STUDIES ON
THE RNA & PROTEIN SYNTHESIS IN THE EMBRYONIC PHASES OF *Asplanchna brightwelli*

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By
VENKATESH. T. R.

BIOLOGICAL SCIENCES GROUP
BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE
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CERTIFICATE

This is to certify that the thesis entitled "Studies on the RNA and Protein Synthesis in the Embryonic Phases of *Asplanchna brightwelli*" and submitted by Shri Venkatesh T.R., ID No. 73S87001, for award of Ph.D. degree of the Birla Institute of Technology and Science, Pilani, embodies original work done by him under my supervision.

7/3/77

(A.K. DATTAGUPTA)
Professor & Head,
Department of Zoology
B.N.Chakravarthy University
Kurukshetra

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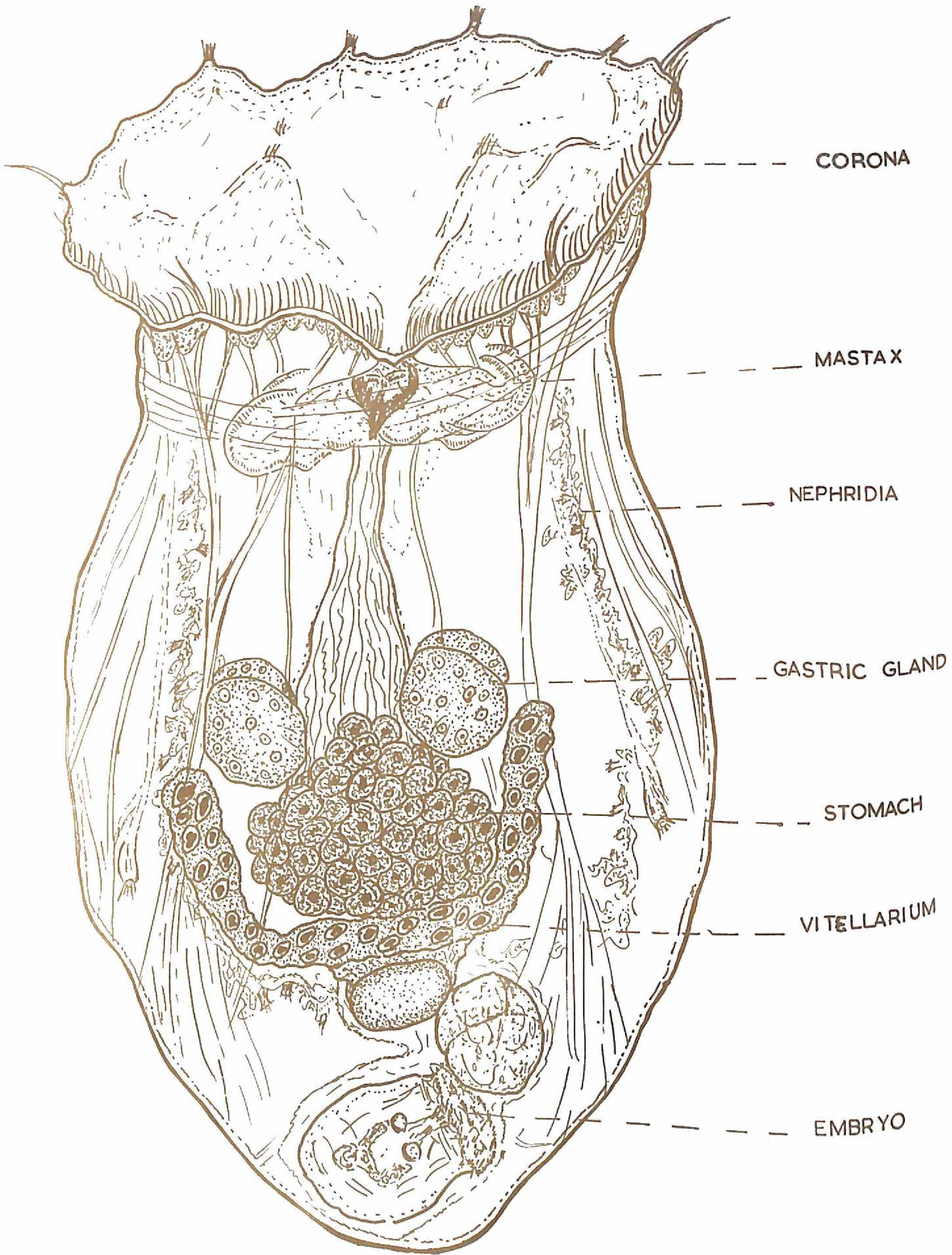

