STUDIES ON THE EFFECTS OF VITAMIN E IN THE SEXUALITY OF ROTIFER ASPLANCHNA

THESIS

submitted in part fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ZOOLOGY

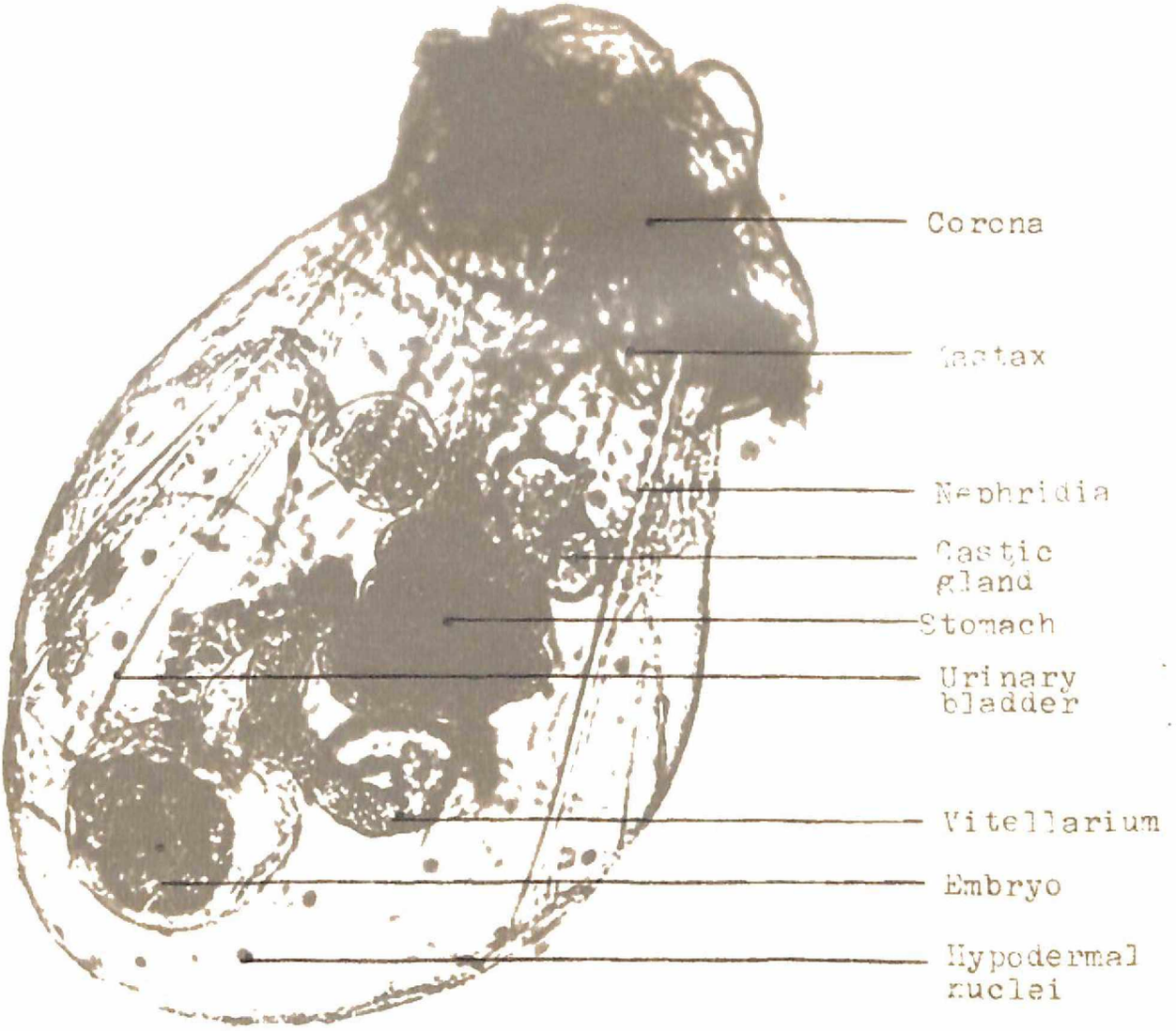
By

VINEY SETH

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE

PILANI, RAJASTHAN

dedicated to my parents



Asplanchna brightwelli

PLATE 1

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SUPERVISOR'S NOTE

The thesis entitled "Studies on the effects of vitamin E in the sexuality of rotifer Asplanchna" is a piece of original work of Shri Viney Seth.

(A.K. Datta Gupta)
Prof. of Zoology and
Dean, Faculty of Science
B.I.T.S., Pilani

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Viney Seth

Viney Seth

C H A P T E R 1

INTRODUCTION

1.1 General Introduction

Rotifers, as a group have attracted the attention of very few workers. Reproduction and sexual transformation in these organisms have received still less attention. The studies of Gilbert and Thompson (1968), Gilbert (1968), and Birky (1967, 1968, 1969) on the sexuality induced in rotifers by administering alpha-tocopherol (vitamin E) along with their food have been of stimulating interest. The discovery of alternation of amictic (asexual) generations by a single mictic (sexual) generation (Ruttner-Kolisko, 1968) has opened newer lines of inquiry. More recently Gopinath (1972) has found almost identical results about mixis phenomenon following Birky's (1967, 1968) method. The present author considered it important to be able to ascertain in quantitative terms the transmission of vitamin E from mother to offspring and to study the nature of mictic males or of the females produced under the mixis stimulus.

Rotifers are exclusively dioecious with a marked sexual dimorphism. These diploid animals usually multiply by female parthenogenesis, but relatively short period of biparental reproduction may intervene which according to Birky (1969) is the mixis phenomenon and, in Ruttner-Kolisko's words (1968) 'alternation of amictic generation by

a single mictic generation'. This alternation of the mode of reproduction has been defined as a change from the amictic female production to mictic female production. Both types of these females are identical morphologically, but differ in the kinds of eggs they produce. The males, on the other hand, are very much different from the point of view of shape and structure. The stimuli which induce the diploid parthenogenetic eggs of an amictic female offspring to develop into mictic female offspring are the environmental factors (Shull, 1913; Tauson, 1925, 1926, 1927; Birky, 1964; Gilbert, 1966, 1968), starvation (Mitchell, 1913), or dietary changes (Whitney, 1929).

Vitamin E is an important algal constituent and acts as a chemical inducer for both sexual transformation and formation of outgrowth on the body wall (BWO) (Gilbert and Thompson, 1968). This sexual transition is effective as long as a definite amount of vitamin E is retained during successive generations.

The present work is on the reproduction of vitamin E fed Asplanchna brightwelli sensu lattissimo (Plate 1) with special reference to sexual transformation. Efforts have been made to study the path of vitamin E during intake, and the amount of H^3-Methyl-D-α-tocopherol transmitted through successive generations of Asplanchna brightwelli. The presumption of haploidy in the males (Birky and Gilbert, 1971) lacks supporting evidence from the study of the chromosomes of these animals.

In the present work some attempts have also been made to ascertain the number of chromosomes in the parthenogenetic and the sexual forms of these animals.

1.2 Review of Literature

The group Rotifers

Rotifers, also called Rotatoria or wheel-animalcules constitute an interesting group of invertebrates. These organisms are fairly widespread and exhibit certain striking phenomena during development. One such interesting phenomenon is cyclomorphosis (Huber, 1906) and, attempts have been made in the past to study cyclomorphosis, cell constancy, and sexual transformation in these animals (Wesenberg-Lund, 1900, 1908, 1930; Voigt, 1904; Birky, 1968, 1969; Edmondson, 1963; Hutchinson, 1967, etc.). Owing to these properties rotifers are increasingly employed for the study of the problems of developmental biology. Computer oriented studies involving symmetry and growth are actively pursued in the laboratories of a competent worker (Levinthal, 1971; personal communications).

Rotifers were first described by Leewenhoek (cited by Hyman, 1951) and were put under Protozoa but later, were grouped under the division Zoophytes (Linnaeus, 1758; Lamarck, 1902; Muller, 1786 - cited by Hyman, 1951). Dutrochet (1812, 1813 - cited by Hyman, 1951) recognized rotifers as a group of animals which are higher than Zoophytes in structure and Ehrenberg (1838) separated them from protozoa but still

regarded them as Infusoria under the group Rotatoria. Many of Ehrenberg's contentions concerning rotifer morphology were amended by Dujardin (1841 - cited by Hyman, 1951) and Huxely* (1853). Hatschek* (1878) promulgated his trochophore theory which assigns great phylogenetic significance to the group. Notwithstanding, a scheme of classification of the group was still awaited, and Hudson and Gosse (1886-89) presented a scheme for the first time, which, with some modifications (Wesenberg-Lund, 1900) is still widely followed. The modification of Wesenberg-Lund (1900) is in the erection of the order Seisonacea . The latter, together with Bdelloidea, form the sub-class Digononta having two gonads; the other rotifers with one gonad were placed under the sub-class Monogononta. Later studies of Harring (1913) and Remane (1933) gave more comprehensive accounts of these animals and also their classification.

Cyclomorphosis

Wesenberg-Lund (1930), Buchner et al. (1957), Edmondson and Hutchinson (1934) studied in detail the phenomenon of cyclomorphosis. Changes in the morphological characters as a result of cyclomorphosis led to confused results in identification and classification of rotifers (Hutchinson, 1967). Studies on cyclomorphosis have earlier been carried out in this laboratory by Nayar (1965a,b,c).

In spite of these works the cause and the significance of this particular phenomenon are still not satisfactorily understood. Hutchinson (1967) has given an account of seven

* cited by Hyman 1951

species of rotifers following the catalogue of Wisznieŵski (1934), leaving apart two species namely, Asplanchna amphora and Asplanchna ebbesborni. The differentiation at the species level under the genus Asplanchna is based either on the hump morphology (Rousselet, 1909), the number of nuclei in the gastric glands and the vitellarium (Sudzuki, 1964[a]) or on the nature of the tooth in the inner margin of the scapus of the mastax (Daday, 1885). Power (1912) and Mitchell (1913[b]), and Mitchell and Power (1914) have studied the population of Asplanchna sieboldi, which are trimorphic and which produce saccate as well as humped and, a very large campanulate forms. According to Mitchell (1913)[a,b], production of the humped forms is due primarily to the nature of food. The hump formation (Power, 1912; Birky, 1964) and the changes in the nuclear number in the vitellarium and the gastric glands (Birky and Field, 1966) in the polymorphic forms, have been experimentally proved to be the effects of dietary changes in the organism. As a result, Gilbert (1968) has included Asplanchna intermedia of Hudson and Gosse (1889) tentatively under the species Asplanchna brightwelli, although de Beauchamp (1951) has erected a supra species Asplanchna brightwelli sensu Lattissimo comprising of Asplanchna ebbesborni, Asplanchna amphora, Asplanchna levdigi and Asplanchna intermedia. The confirmatory studies are yet to be made to ascertain the exact status of these species.

Lauterborn (1898, 1901, 1904) studied in detail the cyclomorphosis (climate induced variation) in Keratella

cochlearis and stated that there can be three possible types of cyclomorphosis which are exhibited simultaneously and independently by the population of this species in a single pond. The first of these 3 types exhibits decreased in the length of the lorica and the posterior spine; the second type is characterized by production of small spinelets all over the lorica, while in the third type there are irregular plaques in the dorsal sculpture.

The phenomenon of cyclomorphosis is more interestingly exhibited by the genus Brachionus. The best account of this has been given by Sachse (1911) and Wesenberg-Lund (1930), besides, there are innumerable field observations by many other workers (Hartman, 1920; Kofoid, 1908; Whitney, 1916; Buchner, 1936; de Beauchamp, 1937, 1938, 1952a,b; Gilbert, 1967, etc.). The morphology of the rotifer Brachionus calyciflorus is extremely variable (Sachse, 1911; Gilbert (Asplanchna) 1967). This species always has two pairs of anterior spines namely, the antero-median and the antero-lateral, and a pair of posteromedian spines. A pair of postero-lateral spines may or may not be present. All of these four pairs of spines vary considerably in length but the postero-lateral is particularly important. The species has two common varieties namely, pala and dorcas, which are distinguished on the basis of shorter or longer length of the antero-median spine. The presence of the postero-lateral spines in the variety is referred to as Brachionus calyciflorus var. pala forma amphiceros, whereas the specimens lacking postero-lateral spines are referred

to as Brachionus calyciflorus var. pala. The specimens of the dorcas variety lacking postero-lateral spines are referred to as Brachionus calyciflorus var. dorcas. The latter variety having postero-lateral spines are called Brachionus calyciflorus var. dorcas forma spinosa (Gilbert, 1967). The variation in the length of postero-lateral spines is due to several factors as have been listed by several authors namely, temperature (Kofoid, 1908), food supply (Sachse, 1911; de Beauchamp, 1937, 1938; Buchner, 1936), annual cycle (Wesenberg-Lund, 1930), and chemical inducer (Whitney, 1916a,). de Beauchamp (1952a,) noted in a culture of Asplanchna brightwelli where Brachionus calyciflorus was provided as a food, that Asplanchna brightwelli tend to show considerable variation in the length of their spines, which were later described as due to 'Asplanchna substance'. The substance accumulated in the water where Asplanchna brightwelli lived. The spine production by Brachionus calyciflorus is considered to be a defensive mechanism from the predator i.e. Asplanchna brightwelli (de Beauchamp, 1952a).

Embryology of rotifers

The rotifers are oviparous, ovoviviparous, as well as viviparous (Hyman, 1951). The members of the order Bdelloidea lack males and their eggs develop parthenogenetically into females. The monogonots have three kinds of eggs, namely, (1) thin-shelled amictic eggs that cannot be fertilized and that develop parthenogenetically into females, (2) smaller

thin-shelled mictic eggs which if not fertilized develop into males, and (3) thick-shelled dormant eggs which are fertilized mictic eggs and invariably hatch into amictic females. Seasonids have eggs of one sort which are required to be fertilized and they hatch into either sex (cited by Hyman, 1951). The best account on the embryology is that of Nachtwey (1925) on the amictic eggs of the viviparous Asplanchna. Tannreuther (1920) also studied the development of Asplanchna. According to these authors the egg undergoes cleavage and, after the primordia of all the organs have been laid down, cell multiplication ceases and there is no further formation of additional cells. The number of the nuclei is fixed in the early embryonic stages and remain constant throughout the life. The histological disposition in these organisms is in the form of syncytium. An indication of their number of cells can be had from the number of nuclei. At the time of hatching, female rotifers of free swimming group are morphologically complete and grow to sexual maturity in a few hours time. The males are generally sexually mature at birth and do not grow. In sessile rotifers the free swimming juvenile form when attached to the substratum, developed into adult females. Meyer (cited by Hyman, 1951) has emphasised the general resemblances between acanthocephalan and rotiferan cleavage pattern. Gopinath (1972) studied in detail in vitro and in vivo developmental patterns of Asplanchna brightwelli. A very interesting phenomenon of eutely, where each organ shows a constancy of number of nuclei, is exhibited to a marked extent by these organisms. Martini (1912)

reported the number of nuclei in Hydatina senta to range from 900-1000 with each organ having a constant number of nuclei. Nachtwey (1925) examined a number of females both humped and saccate forms and confirmed that the arrangement is bilaterally symmetrical. Studies of Birky and Field (1966) have revealed variations in the number of nuclei in the vitellarium and in the gastric glands of Asplanchna. Birky and Power (1968) and Gopinath (1972) have shown nuclear number of other organs, including that of the body wall, to be constant in Asplanchna sp. Levinthal (1971, personal communication) estimated the number of brain tissue of Asplanchna brightwelli to be 182.

