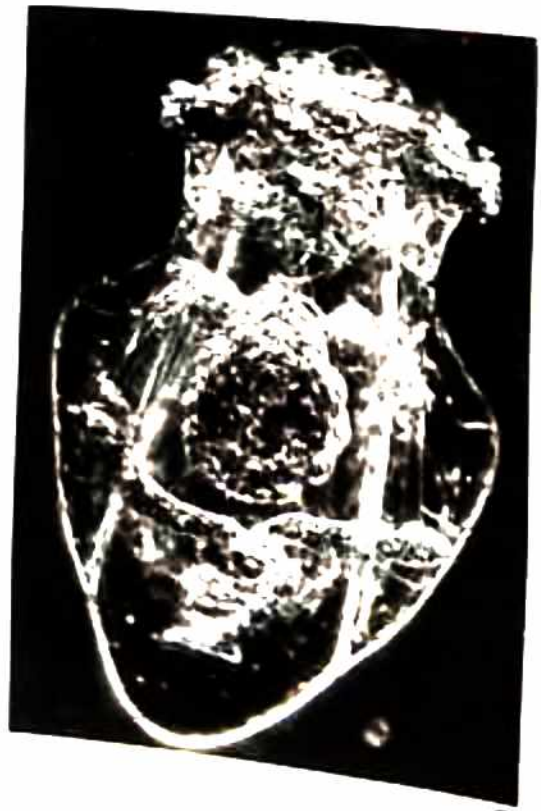

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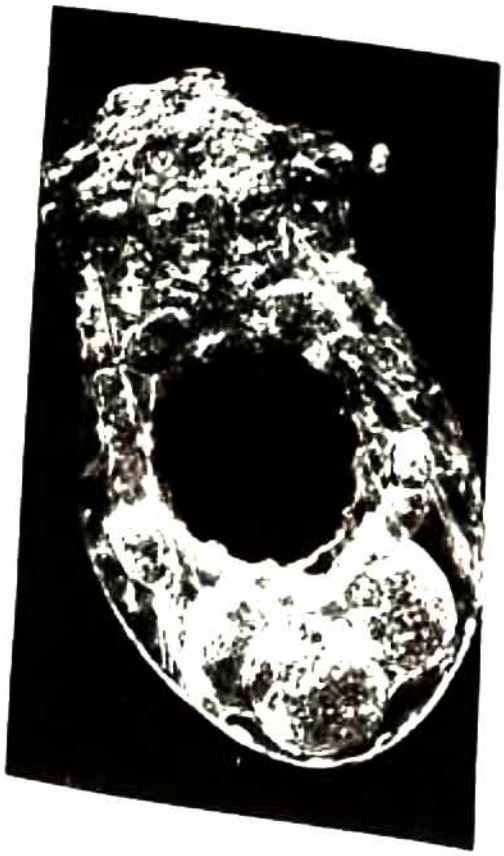

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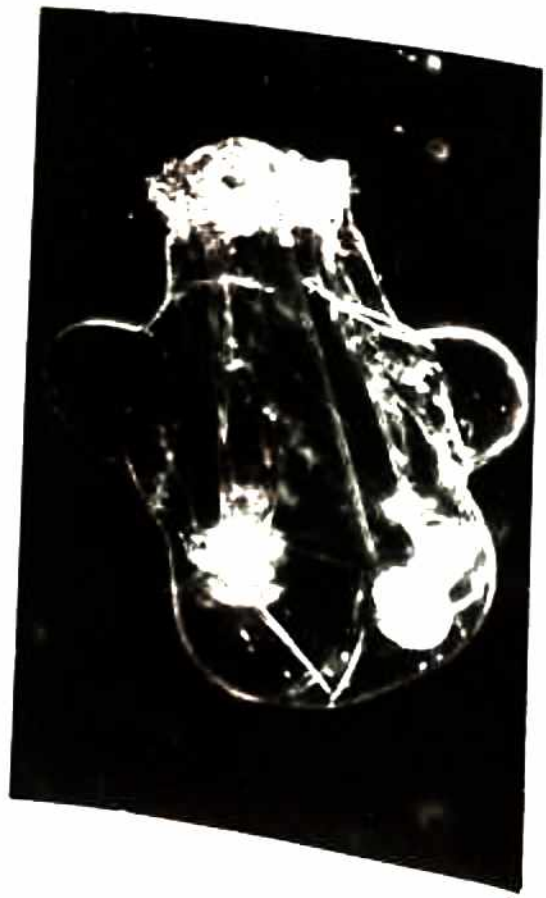