ASPLANCHNA BRIGHTWELLI

CHAPTER I

I. A. General Introduction

The focus of attention in developmental biology is the understanding of how a seemingly simple cell as the egg grows into a multicellular organism with highly differentiated cell types. In order to understand the process of development in molecular terms enumerable studies on the gene activity during development have been made on a variety of organisms. Most of these studies have been carried out on echinoderms and amphibians owing to several advantages which these systems offer for the study of development. In recent years genetic analysis of development is being carried out in the fruit fly, *Drosophila*, by employing the extensive knowledge of genetics of this organism. Rotifers as useful systems of biological study have lately aroused the interests of some workers. Although the development of rotifers presents many interesting features they have not been employed very much in the study of developmental processes. Rotifers possess numerous advantageous characteristics such as parthenogenetic reproduction, relatively short life history, transparent body wall, and eutely. Compared with the echinoderms and the amphibians, rotifers have not been very popular with developmental biologists owing perhaps to the difficulty in handling the animals, also obtaining

adequate number of eggs for biochemical study. Notwithstanding the difficulties, the present study on the gene activity during the development of the rotifer *Asplanchna brightwelli* has been undertaken. The present study deals with some aspects of the RNA and protein synthesis during the embryogenesis of *A. brightwelli*.

I.B. Conserved Messenger RNA and Embryonic Development

In the complex process of development a single cell produces a multicellular organism comprising of a variety of cell types. The various cell types function differentially even though they have a common genome; in other words the cells differentiate. In terms of genetics the process of differentiation leads to the production of different cellular phenotypes from a single cellular genotype (Markert & Ursprung, 1971). In recent years differentiation has been explained in terms of differential gene function. According to the differential gene function theory, different cellular phenotypes arise from a common cellular genotype by "switching on" of specific set of genes. Thus the state of a given cell is determined by what set of genes are on. The idea that differential gene activity could be the basis of differentiation was briefly mentioned by T.H. Morgan in 1934. However, a serious consideration of the subject began in the first part of 1950 (cf Davidson, 1968).

The molecular mechanisms underlying the processes of development and differentiation are still not clear. However, in the last few decades with the advances in molecular biology there have been extensive studies on the gene activity during development and differentiation. In most of these studies the spatio-temporal relationship of the nucleic acid and protein synthesis with the embryonic phases serves as the operational criteria for the study of gene activity during development. Among other things, the timing of the onset of embryo-genome function during early embryonic development has received much attention. The early developmental events include processes like cleavage, blastulation, gastrulation etc. In order to determine whether these early developmental events are under the direct genomic control, development has been studied in enucleated embryos (Fankhauser, 1934; Harvey, 1936; Harvey, 1940; Briggs et al. 1951; Smith & Ecker, 1965; Denny & Tyler, 1964). These experiments have shown that the embryos continue to develop even when their nuclei are physically removed. Further, chemical enucleation studies with Actinomycin D have yielded similar results (Gross & Cousineau, 1963; Gross & Cousineau, 1964; Malkin et al. 1964). Actinomycin D is a drug which binds to DNA and inhibits RNA synthesis. Gross and Cousineau (1963) were the first to utilize Actinomycin D to demonstrate that early developmental events proceed even when RNA