Cohn (1856)* described for the first time the life cycle of Brachionus urceolaris, and later by Maupas (1890a, b; 1891) in Hydatina senta. It was observed for the first time that the eggs of the mictic females of Asplanchna priodonta have two polar bodies, whereas amictic females have only one (von Erlanger and Lauterborn, 1897). Similar observations have been made in many other monogonont rotifers. Tauson (1924) and Storch (1924) suggest that amictic oocytes either undergo only a single maturation division or that one polar body nucleus fuses with the egg nucleus. Birky (1964, 1965, 1967a) studied the physiology and life cycle of Asplanchna brightwelli in details and has supported the views of earlier workers (von Erlanger and Lauterborn, 1897; Tauson, 1924; Storch, 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 forms haploid male progeny.

Sexual transition : Mixis stimuli

The studies on the life cycle of rotifer have shown that the mictic female production is due to the influence of certain environmental factors. The major effective factors which influence mictic production are reported to be starvation (Nausbaum, 1897; Mitchell, 1913b), changes in culture medium (Shull, 1911a,b, 1912, 1915, 1918a,b, 1925; Kahn, 1921), temperature (Maupas, 1891; Moro, 1915), pH (Tauson, 1925, 1926, 1927; Luntz, 1929), photoperiodism (Laderman and Guttman, 1963), the dietary changes (Whitney, 1916, 1929) and population density (Wesenberg-Lund, 1930; Hertel, 1942; Gilbert, 1963). The dietary inclusion of Chalmydomonas resulted in the production of mictic progeny in Asplanchna amphora (Tannreuther, 1919; Whitney, 1929). Buchner and Kiechle (1965) and Kiechle and Buchner (1966) added Chlorella and Euglena to the culture of paramecia fed Asplanchna sieboldi and noticed the formation of mictic animals. Gilbert (1967) extracted certain plant lipids from spinach leaves and found them to have profound influence on the reproductive cycle of Asplanchna. Gilbert and Thompson (1968) isolated and identified alpha-tocopherol (vitamin E) from Scottish Experth grass which proved to be an inducing factor for the mictic female production in Asplanchna.

History of vitamin E

Evans and Emerson, Emerson (1936) isolated two E vitamins, alpha and beta-tocopherol in crystalline form. Within the next two years the constitution of alpha-tocopherol was established and dl-alpha-tocopherol was synthesised. From the researches that followed it became apparent that this substance occurs fairly widely in nature, in plants, in particular.

In this connection it should be worthwhile to quote a passage from the speech of Herbert M. Evans (1962), the discoverer of crystalline vitamin E, on the occasion of the Symposium on vitamin E and metabolism, in Zurich, in 1962.

"The chemical contribution of the Emersons is primarily in the whole vitamin E story (Evans et al., 1936). I well remember their plea to me to suggest a proper name for their purified substance when success crowned their efforts. I promptly invited George M. Calhoun, our professor of Greek to luncheon in Berkeley in our small Faculty Club. "Most scientists, medical men especially", said Calhoun "have been guilty of coining Greek-Latin terms, bastards, of course, and we might have to do this". "What does this substance do?" he asked. "It permits an animal to bear offspring.", I replied. "Well, 'childbirth' in Greek is tocos", he said, "and if it confers or brings childbirth, we will next employ the Greek verb phero. You have also said that the terms have an ending consonant with its

chemical - 'ol', it being an alcohol; your substance is 'tocopherol'.....

Vitamin E and mictic production

The importance of vitamin E compounds and their effects in invertebrates have not been adequately understood. Earlier, Viehoever et al. (1937, 1938) reported that vitamin E enhances vigour and fecundity in cladoceran Daphnia magnum. Further, vitamin E was found to stimulate egg sac formation in copepodes (Jacobi, 1957) and spermatogenic activity in cricket Acheta sp. (Meikle et al., 1965). Asplanchna is the first organism where vitamin E has been found to initiate either a transition from parthenogenetic to sexual mode of reproduction, or a marked change in the body morphology (Gilbert and Thompson, 1968). Birky (1968) stated that vitamin E directly or indirectly controls the number of mitotic divisions in the vitellarium and presumably in the gastric glands whereas the control is differential during the growth of hypodermis. Birky (1969) analysed quantitatively the changes in the morphology induced by vitamin E. The elicitation of three types of responses i.e. mixis, body wall outgrowth (BWO), and reduction in the fecundity of the amictic females by an increase in the level of vitamin E have been studied quantitatively (Birky, 1968, 1969; Birky and Power, 1969). Riggs and Gilbert (1972) measured the labile period of mictic formation and body wall outgrowth induced by tocopherol in the embryos of Asplanchna sieboldi, and claimed that the embryos of this species remain labile

until very late stages of development with respect to differentiating into amictic or mictic females with or without detectable BWO response. They further compared the labile period of Asplanchna sieboldi with Asplanchna brightwelli, the former being of considerably longer duration than that of the latter species.

Birky and Gilbert (1972) have followed the fate of (H^3) alpha-tocopherol fed to the amictic females. These authors have described that in each generation, females lose about 50% of their maternal tocopherol; the remaining material, which is 76-100% undegraded is transmitted almost entirely to their male or female offspring. While discussing the fate of vitamin E these workers claim that successive offspring receive smaller amount of their parental tocopherol. Birky and Power (1969) found it difficult to identify alpha-tocopherol as an intrinsic inducer. Gilbert and Thompson (1968) and Birky (1969) have stated that vitamin E concerns the adaptive significance for the use of this molecule for the production of mictic females. Gilbert (1971) considered that a high level of vitamin E may be an essential constituent for the fertility of male Asplanchna, but not for females. It was further remarked by Birky and Gilbert (1972) that all the rotifer population have evolved mechanism for availing of high level of vitamin E before sexual reproduction is initiated, which should mean that dietary vitamin E is efficiently transmitted without undergoing any degradation from parent to offspring.

Birky (1967) carried out some experiments on the nucleic acid synthesis in Asplanchna brightwelli. Later Gopinath (1972) worked in some details on the nucleic acid and protein synthesis in the developmental stages of Asplanchna brightwelli, applying autoradiographic techniques. According to him, DNA synthesis in the vitellarium, which supplies nourishment to the developing oocyte, indicates polypolidization of its nuclei. Further observations by the said author made on the RNA synthesis in the developing embryos indicate no RNA synthesis in the mitotic phase. But the synthesis is resumed in the post-mitotic embryos, concomitant with the cessation of DNA synthesis. The protein synthesis, however, is a continuous process in the developing embryos as well as in the vitellarium (Gopinath, 1972).

Chromosomes in rotifers

The cytological studies on the Epiphanes senta (Shull, 1921; Whitney, 1909, 1924, 1929) and Asplanchna sp. 'of brightwelli group' (Tauson, 1924) and Asplanchna priodonta (Storch, 1924), have revealed that the amictic oocyte undergoes a single maturation division with no indication of synapsis of the homologous chromosomes or of the reduction in the chromosome number. On the contrary mictic oocytes are known to undergo classical meiosis.

The reports on the chromosome studies are, however, confusing. Whitney (1924) established that the spermatocytes of male Asplanchna intermedia have a haploid number of 26 chromosomes, which develop directly into spermatozoa. However

the author also described that some spermatocytes undergo division to produce cells with 13 chromosomes which develop into 'rudimentary' spermatozoa (cited by Koehler, 1965). Later Whitney (1929) reversed his earlier opinion on the basis of his studies on Asplanchna amphora and stated that the primary spermatocytes contain a haploid number of 13 chromosomes. Tauson (1924, 1927) found a haploid number of 12 chromosomes in Asplanchna intermedia. She claimed that the unfertilized haploid eggs diplodize before the first cleavage so that the mature male is diploid. The interpretation of these workers is further confused by the taxonomic difficulties involved with the identification of the species of Asplanchna due to the formation of morphologically different animals during cyclomorphosis. Bdelloid forms namely, Philodina senta and Habrotrocha tridens in which reproduction is exclusively by parthenogenesis show chromosome number of 13 (Hsu, 1956).

Ruttner-Kolisko (1968, 1969) has questioned biparental reproduction in rotifers and considers it as a case of 'cryptoparthenogenesis'. Birky and Gilbert (1971) have suggested further work for genetical verification of rotifer cycle and confirmation of haploidy in the males of these animals.

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C H A P T E R 2

MATERIAL AND GENERAL METHODS

2.1 Test organism

All experiments have been performed on laboratory cultures of the monogonont rotifer, Asplanchna brightwelli sensu latissimo (Fig. 1, plate 1). Asplanchna brightwelli was collected from an artificial pond in Pilani (Rajasthan) and the cultures of these animals were maintained under laboratory conditions throughout the work from October 1972 to May 1974. Besides, resting eggs of Asplanchna brightwelli, from which fresh cultures were then developed, were obtained from the Faculty of Genetics, Ohio State University, Columbus, Ohio, U.S.A., through the courtesy of Dr. C.W. Birky. The local collections of Asplanchna brightwelli were identified in this laboratory by Dr. N.V. Gopinath and confirmed by Dr. C.W. Birky of Ohio State University and Dr. C.K.G. Nair of Christ Church College, Irmzalakuda, India. Identical results have been obtained in the experiments performed with Asplanchna brightwelli cultured from the resting eggs obtained from Ohio as well as with those from the local collections.

2.2 Culture method

Rotifers have been successfully reared in the laboratory by several workers in appropriate culture media (Needham et al., 1933; Nathan and Landerman, 1959; Dougherty, 1963; Birky,

1964, 1965; Lansing, 1964; Gilbert, 1967, 1968; Gopinath, 1972). The efficiency of these rearing methods on testing (Table 2.1) revealed that the organisms grown on Gilbert's media (GM) (Gilbert, 1967, 1968) were the largest in size (800-1200 µ) and the fecundity of the organisms grown in malted Horlicks in Hay infusion was found to be the maximum (Gopinath, 1972).

In the present work the stock cultures of Asplanchna brightwelli as well as the experimental cultures were maintained in distilled water and Hay infusion in the proportion of 5:2 v/v in cavity blocks of 10 ml capacity with adequate supply of healthy paramecia. In a series of preliminary experiments the most suitable method found for labelling the animals was to label the paramecia first with labelled tocopherol and feed the rotifers on labelled paramecia. Experiments to grow rotifers on other unicellular organisms did not prove satisfactory.

The culture of Paramecium aurelia was prepared on paddy extract (prepared with 50 gms of dried straw of Oryza sativa in one litre of boiled water, adjusted to pH 6.5 with calcium carbonate) containing Aerobactor aerogens. Adequate care was taken to avoid contamination by other organisms. For the purpose of feeding a paramecia concentrate was prepared by filtering the culture through a nylon net of mesh size 5 µm and followed by centrifugation. The settled mass of paramecia was directly transferred to the rotifer culture in the cavity blocks.

Culture medium	Range of size (µm)	Fecundity* per female	Remarks
1. Artificial pond water (Lansing, 1964)	600-850	5.3	
2. Baked lettuce (Birky, 1964)	800-1000	6.33	
3. Gilbert's media (GM) (Gilbert, 1967,1968)	800-1200	7.33	
4. Gilbert media (GM) with Hay infusion (Gopinath, 1972)	800-1000	7.6	1% Hay infusion in Gilbert's media
5. Malted Horlicks in Hay infusion (Gopinath, 1972)	500-800	8.4	0.5 gm Horlicks in 98 ml distt. water and 2 ml of Hay infusion
6. Distt. water and Hay infusion with paramecia	900-1020	5.6	In 5:2 proportion

*Significant at 5% level. $P > .05$ at 5% significante level

Table 2.1 Size and fecundity of Asplanchna brightwelli in different culture media

Increase in the number of paramecia in the rotifer culture was found to have inhibitory effect on the growth of the organisms. Hence, the concentration of paramecia in rotifer culture media was maintained around 10^3/ml of culture medium. The number of rotifers in a cavity block containing 5 ml of culture media was maintained between 15-20 which happened to be the right size of population of the rotifers in each culture block for healthy growth of the organisms. Cannibalism was observed when the size of the population was larger than 15-20 in a culture container.

2.3 Fixation

Asplanchna being delicate and having a sac like structure responds unfavourably to the conventional methods of fixation. So a choice of fixative was found necessary in order to get well expanded animals without the displacement of internal organelles, breakage of muscle strands and nerves, and distortion of cellular structures. Birky (1967) exposed Asplanchna to 0.1% novocaine for about 15 minutes and achieved limited success. Later he (1969) used 0.01% osmic acid for fixation and 70% ethanol for dehydration in order to get well expanded animals with anatomical structures properly displayed. This procedure has been found to be useful and has generally been followed for the gross anatomical studies here. Gopinath (1972) tried different fixatives namely Acetone, Bouin's, Formalin commercial and neutral, Osmic acid, etc., and has compared the efficiency of these fixatives. For anatomical studies in the present work

the fixation has been done in 30% ethanol with gradual upgrading to 70% ethanol. This method has been found to give better fixation besides being economical.

Other fixation methods have been employed with necessary modifications depending upon the type of study involved namely, BWO response determination, localisation of vitamin E by autoradiographic method and chromosome study.

2.3.1 Fixatives and fixing procedure

Ethyl alcohol

Organisms were kept in cavity block of 15 ml capacity containing 10 ml of 30% ethanol for 10 minutes. The animals reacted by sudden contraction. The material was then transferred to 50% ethanol for 3-4 minutes and finally to 70% ethanol for 15 minutes. The contracted specimens gradually returned to their normal shape without distortion. Fixation directly into 70% ethanol did not give this result. On the contrary direct transfer to 70% ethanol results in distortion of muscles and shrinkage of humps. 90-95% of the animals were fixed well.

Osmic acid

Fixation with osmic acid has been particularly suitable for animals labelled with alpha-tocopherol. (H^3) alpha-tocopherol labelled animals were washed with triple glass distilled water and transferred to 2% osmic acid in Gilbert's saline (Gilbert, 1968) and kept for 17 hours at $4^{o}C$ followed

by post fixation with 0.5% $KMnO_4$ for 15 minutes. After rinsing, the animals were rapidly dehydrated in ethanol (50-90%). Care was taken to give minimum time for dehydration. The above procedure was adopted to minimize the loss of labelling and consequently ensuring satisfactory results of the autoradiographic study. Osmic acid of less concentration i.e. 0.5-1.0% reduces the accumulation of the artifacts.

The above method gave 80-90% of the organisms well fixed and was found best for the autoradiographic studies of the rotifers.

Neutral formalin (2 to 5% at 60°C)

This is a modification of Edmondson's hot water method, hot water being replaced by neutral formalin. The method has the added advantage of relaxing and fixing the organisms at one stretch (Gopinath, 1972). Animals were collected in a little volume of water to which the same 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 is followed by rinsing the animals with triple glass distilled water at room temperature and rapid dehydration in ethanol (50-90%). This method was found suitable for studying the BWO response in the labelled animals and also for autoradiographic studies.

Acetic-alcohol 1:3 (Carnoy, 1887)

This standard fixative with slight modifications has been used in the study of chromosomes.

Glacial acetic acid (1 part) and absolute ethanol (3 parts) were mixed in the ratio of 1:3 v/v Animals treated with colchicine were transferred (see Chapter 5, Methodology) to acetic-alcohol mixture for 1 hour at 20-25°C. The animals were examined under stereo-microscope (Olympus) to ensure complete fixation. The rotifers were then transferred to 50% acetic acid in order to soften the animals for squash preparation. The fixed specimens could be preserved for 10-15 days in 70% ethanol.

This method has earlier been found suitable for autoradiographic studies of chromosomes (Ray Chaudhuri et al., 1970; Singh et al., 1970) using H^3-thymidine for the purpose of labelling.

Acetic acid (50%) (Stern and Hotto, 1970)

Animals were transferred after washing with distilled water to 50% acetic acid at room temperature for 1 hour, followed by squash preparation in a drop of 45% acetic acid over a glass slide.