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PLATE -XIII

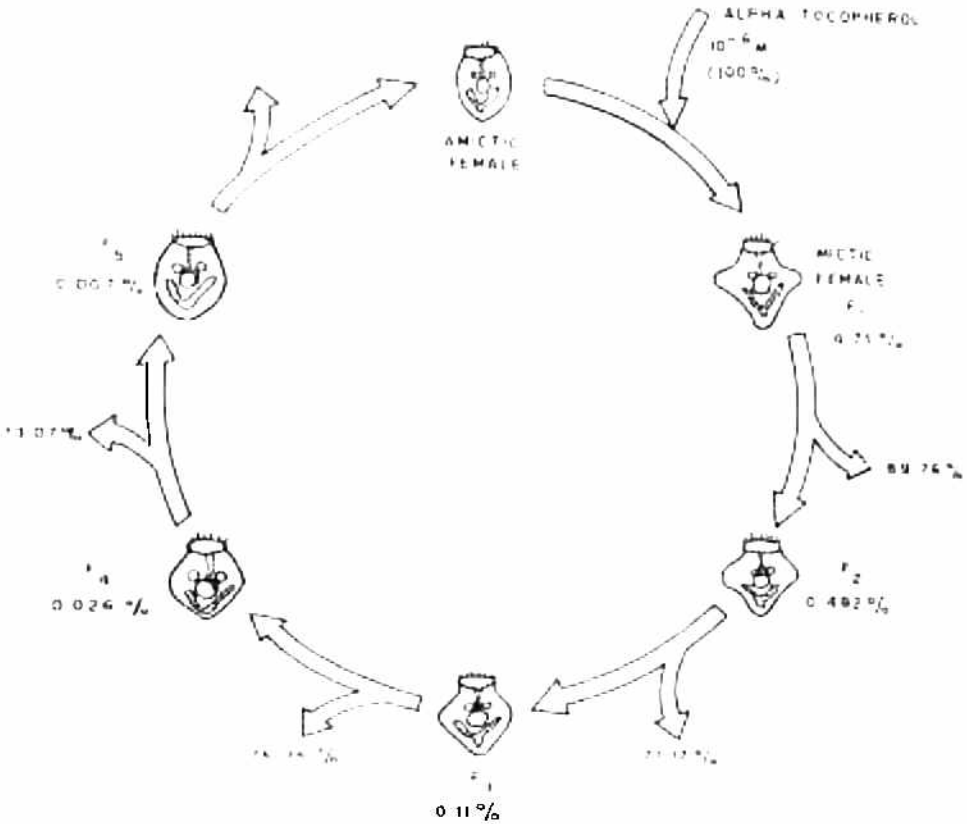
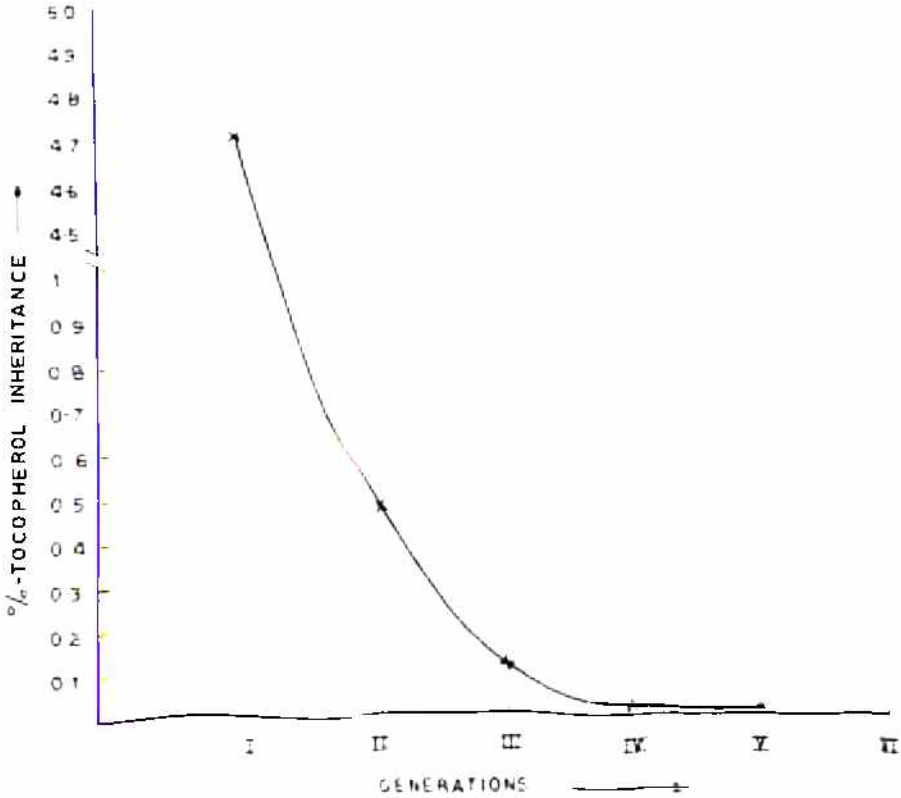


FIG 1 INTAKE AND INHERITANCE OF ALPHA TOCOPHEROL IN ASPLANCHNA BRIGHTWELLI



GRAPH. 4.1. INHERITANCE OF α-TOCOPHEROL IN ASPLANCHNA BRIGHTWELLI - BASED ON H^3- RETENTION %

PLATE. XIV