The results of the application of this method has not been satisfactory since the chromosomes were not found fully stretched. The application of this method in the present

work has been limited to the preliminary studies of chromosomes of Asplanchna.

2.4 Staining

2.4.1 Stain and staining

Toludine blue 0.5% (Pelc, 1956)

It is an effective stain for the autoradiographic studies (Emulsion and strip film technique).

(i) Composition: Toludine blue 0.5 gm
distilled water (2x) 100 ml
adjust to pH 6
} mix well and use without filtering

(ii) Procedure: The autoradiographs were stained in 0.5% aqueous toludine blue for 30-60 seconds followed by washing with 70% ethanol for 5 minutes to remove excess of stain. The stained material was then dried in air and mounted in euparol.

2.5 Labelling of animals

The labelling of the animals was done with (H^3)-D-α-tocopherol with a specific activity of 5.0 Ci/m.mol. and 97% stated purity (Product of Radiochemical Laboratory, Amersham, U.K.). The tocopherol sample was labelled in the 5-methyl group. The compound was stored in benzene, ethanol (9:1) under Nitrogen at $4^{o}C$.

Two types of labelling procedures were followed; namely, in vivo and in vitro labelling.

2.5.1 In vivo labelling

The method employed for the purpose of in vivo labelling of Asplanchna is through its food i.e. paramecia. For the experimental purposes the aliquots of [illegible] labelled tocopherol were dried and extracted in ethanol. This labelled tocopherol in ethanol was injected directly into the hay infusion to form a fine emulsion at a final concentration of 1.2×10^{-5} moles/ml. The emulsion is ingested by the paramecia and subsequently by the rotifers. Random samples of such paramecia were tested in order to ensure that labelled tocopherol has been incorporated in their body. The rotifers were labelled for 10-12 hrs. at 25°C by feeding them on these labelled paramecia.

2.5.2 In vitro labelling

Gopinath (1972) used a modified 'culture chamber' of Birky (1967b) for his studies on in vitro development of embryos. Almost similar method has been employed here in order to bring out the embryos from the gravid females for the purpose of in vitro labelling.

Amictic female rotifers were washed 5 times in sterile water with penicillin and streptomycin (500 mg/litre) 1:1 w/w and transferred on a cavity slide with a drop of distilled water. The water was removed with a fine sterile pipette. The remaining fluid was allowed to evaporate until the animals were slightly wrinkled. The live but dry rotifers were covered with a fine drop of paraffin oil, which prevented

further evaporation. Each female is then carefully ripped open with a fine sterilized microneedle to liberate the pseudocoelomic fluid (PCF), which forms a drop under the oil. Embryos were dissected out into the PCF and the remaining material tissue was removed. A drop (100λ) of tritiated tocopherol (1.2×10^{-5} M) was injected into the oil on the cavity slide and covered with cover slip. The embryos were constantly watched under stereo microscope (6×20) to note their condition, [illegible]. Labelled tocopherol in the embryos was measured by scintillation counting. Care was taken to ensure sterile and conditions at every step of the experiment.

2.6 Autoradiography

The general method of Caro (1964) has mainly been followed with necessary modifications. In the present study non alcoholic fixatives have been employed and the [illegible] duration of the exposure of the autoradiographs has been kept to [illegible] eight weeks. Labelled animals were fixed in the required fixative and the squash was made on thoroughly washed and subbed slides (purified gelatin 5 gm, hot water 1 litre, chrom alum 0.5 gm, and stored in refrigerator) with a drop of acetic acid and covered with silicon coated cover slips. The cover slips were slightly pressed so as to expand the animal completely. In some cases the animals were dissected on a glass slide under the stereo-microscope so as to expose the required organs. Cover slips were flipped off by frosting the slides with liquid air. The slides containing the material were air dried in a chamber free of dust at room temperature. The cover slips which were flipped often contained prepared

tissue. These cover slips were mounted on a slide fixing its edges with quick fix for further use. The prepared slides were covered with strip film AR 10 at 18-20°C or nuclear track emulsion NTB 3 (Kodak) at 43-45°C in the dark room using diffused red safe light (Filter Wratten No. 1). The prepared slides were preserved dry at 4°C for the purpose of exposure in light tight bakelite boxes. Activated silica gel bags were put in the boxes to absorb moisture. Autoradiographs were developed after 8 weeks exposure period.

Developing was done in D-76 (Kodak) or Microdol-X (Kodak) for 5-7 minutes. The autoradiographs were fixed in acid fixing salt solution for 10 minutes and finally washed under running water for 15 minutes and air dried.

Staining was done with 0.5% aqueous toludine blue (pH 6), and the excess of stain was washed with 70% ethanol; mounting was done in euparol.

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C H A P T E R 3

INHERITANCE OF MATERNAL VITAMIN E

3.1 Introduction

While parthenogenesis is the general form of reproduction in rotifers, biparental reproduction is not uncommon, although the latter form of reproduction lasts for a comparatively shorter duration. The sexual transition of the parthenogenetic females has been observed in a few species of monogonont rotifers (Gilbert, 1966). There are a number of environmental stimuli which possibly result in the sexual transition in the natural population of these animals (Wesenberg-Lund, 1908, 1930; Mitchell, 1913a,b; Shull, 1913; Tannreuther, 1919; Tauson, 1925; Gilbert, 1963, etc.). One of these environmental stimuli is the dietary inclusion of tocopherol, an important constituent of algae which is often associated with the diet of these animals. Gilbert and Thompson (1968) first identified the presence of tocopherol in Scottish Experth grass and studied the influence of this substance in the sexual transition of Asplanchna seiboldi.

The sexual transition is associated directly or indirectly with the change of morphogenetic growth of the body wall, the formation of the mictic forms, and an increase in the number of mitotic divisions in certain embryonic organs resulting in proliferation of cells (Gilbert, 1968; Birky and Gilbert, 1972; Gopinath, 1972). The modification and the control of the development are evidently the response of

the organism to extrinsic (environmental) signal namely, the inclusion of vitamin E in the maternal diet. In the present investigation attempts have been made to study :

1. the inheritance of vitamin E by 5 successive generations of Asplanchna brightwelli after its incorporation in the parent body,

2. the inheritance of vitamin E by the embryos of different cell stages of development namely 4, 10, 16, 32 and fully formed embryos,

3. vitamin E required in quantitative terms to induce mictic production, and

4. the relationship between the intake of vitamin E and the formation of body wall outgrowth.

3.2 Design of experiments

The animals have been labelled with H^3-D-α-tocopherol (Chapter 2, pp.23-25).

3.2.1 Inheritance of vitamin E by successive generations

Altogether 4 lines of generations were established with 2 replications in each line. In line I the organisms from F_1 to F_5 were not given vitamin E. The only time organisms received vitamin E in their diet was F_o. The line II is exactly like line I. In line III the culture media contained unlabelled tocopherol in all the generations from F_o to F_5. The line IV served as the control and the organisms were never

given vitamin E from F_o to F_5. The details of the experiments performed with organisms in each of the four lines have been presented in Plate II.

In line I after 10-12 hours when the animals were fully grown and were ready to bear embryos were separated and washed with the culture media. The organisms were then divided into groups for 3 separate experiments run side by side.

Experiment 1: 10-15 animals of group 1 in each replication were transferred to The scintillation vials containing 10 ml of POPOP benzene scintillation fluid (Bray, 1960). The radio-activity in the form of counts per minute (CPM) was noted with the help of α tricarb liquid scintillation counter.

Experiment 2: Identical number of specimens from group 2 were fixed for autoradiographic study (Chapter 4).

Experiment 3: From the rest of the specimens 30 random females were transferred in vitamin E free culture media to breed and give F_1 individuals. The experiments have been repeated in every generation from F_1 to F_4 in line I. Embryos at different stages of their development in the uterine cavity of the F_o females were examined in order to ascertain the extent of labelling of the embryos.

3.2.2 BWO response and vitamin E intake

In line II the labelled animals of the F_o population were washed, rinsed and transferred to tocopherol free culture media to produce F_1 generation as in line I. 15 random

specimens from each of the replications of F_1 generation were fixed (Chapter II, p. 20) in order to measure the distance across the two humps with the help of an Olympus stereo microscope fitted with a stage micrometer. This distance has been regarded as an index of the body wall outgrowth (BWO) response and has been measured in each generation from F_1 to F_5. In F_2 the BWO response as well as the tocopherol concentration of the embryos released in succession by a single female has also been measured. The embryo released first has been named $1F_2$, the second $2F_2$, the third $3F_2$ and so on. Correspondingly 25 specimens of each of the F_1 to F_5 generations were subjected to scintillation counting in order to study the relationship between the level of tocopherol and the BWO response.

3.2.3 Continuous stimuli and mictic response

BWO and mixis response of individuals in every generation of line III have been studied.

3.2.4 Scintillation counting

For the purpose of scintillation counting the animals of the F_o population were labelled and were washed with sterile Gilbert's media (Gilbert, 1966) to remove all unbound radioactivity. 1-50 rotifers were transferred to the scintillation vials containing scintillation fluid and counting was done at 1, 2, and 5 minutes interval using quenched standards and channel ratio method to test the efficiency of the counting.

The counts per minute were taken from the readings obtained and used for data processing. Background counts were taken and these counts were subtracted from the final counts obtained from the samples.

3.2.5 Data processing

The amount of vitamin E retained by each female or male was calculated from the counts recorded (CPM minus Background counts) in terms of moles/individual using the following expression:

(i) Consumption of radioactive vitamin E in moles/individual (X) is given by the relation:

$$X = (C/A) \times B$$

where

C = counts of the sample minus background counts

A = standard counts of the media concentration

B = concentration of the media in moles i.e. 1.299×10^{-5}

(ii) Percentage consumption of vitamin E (P) is given by the relation

$$P = (X/B) \times 100$$

where

X = consumption of radioactive vitamin E in moles/individual

The above expressions were calculated by feeding the data in IBM 1130 computer. The programme is given in the appendix.

3.3 Observation and general considerations

3.3.1 General parameters

The results of the experiments reveal that a significant part of the labelled tocopherol is incorporated into the body of the female parents and is inherited by the successive generations. The response to vitamin E to a varying degree becomes evident in the F_1 individuals by their BWO response although the response is less prominent. These amictic females of F_1 are transitional owing to limited response to the mixis stimulus. Around 25% of the F_2 organisms derived from the F_1 are the mictic forms. Since the developing oocyte attached to the vitellarium and undifferentiated mitotic embryos are easily induced to mictic production than the post mitotic stages of the embryos (Gopinath, 1972), the offsprings in a single generation by a single female are not uniformly mictic or transitional or amictic. Consequently a single female sequentially gives rise to amictic, transitional as well as the mictic forms (Table 3.8). The embryos released last are generally mictic although occasionally they may be transitional. The embryos released first are amictic or transitional. This production of amictic forms followed by transitional and mictic in succession by a single female appears to bear a relationship with the BWO response as well as the quantity of tocopherol (vide infra, pp.40-42). The mictic females are easily recognisable by a fully formed hump and the types of the embryos. The uterus of the mictic female is characterised by the presence of male embryos. Nearly mature male embryos

can easily be identified through the transparent wall of the parent body (Plate III, Fig. 3). The amictic (transitional) females of F_2 are difficult to distinguish morphologically from mictic females until they contain nearly mature embryos whose sex as stated before can be easily identified (Birky and Gilbert, 1971). The uterus of the amictic females contains female embryos only. The F_3 obtained from the amictic forms of F_2 and, F_4 from F_3 show gradual reduction of the BWO response.

3.3.2 Distinction between amictic and mictic forms

The most striking morphological difference between amictic and mictic forms is the formation of BWO in the mictic forms. Birky and Gilbert (1971) mentioned That such distinction is difficult or impossible until the mictic forms contain male embryos. In the present work as well as in earlier works in this laboratory the distinction between the amictic and mictic forms has not been felt as a difficult problem as amictic forms are devoid of any hump formation whereas mictic male and females of Asplanchna brightwelli and Asplanchna sieboldi are always with prominent humps (BWO). The present author has mentioned (vide supra) in this work that the BWO response can be used as a marker of mictic production. There is, however, some difficulty in making distinction between the transitional and the mictic forms since both these forms exhibit prominent BWO response although the response in the transitional forms is comparatively less. The mictic females can be confirmed by male embryos in their body as Birky and Gilbert havementioned,

since only mictic females are capable of producing males which develop parthenogenetically from haploid eggs. The amictic and transitionals reproduce only females.

3.3.3 Inheritance of maternal vitamin E

The concentration in terms of moles/female has been estimated (Chapter II, p. 31) in every generation from F_o to F_5 (Table 3.1, Plate V, Fig. 1). While the concentration in F_o is of the order of 1.02×10^{-5} M/female, in F_1 the concentration is 0.894×10^{-5}. These quantities are 77.8% and 68.8% of the concentration of the media respectively. The concentration in the F_2 individuals is 0.686×10^{-5}. Apparently this quantity is 52.8% of the original concentration of the media. The relative concentrations in the F_3, F_4, and F_5 individuals are 0.445×10^{-5}, 0.168×10^{-5}, and 0.773×10^{-6} respectively. The percentage concentration in terms of the original concentration of the media are 34.2, 12.9, and 5.95% respectively. The reduction in the concentration is owing to the loss to the media along with the parent body. The oocyte extruded from the vitellarium in each generation contains labelled tocopherol (Fig. 3 , Plate VIII). In this respect the observations made by Birky and Gilbert (1972) are different. According to them vitamin E passes into the embryo from the uterus only. It is, however, true that the emrbyos contained in the uterus are in an environment of vitamin E since there is the accumulation of vitamin E in the uterus.

S. No.	Generation	Amount of vitamin E transmitted in moles/female	% transmission of vitamin E	S.D.	S.E.
1.	F_o	1.020×10^{-5}	77.70%	±1.31	±0.30
2.	F_1	0.894×10^{-5}	68.8%	±2.44	±0.48
3.	F_2	0.686×10^{-5}	52.8%	±1.01	±0.20
4.	F_3	0.445×10^{-5}	34.27%	±1.75	±0.35
5.	F_4	0.168×10^{-5}	12.99%	±0.37	±0.07
6.	F_5	0.773×10^{-6}	5.95%	±0.41	±0.08

Table 3.1. The amount and percentage transmission of vitamin E in the successive generations of Asplanchna brightwelli

The estimation of the concentration of vitamin E has also been made in the mictic males, mictic females, and the resting eggs (Table 3.2, Plate VI, Fig. 3). The concentration of vitamin E in the mictic females of the F_3 generation is 0.436×10^{-5} M/female which is close to the corresponding concentration of the amictic females of the same generation. In the males the concentration is 0.299×10^{-5}, and in the resting eggs 0.290×10^{-6}. The males of F_3 thus appear to have much lower concentration of vitamin E as compared with the mictic females of the same generation. The concentration of vitamin E in the resting eggs is extremely low compared with the other F_3 individuals.

S. No.	Individuals	Vitamin E intake : M/individual	% intake/ organism	S.D.	S.E.
1.	Amictic female	0.445×10^{-5}	34.27	±1.75	0.35
2.	Mictic female	0.436×10^{-5}	33.62	±0.55	±0.11
3.	Mictic males	0.299×10^{-5}	23.03	±0.32	±0.06
4.	Resting eggs	0.290×10^{-6}	2.23	±0.23	±0.04

Table 3.2. The intake of vitamin E by amictic, mictic females, mictic males and resting eggs

3.3.4 Inheritance of vitamin E by the embryos in vivo

Attempts have been made to study the intake of vitamin E by the embryos in their different stages of development in the uterine cavity. The smallest embryo that has been studied for the purpose is of 4 cell stage and the largest embryo is the nearly mature one before hatching (10-12 hours). The embryos of different stages were collected by dissecting the gravid females and were classified according to the number of cells comprising their body namely, 4, 10, 16, 32, and nearly mature. The concentration of vitamin E in the embryos of each of these stages was estimated by taking the counts (Table 3.3). The concentration of vitamin E in all the different stages is close to uniformity (Plate VI, Fig. 1).

S. No.	Cell stage	Amount of vitamin E intake in M/embryo	% intake of vitamin E	S.D.	S.E.
1.	4	0.883×10^{-5}	68.0	±0.37	±0.08
2.	10	0.879×10^{-5}	67.6	±0.11	±0.02
3.	16	0.877×10^{-5}	67.5	±0.27	±0.06
4.	32	0.877×10^{-5}	67.5	±0.22	±0.05
5.	Full developed embryo	0.895×10^{-5}	68.9	±0.16	±0.03

Table 3.3. Inheritance of vitamin E by embryos (F_1) of different stages of development of Asplanchna brightwelli

3.3.5 Inheritance of vitamin E by the emrbyos in vitro

In the present work the in vitro embryos survived upto 16 cell stage. Earlier Birky (1967) and Gopinath (1972) succeeded in maintaining in vitro embryos upto 32 cell stage. The counts obtained from 4, 10, and 16 cell stages are very low, and sometime very close to the background counts obtained from the scintillation fluid. Studies on the nearly mature embryos could not be performed since the embryos did not survive the in vitro conditions. However, in utero studies reveal (vide supra) that the level of concentration of vitamin E in the various stages of development including the fully formed embryo is nearly uniform. The result of the in vitro experiment suggests that negligibly small amount of vitamin E, if at all, finds its way into the body of the fully developed embryo from the surrounding environment (Table 3.4).

S.No.	Cell stage	Background counts (CPM)	Count of the sample (CPM)
1.	4	30	35
2.	10	30	34
3.	16	30	37
	Embryos did not survive after 16 cell stage		

Table 3.4. Radioactivity (CPM) of embryos of Asplanchna brightwelli labelled in vitro

3.3.6 Level of vitamin E to induce mixis

The results of the experiments indicate a level of concentration of tocopherol in the amictic female to which the organism responds by way of mictic behaviour. This level of concentration is of the order of 0.686×10^{-5} moles/female (c.f. Table 3.1, F_2). The concentration of Vitamin E in F_3 transitional (amictic) females, which give rise to the amictic forms of F_4, is 0.445×10^{-5}M. The present mictic induction is a function of the dietary tocopherol whose concentration in the mictic individual females should be between 0.445×10^{-5} and 0.686×10^{-5}M. A concentration of the order of 0.761×10^{-6}M/female evidently does not produce any response by way of BWO or mixis (c.f. Table 3.7).

3.3.7 Response to continuous mixis stimuli

Asplanchna brightwelli reared in tocopherol containing culture media in every generation (Line III) are transitional

in F_1 but continues to remain mictic from F_2 to F_5 studied (Gopinath, 1972) (Table 3.5).

Experiment	Generations					
	F_o	F_1	F_2	F_3	F_4	F_5
Line I	AM	T	M	M	T	AM
Line II	AM	T	M	M	T	AM
Line III	AM	T	M	M	M	M
Line IV	AM	AM	AM	AM	AM	AM

Table 3.5. Mixis in Asplanchna brightwelli in response to tocopherol. Generations in the box received tocopherol.

The BWO response of the organisms from F_2 to F_5 is of the same magnitude (Table 3.6; Fig. 2,Plate VI). Mictic phase continues under continuous mixis stimuli.

S.No.	Generations	BWO response (μ)
1.	F_o	673
2.	F_1	761
3.	F_2	818.5
4.	F_3	817.5
5.	F_4	815.5
6.	F_5	811

Table 3.6. BWO response in successive generations of Asplanchna brightwelli continuously exposed to tocopherol

3.3.8 Body wall outgrowth response

The BWO response has been measured in the organisms of Line II. The transitional females which constitute the F_1 progeny exhibit the beginning of the BWO response by way of hump formation which, compared with the fully formed humps in the mictic forms, is much less (Table 3.7; Plate V, Fig. 1). The transitional nature of the F_1 forms with less

S.No.	Generations	BWO response (µm)	Vitamin E intake (moles)
1.	F_0	674	0.990×10^{-5}
2.	F_1	760	0.894×10^{-5}
3.	F_2	820	0.685×10^{-5}
4.	F_3	750	0.443×10^{-5}
5.	F_4	700	0.169×10^{-5}
6.	F_5	680	0.761×10^{-6}

Table 3.7. BWO response and vitamin E intake by successive generations of Asplanchna brightwelli

prominent humps can be confirmed by looking at the embryos contained in their uterine cavity, namely the absence of the male embryo. The mictic males and the mictic females which constitute a major part of the F_3 population are distinguishable by their respective characteristics. The males are characterized by their prominent postero-lateral humps while the mictic females by antero-lateral humps (Plate III, Figs. 3 and 4). A significant number of the F_3 organisms

is similar to the F_1 transitional females with less prominent BWO response. It may be recalled that under mixis stimulus a female sequentially gives rise to amictic, transitional, and mictic forms; the frequency of transitional and mictic being larger (Table 3.8). The BWO response of the embryos released

Experiment series	Offsprings of F_1 generation				
	$1F_2$	$2F_2$	$3F_2$	$4F_2$	$5F_2$
1	AM	AM	T	T	M
2	AM	AM	T	T	T
3	T	T	T	T	M
4	T	T	T	M	M
5	T	T	T	M	M
6 Control	AM	AM	AM	AM	AM

Table 3.8. Effect of vitamin E on the amictic generations of Asplanchna brightwelli

in succession by a single female of F_1 has been measured. The BWO of the embryo released first is less compared with those released later (Table 3.7; Plate V, Fig. 2). The response of the embryo which is released last of all exhibits maximum BWO response. Birky and Gilbert (1972) observed similar events in their experiments with Asplanchna seiboldi. Evidently the incorporation of vitamin E in the parent body results in the transitional females of F_1 which in turn produces the mictic forms in F_2. The transition from amictic to mictic and later to sexual forms can be traced by the BWO response.

While the maximum breadth of the normal amictic females is of the order of 675 µm, the distance across the humps of the transitional females (F_1) is 780 µm (Plate IV, Figs. 1 and 2). The response increases further in F_2 individuals where the maximum breadth across the humps is 820 µm which is also the maximum BWO response of the individuals from F_1-F_5. The concentration of vitamin E has also been observed to be maximum in the 4th and 5th individuals of the F_2 compared with the other individuals of the same generation (Table 3.9; Fig. 2, Plate V). Thus, an apparent

S.No.	Offsprings of F_1 generation	BWO response (µm)	Vitamin E intake (moles)
1.	$1F_1$	785	0.603×10^{-5}
2.	$2F_2$	795	0.634×10^{-5}
3.	$3F_2$	802	0.647×10^{-5}
4.	$4F_2$	812	0.666×10^{-5}
5.	$5F_2$	820	0.667×10^{-5}

Table 3.9. BWO response and vitamin E intake by offsprings of F_1 generation of Asplanchna brightwelli

relationship exists between the BWO response and the concentration of vitamin E in Asplanchna brightwelli and, the responses have been observed to decrease gradually from F_3-F_5. The response is the lowest in F_4 individuals while F_5 hardly exhibits any BWO (Plate IV, Figs. 1 and 2).

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C H A P T E R 4

INTAKE PATHWAY OF VITAMIN E DURING MICTIC DEVELOPMENT

4.1 Introduction

The knowledge of the embryonic development of the rotifer is based mainly on the studies on Asplanchna, although the embryology of Asplanchna may not be typical of the rotifer group in general (Hyman, 1951). Gopinath (1972) identified three phases of the embryonic development of Asplanchna brightwelli, and they are gametogenetic, mitotic and post mitotic.

The female reproductive system of rotifer Asplanchna consists of a single syncytial ovary and a syncytial vitellarium bound together by a common membrane that continues as uterus or uterine cavity (Plate III, Fig. 1). In Asplanchna only one ovum develops at a time in the ovary. The matured oocyte moves to the base of the vitellarium, which nourishes the developing embryo. With the onset of the first cleavage the detachment of the embryo from the vitellarium takes place. Further development of the embryo takes place in the uterus. After the detachment of one embryo, initiation of the development of another oocyte starts in the vitellarium within a period of 12-15 minutes (Gopinath, 1972).

The mictic transformation under the influence of vitamin E involves triggering the meiotic events and

fertilization to form resting eggs, as well as haploid parthenogenesis to give rise to the males. Vitamin E enters the body of the rotifer along with paramecia and, for the vitamin E to make its way into the body of the embryo it should presumably pass through the vitellarium. The basis of this presumption is that the vitellarium is the organ which supplies nourishment to the oocyte. Experiments have been performed here in order to study the course of vitamin E through the body of Asplanchna.

4.2 Design of experiments:

4.2.1 Labelling of Asplanchna

Two types of labelling procedures have been employed i.e. in vivo and in vitro (c.f. Chapter 2, pp. 24-25). For the present study the general method of in vivo labelling has been modified and is described under the heading 'pulse labelling'.

Pulse labelling: In vivo labelling of rotifers was done by directly injecting the labelled vitamin E in the culture media having paramecia concentrate. Paramecia were labelled for 2 hours at 25°C. Nearly 200 specimens of Asplanchna brightwelli, as soon as they were hatched, were transferred to 10 cavity blocks each having culture media with labelled paramecia. 5 animals were withdrawn after every 30 minutes interval for nearly 18 hours for autoradiographic study.

4.2.2 Fixation of labelled rotifers

The labelled animals were fixed in non-alcoholic fixative for reason stated before (Chapter 2). The fixatives employed were 0.4% osmic acid, or 2.5% formalin. The procedure for fixation of labelled animals has already been described (c.f. Chapter 2, pp. 20-21). These fixed animals were dehydrated by passing quickly through alcohol series (70%-absolute). Alcohol helped removal of adsorbed tocopherol on the body of the animals.

4.2.3 Preparation of squash

The fixed animals were placed on subbed slides with a drop of cold acetic acid. The slides were covered with well washed and dried cover slips and squash preparations were made. In some cases the animals were dissected so as to expose the required organ for study. The cover slips were flipped off by frosting in liquid air. The cover slips so removed occasionally contained some tissue attached to it. These cover slips were mounted on well cleaned slides with quick-fix. The slides with squash material were air dried in a dust proof chamber at room temperature.

4.2.4 Autoradiography

Autoradiographs were prepared mainly following the method of Caro (1964) with some modifications for the present piece of study (c.f. Chapter 2, pp. 25-26). Either Kodak NTB 3 liquid track emulsion at 43-45°C, or Kodak AR-10 strip films

at 18-20°C were employed for coating the material on the slides. The slides were air dried and stored in light proof boxes at 4°C for a period of 8 weeks exposure. The autoradiographs were developed for 5-7 minutes at 25°C in Kodak D-76 developer followed by fixation in acid fixing salt for 10 minutes. Fixed autoradiographs were washed under running water for 15 minutes. Staining was done with 0.25% aqueous toludine blue (pH 6). Excess of stain was washed in 70% ethanol. Mounting of the slides was done in Euparol.

Autoradiographs were observed and photographed under Carl Zeiss phase contrast photomicroscope.

4.3 Observation and general considerations

4.3.1 Path of vitamin E during ingestion and development

Path of vitamin E starting from the point of its ingestion by the parthenogenetic females to the point of hatching of the fully formed embryos has been traced by pulse labelling of Asplanchna brightwelli and studying the autoradiographs at suitable [illegible] intervals. The animals labelled for the first 40 minutes show only the coronal region well labelled (Fig. 1, Plate VII), whereas in a few autoradiographs the presence of tocopherol has also been observed in the mastax region (Fig. 2, Plate VII). The labelling of mastax and the anterior region of stomach cells is more prominent in the animals fixed after $1\frac{1}{2}$ to 2 hours of labelling.

It takes 4 hours to label all the cells of the stomach. It has been observed that each cell of the stomach shows high labelling in the cytoplasmic region (Fig. 3, Plate VII). The gastric gland is totally devoid of any labelling (Fig. 4, Plate VII) with the exception of very few autoradiographs which exhibit very mild labelling in this gland. The animals continuously labelled for 17-18 hours also reveal complete absence of labelling in the gastric glands.

The various internal organs are surrounded by the pseudocoelomic fluid (PCF) and, the inducer molecule (vitamin E) moves into this PCF. Labelled tocopherol has been localised in the area below the stomach and above the vitellarium after 5 hours of labelling (Fig. 1, Plate VIII). Most of the tocopherol extruded from the stomach is taken up by the vitellarium (Fig. 2, Plate VIII). Young oocyte develops at the base of the vitellarium (Fig. 3, Plate VIII) and receives nourishment from it. High degree of labelling has been observed in oocytes which detach from the vitellarium after 10 hours of labelling (Fig. 4, Plate VIII). Meanwhile more tocopherol flows into the vitellarium through the PCF from the stomach and passes on to the next developing oocyte at the base of the vitellarium.

A part of vitamin E enters the uterine cavity through the PCF since there is no direct passage to the uterine cavity (Fig. 1, Plate IX). The amount of tocopherol is more or less constant in the early embryonic stages, the two celled embryo, however, contains slightly less compared with

the succeeding stages.

It is interesting to note that the detached embryos do not draw any nourishment during development or at least there is no mechanism for the purpose. The embryo draws nourishment from the vitellarium before it is detached. While the labelling from 4 cell Stage to near maturity appears to be of identical (Figs. 2, 3, 4, Plate IX) intensity, the labelling of the fully developed embryos still inside the body of the mother indicates a comparatively more heavy labelling (Figs. 1,2,3, Plate X). Fully formed embryos have been observed to move about inside the uterine cavity and pseudo-coelomic fluid. It is possible that fully formed embryo by assuming its function while still inside the body of the mother before it is released draws the labelled tocopherol either from the uterine cavity or from the PCF.

Organisms labelled continuously for 16-18 hours show high degree of labelling in corona, mastax, stomach, vitellarium, uterus and developing embryos (Fig. 4, Plate X).

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C H A P T E R 5

CHROMOSOMES IN ASPLANCHNA BRIGHTWELLI

5.1 Introduction

The earliest known work in the field of chromosomes of rotifers is that of Whitney (1924), who found 26 haploid number of chromosomes in the spermatocytes of *Asplanchna intermedia*, and 13 haploid number of chromosomes in the spermatocytes of *Asplanchna amphora* (Whitney, 1929). Tauson (1924, 1927), however, reported a haploid number of 12 chromosomes in *Asplanchna intermedia*. It may be recalled here that *Asplanchna intermedia* of Hudson and Gosse (1889) has been included tentatively by Gilbert (1968) under *Asplanchna brightwelli* along with the species *ebbesborni*, *amphora*, and *levdigi* of the genus *Asplanchna*. The present author has not come across any other work on the chromosomes of these organisms. Evidently the reports on the chromosomes are extremely meagre besides being conflicting in themselves. An idea about the number or the nature of the chromosomes in rotifers is thus completely lacking. Competent workers in the field of rotifers have strongly felt the possible association of haploidy with the sexual transition in these organisms. An attempt has been made in the present work in order to ascertain the number of chromosomes in the mictic as well as in the amictic forms although the work originally was not intended to incorporate a chromosome study of the animal.

5.2 Preparation of specimens for the chromosome study

Specimens of Asplanchna brightwelli were treated with colchicine in order to arrest the metaphase chromosomes. Asplanchna brightwelli treated directly with colchicine did not give satisfactory result in arresting metaphse. Hence an indirect method similar to the labelling process has been employed here.

Owing to the lethal effect of colchicine, screening experiments were performed to determine a suitable concentration for the treatment which the animals will survive. 5 concentrations namely, 0.25, 0.5, 1.0, 1.5, and 2.0% solutions were prepared in distilled water and, 10 ml of each of the concentrations were put in 5 different cavity blocks together with a control with distilled water only. 2 ml of hay infusion with paramecia concentrate (10^3/ml) were added to each of the cavity blocks with the test solutions and observed for 15 minutes for the lethal effect of colchicine. 15 specimens of Asplanchna brightwelli were transferred to each of the cavity blocks containing paramecia in colchicine solution. The animals were observed every half hour for 24 hours. Apparently the animals survive in 0.5% colchicine although a concentration of 0.25% has been found most suitable for both survival as well as good metaphase plates. Concentration higher than 0.5% appeared lethal for the organisms.

2 ml of paramecia concentrate (10^3/ml) were treated with 0.25% colchicine for 1 hour at 25°C; and the treated paramecia were given to 15 specimens of Asplanchna brightwelli to feed upon for 3 hrs at 25°C. The rotifers thus treated were washed in distilled water and fixed in aceto-alcohol (1:3) for 1 hour. The fixed animals were then transferred to 50% acetic acid to ensure softening of tissues.

5.3 Preparation of squash

3 fixed animals were placed on a clean slide with a drop of 50% acetic acid and covered with a clean cover slip. The gravid females were ripped open in order to release the embryos. The excess of the acetic acid leaking along the edges of the cover slip was removed with a filter paper. The squash of the animals was prepared by tapping on the cover slip with reasonable force with the help of a rubber tipped rod. Similar squash preparations were made using only embryos. The method employed for removing the cover slip for the purpose of staining the squashed tissue was that of Raychoudhuri (1973) (personal communications).

The slides with the squash preparations were kept together with a piece of filter paper soaked in 50% acetic acid in horizontal coupling troughs covered with a glass lid for overnight at 4°C. These troughs were then brought to room temperature and were filled with aceto-alcohol (1:3). The cover slips easily separated from the slides. The

squashed tissues on the slides were air dried in dust free container before staining.

5.4 Staining procedure

Geimsa and aceto-orcein have been used and both these stains gave satisfactory result.

Geimsa: The stain has been used in the form of buffer at pH 6.6. The method employed here is a modification (Raychoudhuri, personal communication, 1974) of the original technique (Gelei, 1921).

Stock geimsa solution

Giemsa powder	280 mgm	Dissolve, keep overnight
Glycerol	25 ml	at room temperature and
Methanol	25 ml	filter, store at 4°C

Working solution

Geimsa stock soln.	5 ml
Methanol	3 ml
Citric acid (0.1M)	3 ml
Distilled water (3×)	100 ml

Adjusted to pH 6.6 with 0.2M disodium hydrogen phosphate

The material was stained in working giemsa solution for 30 minutes at room temperature. The stained material was air dried after washing with triple distilled water. Mounting was done in euparol. Each staining operation was done with fresh geimsa working solution.

Aceto-orcein: The material was stained in 1.5% aceto-orcein solution for 15 to 30 minutes at room temperature. The stained preparation after washing with absolute alcohol was mounted in euparol.

Microscopical preparations of the chromosomes were studied and photographed under an Olympus photomicroscope (×1500) using green filter.

5.5 Observations and general considerations

Altogether 400 specimens have been examined. Observation has been made on at least 10 metaphase plates of each of the mictic or of the amictic forms of Asplanchna brightwelli.

The chromosomes of Asplanchna brightwelli are extremely small in size, nevertheless they are stained with uniform intensity and are visible clearly under high magnification (×1500). Each chromosome shows centromeric constriction around its middle. In some plates the pairing of the chromosomes has been observed although generally pairing of the chromosomes has been found to be absent (Tauson, 1929).

Amictic forms - An examination of random metaphase plates reveals that the diploid number of chromosomes in the embryonic cells of the amictic (parthenogenetic) females of Asplanchna brightwelli is 22 ($2n$) (Plate XI, Fig. 1). The number is, however, different in the vitellarium. There are generally 33 ($3n$) or 44 ($4n$). In some cases as many as 66 ($6n$)

chromosomes have been observed (Fig. 2, Plate XI). The cells of the vitellarium thus typically contain polyploid number of chromosomes.

The embryonic cells of Asplanchna brightwelli could be recognized by their cell boundaries. Contrary to this the somatic cells are syncytial. The nuclei of the vitellarium are large and are characterized by polyploid chromosomes. Polyploidization and polytenization are common in nurse cells, especially in insects (King, 1964; Raven, 1961). The cells of the vitellarium of rotifers may be regarded as analogous to the nurse cells of other organisms (Hyman, 1951). The presumption of Birky (1967) and Gopinath (1972) with regard to polyploidy in the vitellarium cells is thus apparent from the present observation.

Mictic forms - The chromosomes of the mictic forms are identical to those of the amictic forms in every respect except the number. The results of the mictic males reveals the presence of a haploid number of 11 chromosomes in the spermatocytes (Fig. 4, Plate XI). The corresponding number in the somatic cells is also 11. In the mictic females the chromosomes are 2n = 22 (Fig. 3, Plate XI). The oocyte chromosome number could not be ascertained. The resting eggs give rise to normal amictic females. Evidently in the males as well as the females the germ cells contain haploid number of chromosomes.

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C H A P T E R 6

DISCUSSION

6.1 General discussion : Significant points of observation

Tocopherol (vitamin E) has been known to be a mixis inducing substance in Asplanchna brightwelli and a few other related forms of monogonont rotifers. In the present work the inheritance of D-α-tocopherol and its bearing on the sexuality of Asplanchna brightwelli has been studied. There are a few points of observation which are very significant in the present context of mictic phenomenon and associated sexuality. Mictic phase intervenes the normal amictic generations of Asplanchna brightwelli as a result of the organism's response to vitamin E (D-α-tocopherol). In quantitative terms the amount of tocopherol that should induce mictic production lies within a range from 0.445×10^{-5}M to 0.686×10^{-5}M/female. A concentration lower than this range evokes only limited response. The change from the amictic to the mictic phase is associated with the body wall outgrowth response, which can serve as a visible marker of this transition. Asplanchna brightwelli grown in α medium without tocopherol continues to remain amictic.

The amictic and the mictic females differ physiologically in their reproductive parameters (Miller, 1931; Buchner, Kiechle and Hamm, 1965; Birky and Gilbert, 1971). The amictic females produce only daughters (amictic females) by

parthenogenesis and the mictic females produce haploid gametes which give rise to the resting eggs if fertilized or males if unfertilized. Indeed strong suggestions in favour of the production of haploid gametes and male haploidy have been made by several workers in the field of parthenogenesis of rotifers (Maupas, 1890[illegible], 1891; Birky, 1965; Birky and Gilbert, 1971; Ruttner-Kolisko, 1968, 1969; Gopinath, 1972; Sreedharan, 1973). While the presumption is but logical, supporting evidence in its favour has been extremely limited. Experiments performed in this work with a view to ascertain the chromosome number in Asplanchna brightwelli indicate $2n$ = 22 in females and $1n$ = 11 in males. To the present author's knowledge the information on the chromosomes of monogonont rotifers are those of Shull (1921,1925), Tauson (1924, 1927) and Whitney (1924, 1929). Between then and now Hsu (1956) gave the chromosome numbers in two Bdelloid rotifers.

6.2 Role of vitamin E in the process of transition

There are reports which claim that vitamin E enhances vigour and fecundity in Daphnia (Viehoever et al., 1937,1938). In Copepoda vitamin E's possible role in egg sac formation has been suggested by Jacobi (1957). Meikle et al. (1965) reported spermatogenetic activity in cricket Acheta in response to vitamin E. Vitamin E's role other than sexual transition in Asplanchna brightwelli is tacheauxetic (excess) growth of specific cells in the embryonic hypodermis (Gopinath, 1972).

Birky and Gilbert (1971) considered the necessity of a high level of dietary vitamin E for mictic production. The production of the male is a consequence of mixis and the male fertility is a function of vitamin E in the males. Autoradiographic studies in the present work reveal the presence of tocopherol in the testis of the males (Plate XI, Fig.4.) which suggests vitamin E's role in the production of mature sex cells. Vitamin E, as has been estimated in the body of the mictic female, is 0.436×10^{-5}M/individual whereas the concentration in mictic male is 0.299×10^{-5}M/individual. Since the males do not feed they must necessarily derive the vitamin entirely from their mothers. It is assumable that there exists some mechanism which protects degradation of vitamin E (Birky and Gilbert, 1972) during the process of its transfer through successive generations.

6.3 Concentration of tocopherol required for mictic induction

Birky and Gilbert (1971) hinted about the evolution of an 'absolute requirement' of vitamin E for mictic female. Tocopherol is fairly readily available in the natural environment of rotifers and it is likely that rotifers ingest some tocopherol along with their diet. The mictic production is indeed dependent upon a particular level of concentration of vitamin E which in Birky and Gilbert's term is an 'absolute requirement'. The present studies indicate that the requirement for Asplanchna brightwelli lies somewhere in the range between 0.445×10^{-5}M and 0.686×10^{-5}M/female. Males develop

from the unfertilized eggs of the mictic females. The results of the autoradiographic experiments clearly show that the maternal vitamin E passes into the body of the embryo by way of vitellarium and young oocyte. The males derive the vitamin E during the early embryonic stage and, for their fertility the males evidently need a concentration of vitamin E of the order of 0.299×10^{-5}M/individual. The mictic mothers of the male should have in their body adequate vitamin E to ensure that their [illegible] male offsprings are fertile. Mictic production should, therefore, ensure male production and male fertility. On part of the monogonont rotifers the mictic production is a device to withhold the activity of the population temporarily by way of dormant eggs or at least to control the size of the population. The organisms remain in a dormant stage in the resting eggs to emerge under favourable conditions. Making of fertile males is therefore an absolute necessity and a significant consequence of mictic production. Birky and Gilbert's (1971) indication of an 'absolute requirement' of vitamin E for mictic production appears very significant to the present author. However, the results of the present experiments could not be more indicative than the range given where this 'requirement' may lie.

6.4 Reversibility of amictic generations

As a sequel to the mictic production males and resting eggs are produced. The resting eggs are covered with a hard

covering whose exact nature in terms of composition has not been understood. Since rotifer clones appear in puddles after prolonged draught it is assumable that the protective hard covering of the resting eggs is resistant to desiccation (Ruttner-Kolisko, 1968). The resting eggs give rise to normal parthenogenetic (amictic) females both in natural as well as in laboratory conditions. Thus, the amictic generations, which are intervened by a mictic generation, revert back to the amictic generations.

The mictic production in Asplanchna brightwelli is a function of the appropriate level of concentration ('absolute requirement' : Gilbert and Birky, 1971) of vitamin E in the mictic female who inherits it from the mother (transitional female). The first oocyte of the transitional female generally develops into a transitional female (Table 3.8) with less prominent BWO response. Obviously the level of concentration in these forms is less than what is necessary for the mictic production hence, the transitional females (Table 3.9). These transitional forms of F_3 produce amictic females.

6.5 Transfer of maternal vitamin E into the body of embryo

A significant part of the dietary vitamin E is incorporated in the body of the female of F_0 population (Table 3.1). Autoradiographic experiments have been performed in order to study the course of vitamin E inside the body of the amictic female. The study was intended to ascertain the path through which the maternal vitamin E is transferred to the offsprings.

Evidently it takes about 5 hours for vitamin E to reach the vitellarium of the organism. In about 7-8 hours the first oocyte begins to extrude out from the vitellarium, and autoradiographs show labelling of the cytoplasm of the young oocyte (Plate VIII, Fig. 3) as well as its junction with the body of the vitellarium. The oocyte inherits the maternal vitamin E from the vitellarium, as Table 3.3 (A) indicates that the level of vitamin E in the body of the embryos is almost constant. There is, however, the indication of a slight increase of the vitamin in the fully formed embryo. Evidently vitamin E, either from the uterine cavity or from the pseudocoelomic fluid (PCF), does not enter into the body of the embryo although labelled tocopherol has been found to be present both in the uterine cavity as well as the PCF. Compared with the preceding embryonic stages the slight increase of vitamin E in the body of the fully formed embryo can be explained. The coronal movement of the fully developed embryo inside the uterus or in the PCF is clearly visible through the transparent body wall of the mother. It is possible that some PCF containing labelled tocopherol enters the body of the fully formed embryo by the coronal movement. Until this stage is reached, the embryo develops at the expense of the food supplied by the vitellarium during the oocyte formation. There is no other mechanism, which the author is aware of, by which the developing embryo of Asplanchna brightwelli can get nourishment except that supplied by the vitellarium of the mother. In Ruttner-Kolisko's view

Birky and Gilbert's (1971) opinion is difficult and, the difficulty appears to be largely owing to the uncontrolled experiments performed. There are some forms of rotifers which, according to the latter authors, are not capable of producing mictic females. In mictic production the quantity and the type of food, temperature, certain chemicals, also the genotype and the age of the females can influence the production of mictic offsprings, but the most consistently effective inducer of mictic production, as Birky and Gilbert (1971) have rightly pointed out, is "a switch of major food organism from the colourless alga to the green alga" (Table 6.1).

Author	Year	Rotifer sp.	Food/environmental stimuli	Remarks
1. Punnet	1906	E. senta	Starvation and change in food	No mictic induction
2. Mitchell	1913	A. amphora	Starvation and Euglena as food	Mixis
3. Shull	1913	A. intermedia	pH change of the medium Alga as food	Mixis
4. Tauson	1925 1926 1927a,b	Asplanchna sp.	Change in oxygen concentration, carbonate concentration	Mixis
5. Whitney	1929	A. amphora	Chlamydomonas as food	Enhancement of amictic and mictic production

(contd.)

	Author	Year	Rotifer sp.	Food/environmental stimuli	Remarks
6.	Gilbert	1963	B. calyciflorus	Rotifer density green and colorless. Euglena as food.	Mixis
7.	Pourriot	1963 1965	-----	Exposure to light.	Mixis
8.	Ruttner-Kolisko	1964	B. rubens	Cold shock to amictic culture at 6-10°C for 2 hours.	Increase in mictic production.
9.	Birky	1964	A. brightwelli	Eudorina sp. as food.	Mixis
10.	Buchner and Kiechle Kiechle and Buchner	1965 1966	A. sieboldi	Chlorella or Euglena as food	Mixis
11.	Gilbert	1963	A. brightwelli	pH change in phosphate buffer.	No mixis
12.	Lechner	1966	A. girordi	i. Lack of P. caudatum ii. High algal density. Aging of culture. iii. Increase in rotifer density.	Mixis
13.	Gilbert	1968	A. brightwelli	Chlamydomonas and Euglena as food.	Mixis
14.	Gilbert	1968	A. brightwelli	Acetone soluble fraction of Euglena and Chlamydomonas in media.	Mixis
15.	Gilbert and Thompson	1968	A. sieboldi A. brightwelli	Vitamin E	BWO and Mixis.

Table 6.1. Various stimuli listed by different workers for induction of mixis phenomenon.

A significant departure from the aforesaid generalization of Birky and Gilbert is Gilbert's experiment (Gilbert, 1963) with amictic Brachionus calyciflorus. Gilbert found greater percentage of mictic female offspring if the density of Brachionus calyciflorus in the culture was 4 females/ml. Density in Gilbert's opinion possibly results in the accumulation of some compounds which are specific in mictic female production. The effect of the unknown substance accumulated owing to density in Gilbert's experiment is comparable with that of vitamin E; the two substances possibly act in the same way.

Mitchell (1913) failed to induce mictic female production in Asplanchna without adding green algae to the Paramecium diet of the organism. Gilbert (1968) demonstrated that in an appropriate culture fluid no mictic females are produced unless green algae are present in the diet. The specific substance of the green plant material responsible for induction of mixis was identified by Gilbert (1967) as α-tocopherol (vitamin E) and later described by Gilbert and Thomson (1968) as an active ingredient. This derivative of the green plants has been found to be absolutely essential for both mictic as well as BWO responses (Gilbert and Thomson, 1968; Birky, 1968).

The findings here and elsewhere point to the fact that the sexual reproduction in rotifers and the associated BWO response are stimulated in the presence of green algae or in

other words one of its derivatives, vitamin E in the food of the organism. The oocytes of amictic female undergo a single equational division (extrusion of one polar body : Birky and Gilbert, 1971; Gopinath, 1972) which allows amictic generations of genetically identical clones of Asplanchna brightwelli to continue. The mode of reproduction occurs by diploid parthenogenesis. The oocytes of mictic female undergo meiosis resulting in haploid eggs. In response to vitamin E the production of sexual female and [illegible] male as well as the production of fertile gametes suggests the action of vitamin E at more than one level. Solberg and Dougherty (1959) reported failure of Brachionus variabilis to produce mictic daughters irrespective of the diet given which the authors have described as a result of mutation.

6.7 Mictic adaptation

The mictic phenomenon as in Asplanchna brightwelli can be viewed as a physiological adaptation on the part of the organism in order to suit special needs. Mictic generations are extremely temporary and the adaptation on the part of the organism manifests in the production of resting eggs which invariably hatch into normal parthenogenetic (amictic) females. Mictic production, therefore, can be visualized as a device for control over the total size of the population (vide supra), also for protection from extreme unfavourable conditions. The mictic female production as has been stated before is dependent upon an 'absolute requirement' of vitamin E and

when this level of requirement is reached in the body of the organism, the latter responds in a detectable manner. The functions of these mictic females are:

1. production of haploid gametes,
2. development of a mechanism by which fertilization can be effective, and
3. development of a hard covering, very likely resistant to desiccation, around the resting egg.

Experiments employing labelled tocopherol show that mictic males contain 0.299×10^{-5}M/individual and mictic females 0.436×10^{-5}M/individual. The concentration of tocopherol in resting egg is 0.296×10^{-6}M/egg and the amount of tocopherol in the resting egg must have been contributed by the mictic mother, from its vitellarium to the young oocyte. It cannot be said with certainty if a part of this tocopherol of the resting egg is a contribution of the mictic father by way of sperm cell. With the formation of the resting eggs the mictic phase is apparently over, hence the resting eggs inherit the least of the parental vitamin E.

It is evident from the foregoing account that the requirement of vitamin E at different levels of mictic production is not uniform. The requirement for a mictic mother to produce mictic daughter is different from the requirement of the mictic male to produce fertile spermatocytes.

It has been seen in the present work (Table 3.8) that the different offsprings of a single tocopherol-fed female

are not uniformly mictic or amictic or transitional. The transitional forms give rise to transitional as well as mictic forms. Since the production of mictic daughter is a function of the appropriate concentration of vitamin E in the body of the mother, the mixis can be conceived as a potentiality on the part of the female parent to produce mictic forms as a result of successful transfer of the required amount of vitamin E to the developing oocyte.

6.8 Significance of BWO response

Gilbert (1973) classified the morphotypes in Asplanchna seiboldi as saccate (normal amictic), cruciform (intermediate), and campanulate (mictic) females. There are suggestions (Birky and Gilbert, 1971; Hurlbert et al., 1972; Gilbert, 1973) that the humps (BWO) in both the males as well as in the females prevent their ingestion by conspecific females (cannibalism). Gopinath (1972) has observed the displacement of certain hypodermal nuclei in the humped forms without causing any change in the nuclear number in Asplanchna brightwelli. Increase in the number of mitotic divisions and a consequent increase in the nuclear number has been found to be an effect of vitamin E (Gopinath, 1972; Birky, 1968) on morphogenetic growth; but humps without altering the nuclear (cell) number is a manifestation of reorientation of the hypodermal cells. BWO response in Asplanchna is thus a morphotypic variation associated with the mictic development. In the males, however, the reproductive duct from the testis after merging with the

prostatic duct enters the hump and opens at its tip. The body wall around the gonopore can assume a tubular form and acts as a copulatory organ (Hyman, 1951).

6.9 Other related response to environmental stimuli

Rotifers exhibit cyclomorphosis, a variation from the parent forms induced by the changes in the environment, and Hutchinson (1967) considers it to be of 'adaptive significance'. Asplanchna priodonta attains maximum length of the body when the population becomes dense (Schreyer, 1920). Besides, these animals with increased length of the body have been found by the author to reproduce sexually although the organism normally reproduces parthenogenetically. The sexual phase of Asplanchna priodonta can be called mictic and the mictic phenomenon as has been found by Schreyer (1920) appears to be density dependent. Wesenburg-Lund (1900, 1908, 1930) and Voigt (1904) on the other hand observed a different type of cyclomorphosis in Asplanchna priodonta. Some of the offsprings carried by the mother were more elongated than the parent and these extreme forms were found to be sterile. Besides, the fertility of the organism decreased with the initiation of elongation. The pertinent observation of Wesenburg-Lund and Voigt can be interpreted as presence of dormant eggs with few adult organism. Few or none mature organism in the offsprings can be described as a case of decreased fertility or sterility.

Among the monogonot rotifers the cyclomorphic changes appear to be associated with the mictic production. Power (1912) and Mitchell (1913) observed Asplanchna seiboldi to be trimorphic namely, saccate, humped, and campanulate which according to Gilbert (1972) are saccate, cruciform, and campanulate respectively. Mitchell and Power (1914) considered the production of humped forms owing to the nature of food supplied. According to Mitchell (1913) saccate forms grow in a culture containing Paramecia while the humped forms develop when Euglena and Oxytricha are given as food. The transformation from saccate to campanulate form takes place with the formation of the intermediate forms. Mitchell (1913) found that some lines of Asplanchna seiboldi never produced humped individuals and he considered such lines generally 'unenergetic'. Solberg and Dougherty (1959) regarded such a failure in Brachynus variabilis as a result of mutation. So far cyclomorphic changes accompanied by formation of the mictic individuals are limited to Asplanchna. In the present work the induction of morphological changes in the laboratory culture of Asplanchna brightwelli by dietary tocopherol resemble closely the cyclomorphic change in natural populations of Asplanchna.

6.10 Significance of sexual reproduction in Asplanchna

The sexual mode of reproduction in Asplanchna brightwelli appears dispensable since the organism reproduces parthenogenetically almost at an exponential rate. This

nearly exponential growth can take place under conditions that ensure unlimited food supply and least intraspecific competition. Sexual reproduction is essential, as Doagherty argued (cited by Birky and Gilbert, 1971), for the evolutionary success of any organism. Asplanchna brightwelli must have its own mechanism to acquire and maintain genetic variability. The intervention of sexual phase should help production of genetic variability and consequently evolutionary flexibility (Birky and Gilbert, 1971).

Ruttner-Kolisko (1968, 1969) has questioned whether mictic production in rotifers does involve effective fertilization and genetic recombination. Her results of interspecific cross between female Brachionus urceolaris and male Brachionus quadridentatus reveal that for six successive generations the progeny resembles Brachionus urceolaris. According to her, this represents a case of 'cryptoparthenogenesis' in which the sperm in the original cross fails to contribute chromosomes to F_1. She considers that the contribution of sperm is towards the making of the hard shell of the resting eggs.

The sexual reproduction in Asplanchna brightwelli does not appear to be a case of 'cryptoparthenogenesis' and is significantly important because it results in the production of resting eggs. Fertilization helping production of genetic variability in Asplanchna brightwelli may be of limited scope owing to inbreeding. But the formation of resting eggs is a very good mechanism for dispersal and consequently for outbreeding (Birky and Gilbert, 1971).

6.11 Scope of further work

Interesting problems arise from the observations made in the present work. Evidently the requirement of vitamin E is different at different levels of mictic production, also there are good reasons to believe that undegraded vitamin E passes from one generation to another. It would be interesting to ascertain more specific role of vitamin E during mictic development. Sexual reproduction results in genetic variability. The extent of this variability in monogonont rotifers would be informative and its comparison with the evolutionary fate of species like Asplanchna brightwelli under continuous parthenogenesis should be interesting. Besides, since fertilization in Asplanchna brightwelli has to take place within a couple of hours of hatching of the female before the cuticle gets hardened, Asplanchna brightwelli is possibly forced to inbreeding. It should be interesting to ascertain the extent to which the resting eggs are dispersed resulting in outbreeding and consequently to genotypes different from the parents. Some of these problems are, in view of the author for further investigations.

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S U M M A R Y

S U M M A R Y

Inheritance of dietary vitamin E and its bearing on the sexuality of Asplanchna brightwelli has been studied. Mictic phase intervenes the normal amictic generations of Asplanchna brightwelli as a result of the organisms response to vitamin E.

The concentration of vitamin E in Asplanchna brightwelli in each of the F_0 to F_5 generations has been measured by scintillation counting technique. The requirement of vitamin E for mictic production lies in the range between 0.445×10^{-5} and 0.686×10^{-5} M/individual.

Autoradiographic studies reveal that the maternal vitamin E passes into the body of the embryo by way of vitellarium into the oocyte. In vitro studies reveal that the developing embryos do not draw vitamin E from their immediate environment.

The concentration of vitamin E in the embryos from the oocyte stage to the fully formed stage appears more or less constant.

Production of the males is a significant consequence of mictic production and for its fertility the males evidently need a concentration of vitamin E of the order of 0.299×10^{-5} M/individual.

Mictic production in Asplanchna brightwelli is associated with BWO response. An apparent relationship exists

between the concentration of vitamin E and the BWO response. BWO response, thus appears to be morphotypic variation associated with mictic production.

In the amictic as well as The mictic females the diploid chromosome number is 22. The nuclei of the vitellarium are polyploid; polyploidization ranges from 3n to 6n as has been observed. The chromosome number in the males is 11.

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A P P E N D I X

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     1N ASPLANCHNA)
      READ(8,[illegible])K
 1[illegible] FORMAT(I[illegible])
      DO [illegible] KHS=1,K
C        SPECIFICATIONS FOR THE EXPERIMENTAL SET
      WRITE (5,1[illegible])KHS
 1[illegible] FORMAT([illegible]H1,2[illegible](//),[illegible]2X,'EXPERIMENTAL SET',1X,I2)
      READ(8,[illegible])NK
    5 FORMAT(I[illegible])
      READ(8,[illegible])(N(I),I=1,NK)
    [illegible] FORMAT([illegible]I4)
      DO [illegible] I=1,NK
C        SPECIFICATIONS FOR THE SUB SET
      WRITE(5,[illegible]4)I
 [illegible]4 FORMAT(1H1,////,6[illegible]X,'SUB SET',1X,I2)
C        SPECIFICATIONS FOR THE SAMPLE NUMBER, CONSUMPTION RADIO-
C        ACTIVITY IN MOLES AND PERCENTAGE CONSUMPTION
      NN=N(I)
      READ([illegible],[illegible])(Z(LTS),LTS=1,NN)
    2 FORMAT([illegible]I4)
C        INITIALIZING VALUES
      SUMZ=[illegible]
      SUMC=[illegible]
      SUMP=[illegible]
      SSQZ=[illegible]
      SSQC=[illegible]
      SSQP=[illegible]
      WRITE(5,6[illegible])
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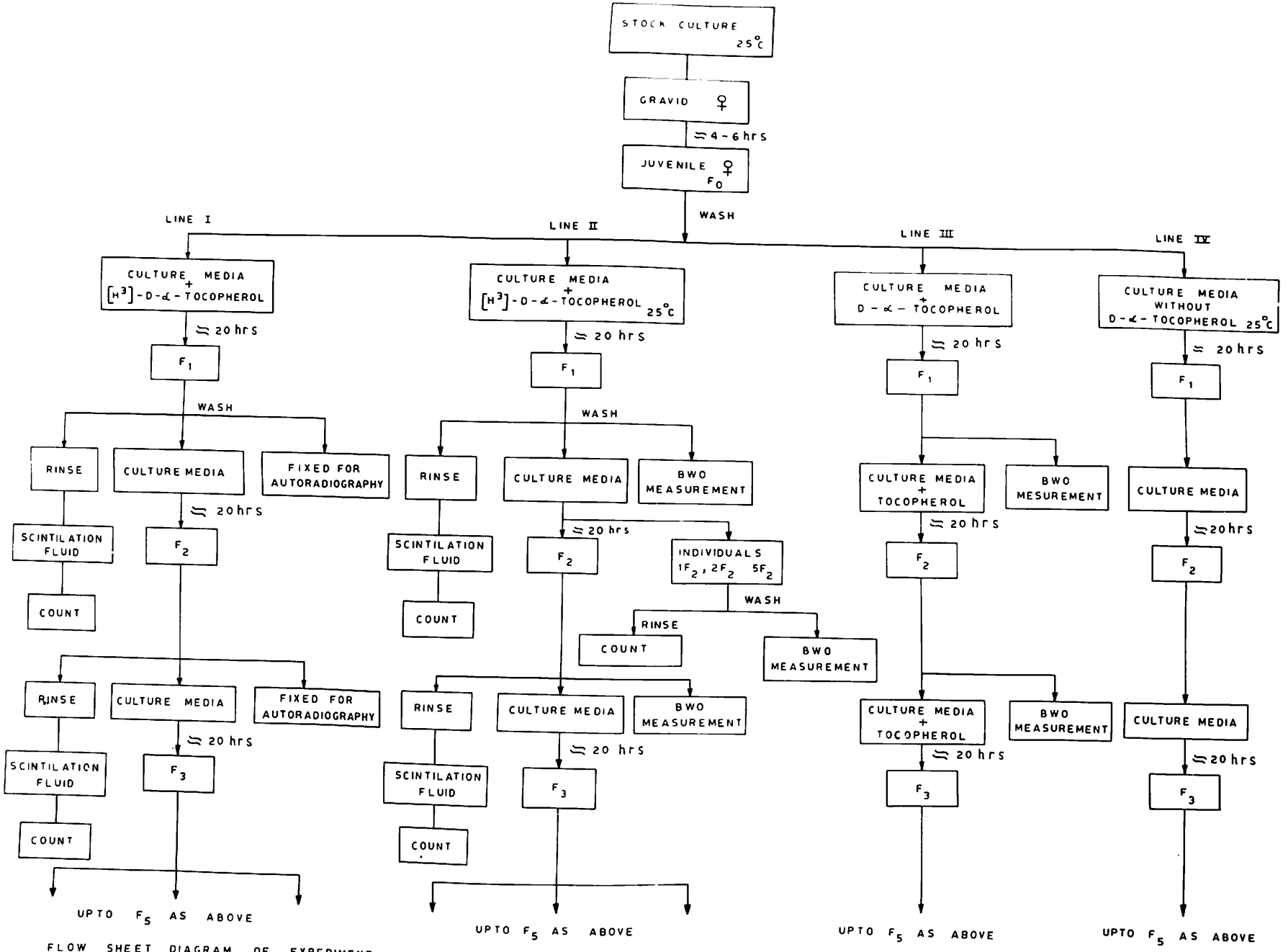


FLOW SHEET DIAGRAM OF EXPERIMENT DETERMINING THE INTAKE AND TRANSMISSION OF VITAMIN E IN ASPLANCHNA BRIGHTWELLI.

PLATE II

PLATE III

Phase contrast photographs

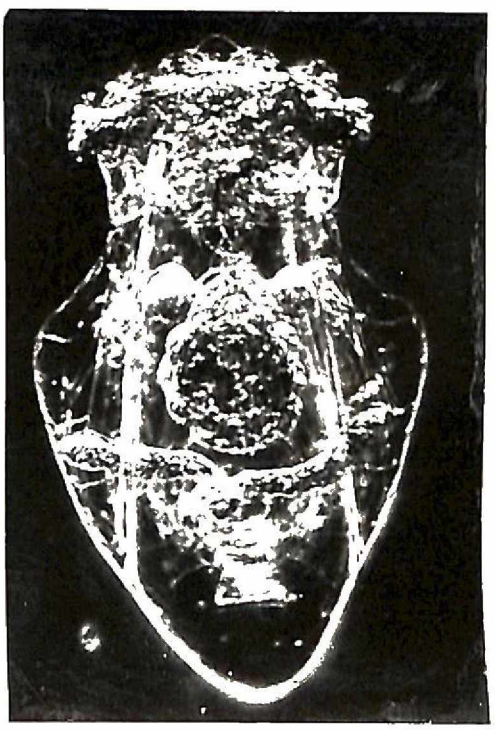
Fig. 1. Asplanchna brightwelli amictic female
×300

Fig. 2. Asplanchna brightwelli transitional female (low humped)
×300

Fig. 3. Asplanchna brightwelli mictic female with male embryo inside the uterus
×300

Fig. 4. Asplanchna brightwelli male
×500

1



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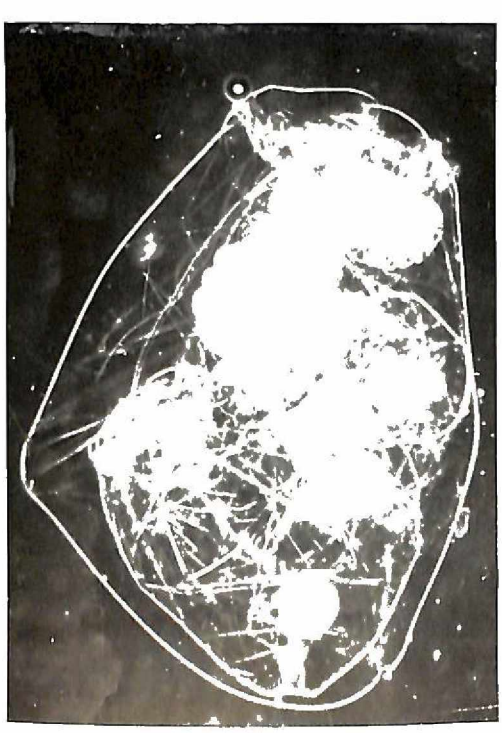

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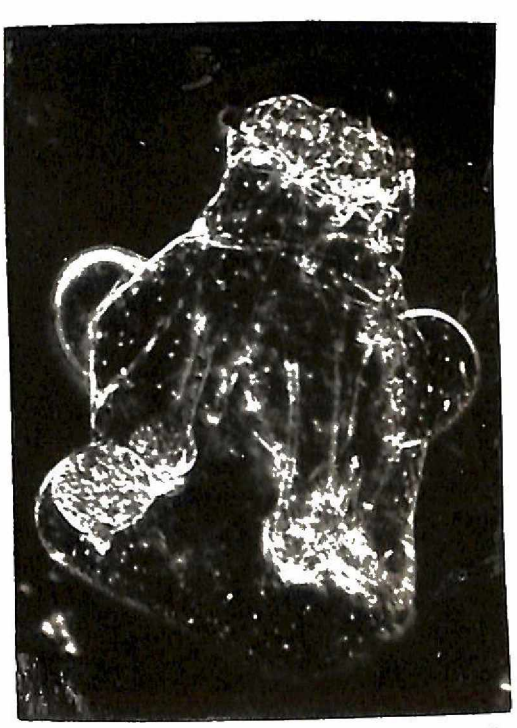


4

PLATE III

PLATE IIIA

Humps in Asplanchna brightwelli

Fig. 1. Transitional low humped form

×300

Fig. 2. Fully humped form with lateral humps (LH)

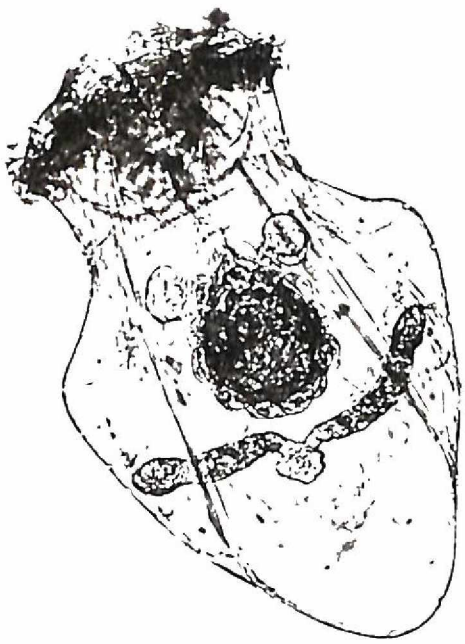
×250

Fig. 3. Fully humped mictic female with lateral (LH) and dorsal (DH) humps

×250

Fig. 4. Mictic male with prominent humps, copulatory organ (CO) and Testis (T)

×500



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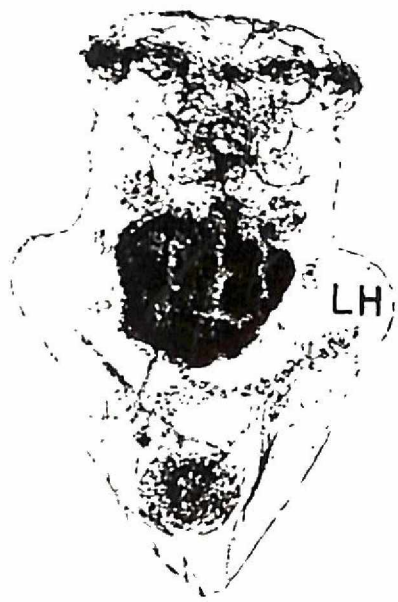

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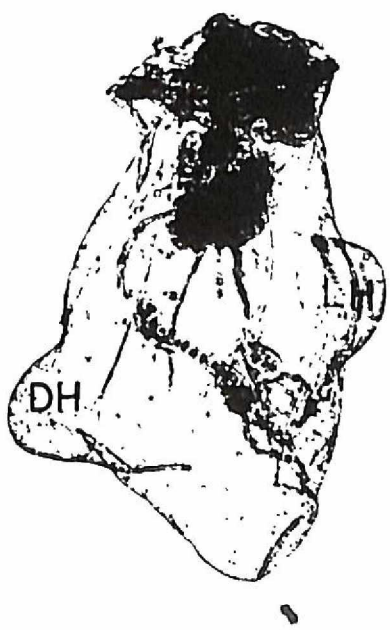

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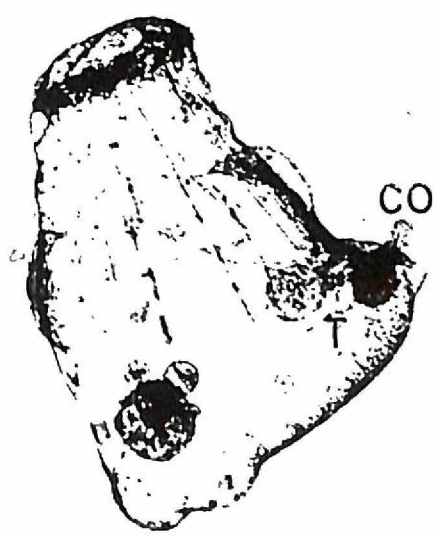

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PLATE IIIA

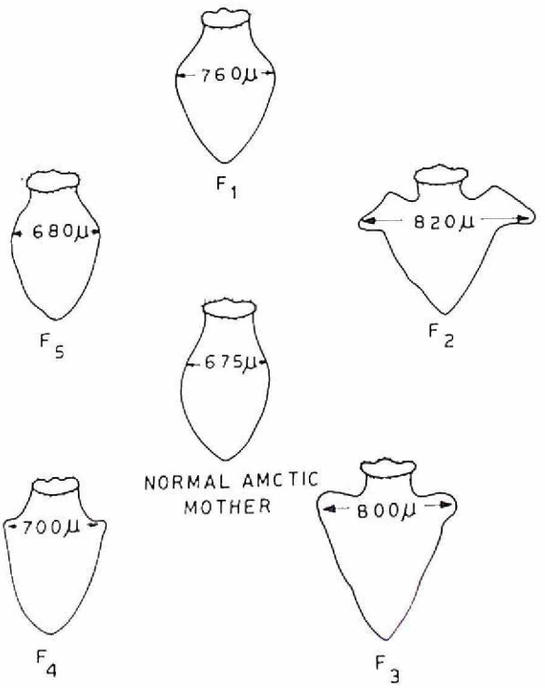


FIG 1 DIAGRAM SHOWING BODY WALL OUT GROWTH RESPONSE IN SUCCESSIVE GENERATIONS OF A BRIGHTWELLI

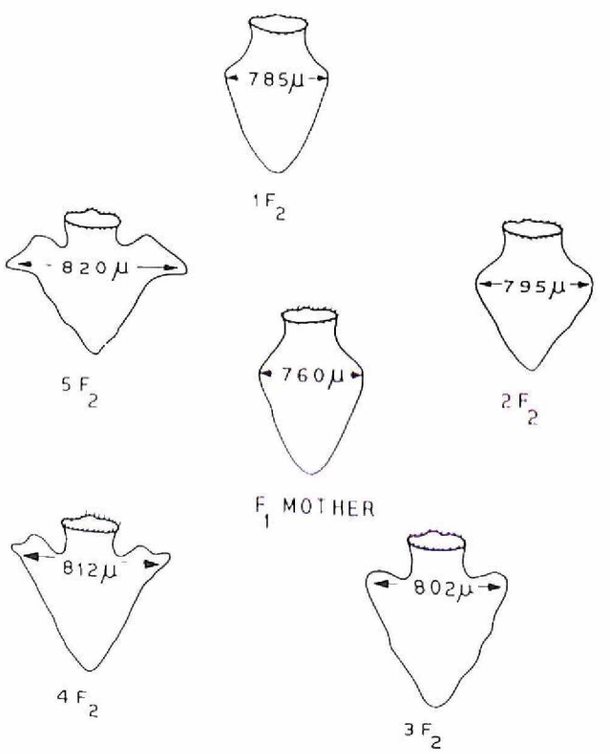


FIG 2 DIAGRAM SHOWING BODY WALL OUT GROWTH RESPONSE IN THE INDIVIDUAL OF F_2 GENERATION OF A BRIGHTWELLI

PLATE IV

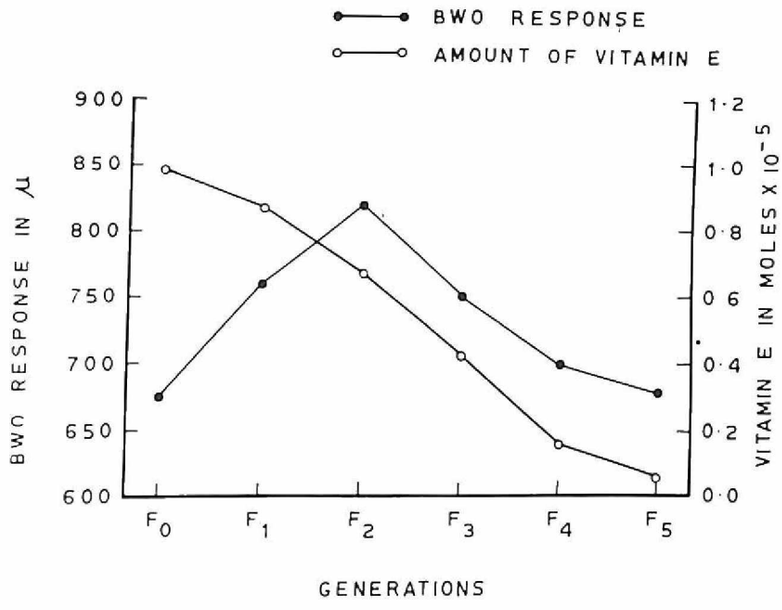


FIG.1 BWO RESPONSE AND VITAMIN E INTAKE BY SUCCESSIVE GENERATIONS OF A. BRIGHTWELLI.

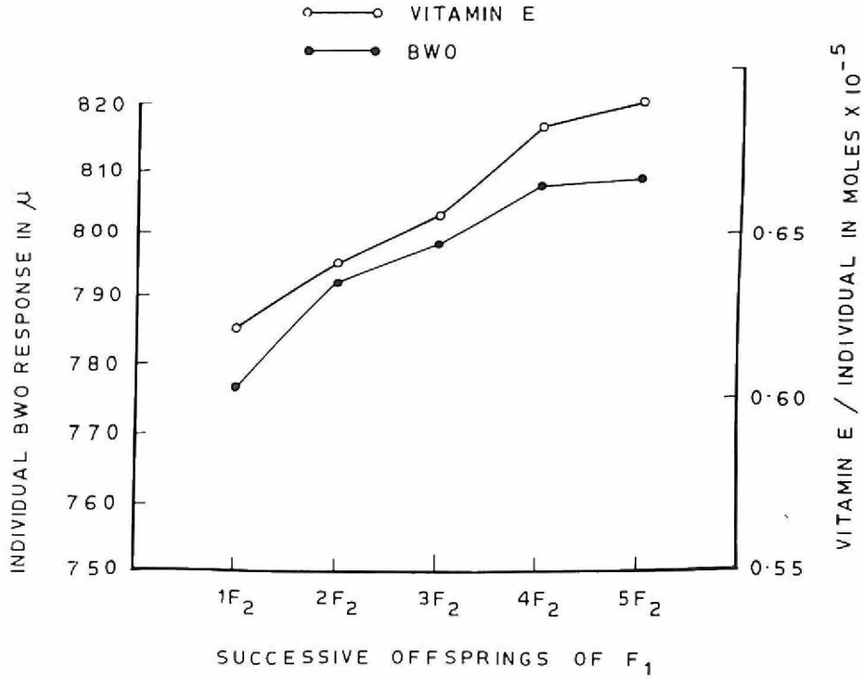


FIG. 2 BWO RESPONSE AND VITAMIN E INTAKE BY SUCCESSIVE OFFSPRINGS OF F_1 GENERATION IN A. BRIGHTWELLI.

PLATE V

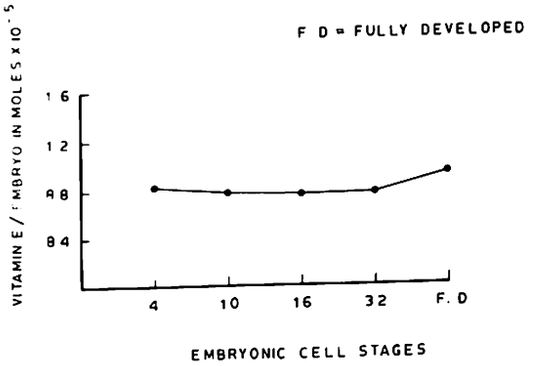


FIG 1 VITAMIN E INTAKE BY DIFFERENT EMBRYONIC CELL STAGES IN F_1 GENERATION OF A. BRIGHTWELLI.

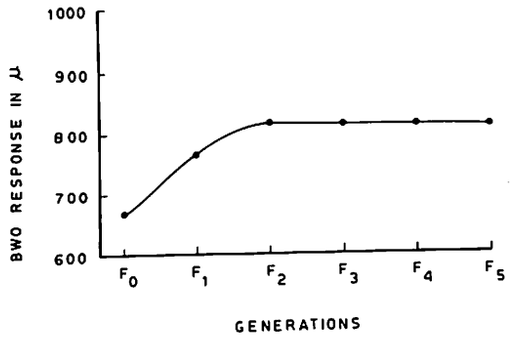


FIG. 2 BWO RESPONE DURING SUCCESSIVE GENERATIONS OF A. BRIGHTWELLI CONTINOUSLY EXPOSED TO TOCOPHEROL.

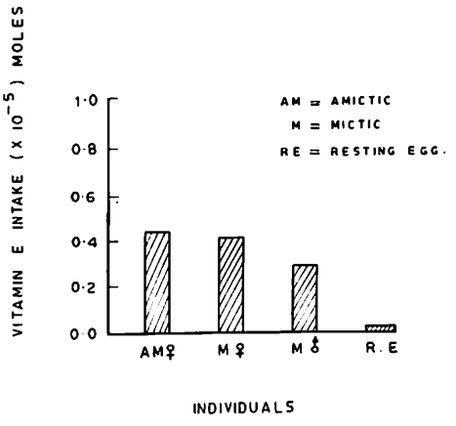
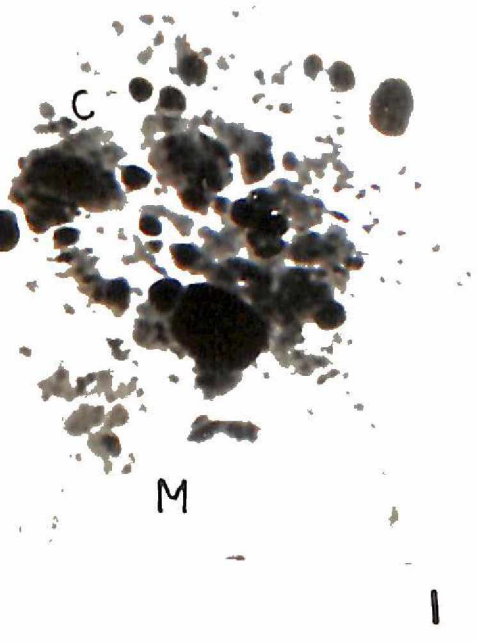


FIG 3 VITAMIN E INTAKE BY INDIVIDUALS OF F_3 GENERATIONS OF A. BRIGHTWELLI

PLATE VI



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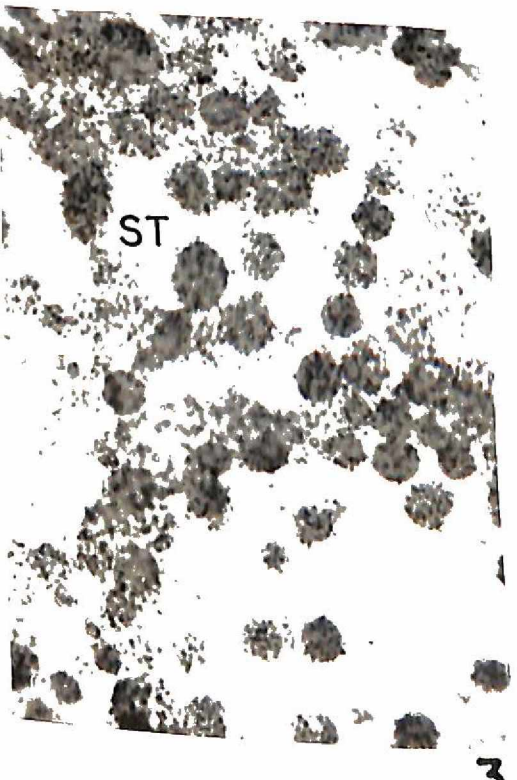

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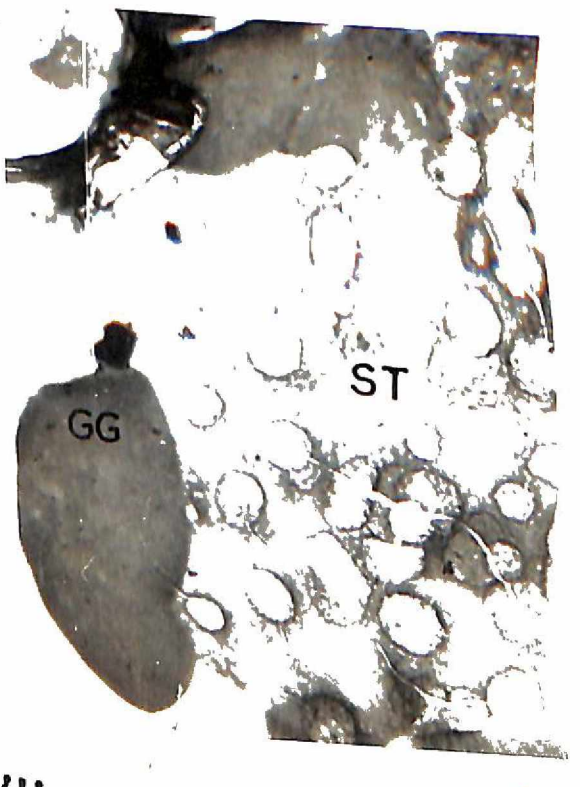


4

PLATE VII

PLATE VIII

Fig. 1. Autoradiograph of the region between stomach and vitellarium of A. brightwelli labelled for 5 hours with (H^3)-D-α-tocopherol. Dense labelling in the region above vitellarium (R).

×1000

Fig. 2. Autoradiograph of the vitellarium of A. brightwelli labelled for 6 hours with (H^3)-D-α-tocopherol. Heavy labelling in the vitellarium.

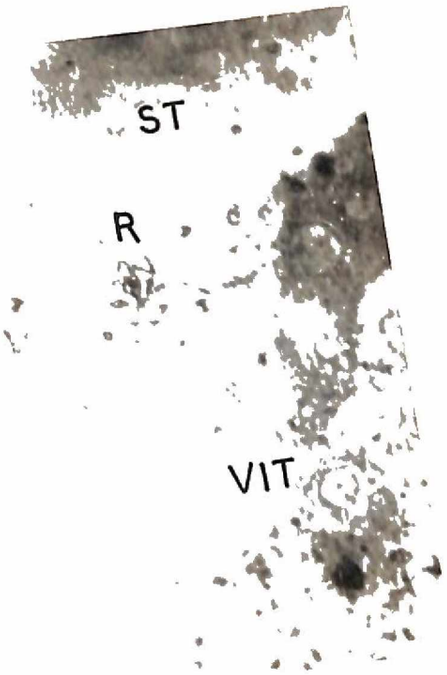
×800

Fig. 3. Autoradiograph of the oocyte (OO) attached with vitellarium of A. brightwelli labelled for 7 hours with (H^3)-D-α-tocopherol. Heavy labelling in the oocyte (OO) and vitellarium (VIT).

×800

Fig. 4. Autoradiograph of the oocyte detached from the vitellarium labelled for 10 hours with (H^3)-D-α-tocopherol. Heavy labelling in the oocyte (OO) and vitellarium (VIT).

×800



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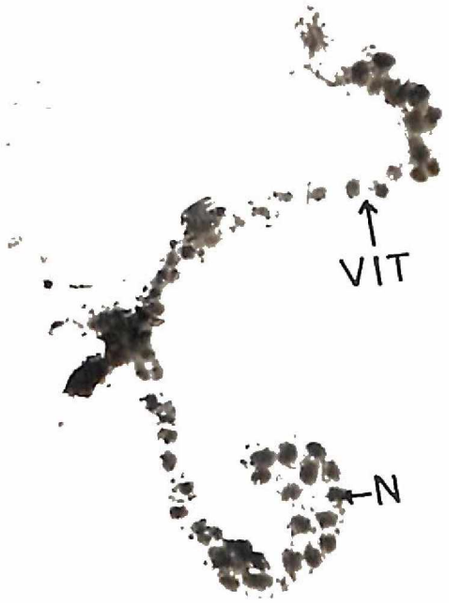


2

3

4

PLATE VIII

PLATE IX

Fig. 1. Autoradiograph of uterine cavity of *A. brightwelli* labelled for 5-6 hours with (H^3)-D-α-tocopherol. High labelling in uterine cavity (UT).

×1000

Fig. 2. Autoradiograph of dividing embryo of *A. brightwelli* labelled for 12 hours with (H^3)-D-α-tocopherol. Uniform labelling in the embryo (EM).

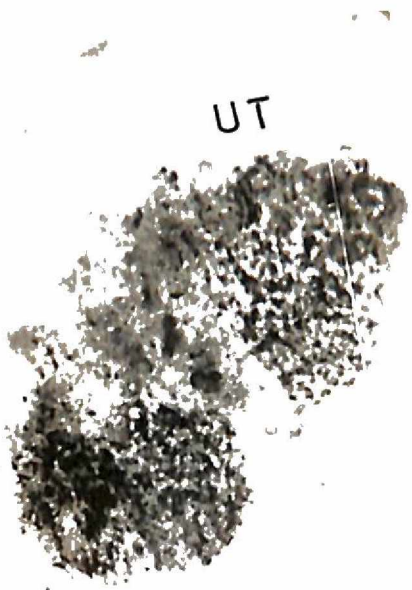
×800

Fig. 3. Autoradiograph of developing embryo of *A. brightwelli* labelled for 14 hours with (H^3)-D-α-tocopherol. Uniform labelling in the embryo (EM).

×1000

Fig. 4. Autoradiograph of the embryo in the post mitotic stage of *A. brightwelli* labelled for 16 hours with (H^3)-D-α-tocopherol. Uniform labelling in the embryo (EM).

×600



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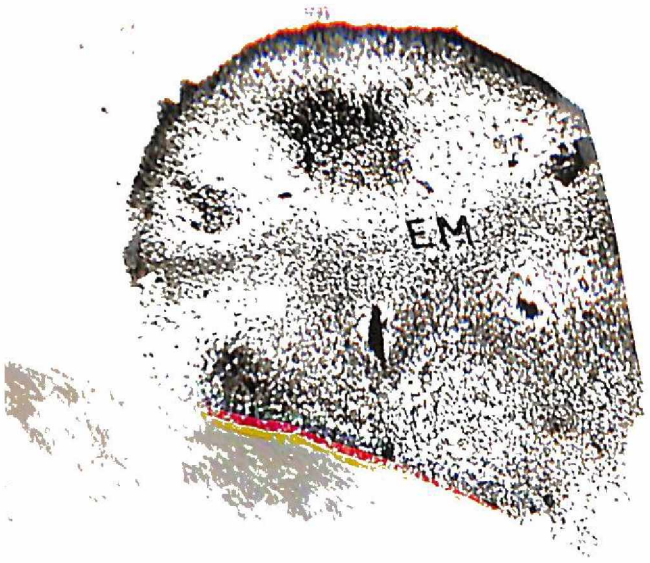


3

4

PLATE X

Fig. 1. Autoradiograph of the continuously labelled A. brightwelli for 18-20 hours with (H^3)-D-α-tocopherol. Embryos (EM) in different stages heavily labelled.

×500

Fig. 2. Autoradiograph of the continuously labelled A. brightwelli for 20 hours with (H^3)-D-α-tocopherol. Heavy labelling in stomach (ST), Pseudocoelomic fluid (PCF) and Vitellarium (VIT).

×400

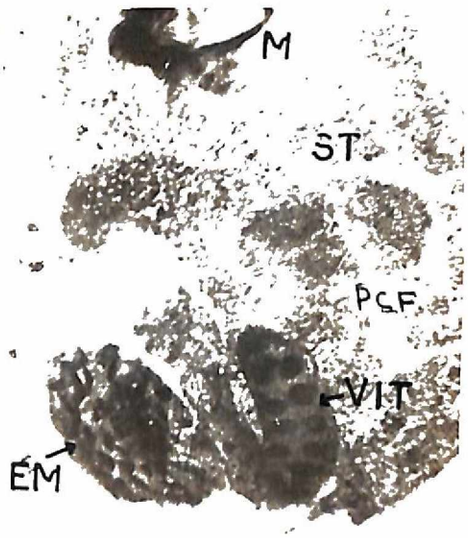
Fig. 3. Autoradiograph of continuously labelled A. brightwelli for 20 hours with (H^3)-D-α-tocopherol. Heavy labelling in Pharynx (PH), Vitellarium (VIT) and Embryos (EM). Gastric glands without any label(GG).

×400

Fig. 4. Autoradiograph of male A. brightwelli labelled with (H^3)-D-α-tocopherol. Heavy labelling in Testis (T) and Copulatory organo (CO).

×400

1



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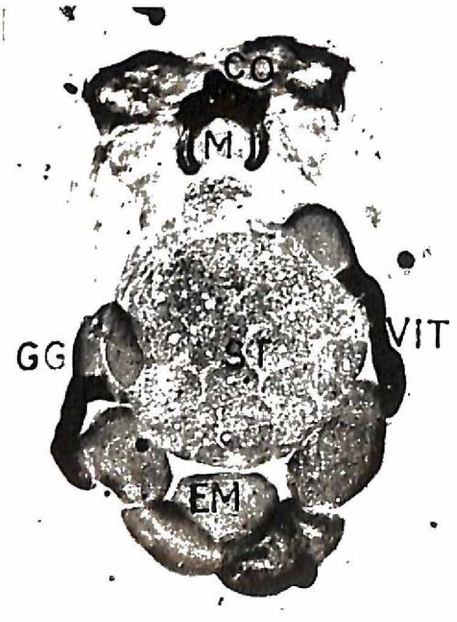

3

4

PLATE X

PLATE XI

Fig. 1. Chromosomes of amictic female *A. brightwelli* (2N = 22).

×2000

Fig. 2. Chromosomes of polyploid vitellarium cell of *A. brightwelli* (6N)

×2000

Fig. 3. Chromosomes of mictic females *A. brightwelli* (2N = 22).

×1500

Fig. 4. Chromosomes of mictic male *A. brightwelli* (N = 11)

× 1500

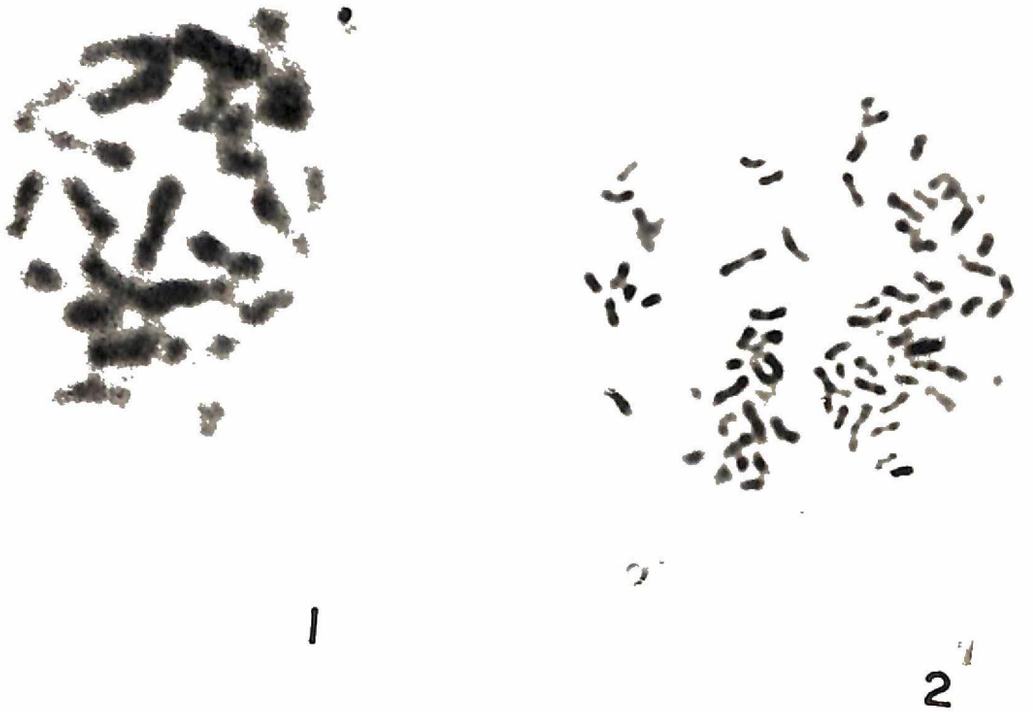

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PLATE XI