synthesis was blocked with the drug. Such a result has been obtained in experiments in a wide variety of organisms, (Brachet et al, 1964; Brachet & Denis, 1963; Collier, 1966; Sargent & Raff, 1976). In almost all the cases development proceeded upto gastrulation in the presence of Actinomycin D. These experiments led to the view that "development through cleavage and upto the point of first functional differentiation is independent of the embryo gene action" (cf. Davidson, 1968). In this connection other experiments have shown that protein synthesis is necessary for cleavage and morphogenesis, also fertilization markedly stimulates protein synthesis in the eggs of many organisms (Hultin, 1961; Nemer, 1967; Bramachary, 1968; Gross, 1967; Bell and Reeder, 1967; Mano, 1966; Marcus & Feeley, 1964). It thus became evident that the early embryonic protein synthesis and the consequent developmental events proceed without concommitant RNA synthesis.

Based on these observations the concept of '<u>Conserved m RNA</u>' or "<u>Masked maternal messenger RNA</u>" has emerged (Gross, 1964 ; Spirin, 1966; Tyler, 1968; Gross et al, 1967, Nemer, 1967). Here it is envisaged that the egg during the course of oogenesis inherits a stock pile of stable mRNA and other components of the protein synthesizing machinery, in addition to the nutrient material. Further, the conserved maternal mRNA is utilised in the early embryonic protein synthesis. In the recent years the presence

of stable, conserved maternal mRNA and its utilisation during early development has been demonstrated in a wide variety of systems namely - eggs, dormant embryos (Davidson et al, 1965; Davidson et al 1966; Slater and Spiegelman, 1966) and plant seeds (Dure and Watters, 1965; Barker and Rieber, 1967; Bhat and Padayatty, 1974). The main approach in most of these experiments has been to demonstrate that the system concerned contains the necessary machinery to carry on protein synthesis. Cell-free systems derived from the eggs or seeds have been shown to stimulate endogenous amino acid incorporation into proteins. Also mRNA fractions have been isolated and purified from the eggs and seeds of various systems. These mRNA fractions have been translated <u>in vitro</u> in a cell-free system and the protein products have also been characterised in certain cases (Gross et.al. 1973; Gross et.al., 1973a; Gabrielli and Baglioni, 1975; Grosteld and Lattaller, 1975).

Thus, presently there is ample evidence to show that most eggs contain stable maternal mRNA, conserved during oogenesis and utilized during early embryogenesis. However, the fact that the egg accumulates mRNA is not very surprising, since the egg accumulates a very large variety of materials like ribosomes, yolk etc. during oogenesis. But some of the important questions that are yet to be answered

are (i) what is the mechanism of storage of these messengers which renders stability to the molecules ? (ii) what is the location and the nature of distribution of these messengers inside the cell, and (iii) how are these molecules sequestered during development. Spirin (1966) has proposed that the messengers are stored in the form of ribonucleoprotein particles called '<u>Informosomes</u>'. There are many reports where informosome like particles have been characterised from eggs and embryos (Spirin and Nemer, 1965; Spirin, 1966; Mano, 1966). Finally as Gross and Gross (1973) put it, the whole concept of maternal messengers is relevant from the point of view of the '<u>economics of developmental information flow</u>'.

However, the presence of maternal messengers and their utilization during early development does not imply that the embryo genome is completely silent during this period. It is now well known that many embryos start the synthesis of informational RNA (mRNA and Heterogenous nuclear RNA: HnRNA) very early during development (Nemer, 1963; Nemer and Infante, 1965; Gross et.al. 1965; Brown and Littna, 1966; Brown and Littna, 1966a; Whitely et.al. 1966; Glisin et.al. 1966; Kaulenas and Fairburn, 1966; Davidson, 1968). Further, there is evidence to suggest that some of the informational RNA synthesised during the early stages of

development are translated into proteins. But it is still not clear as how much of this synthesised mRNA is stored for later utilization and how much is utilized immediately.

In the past few years the presence of polyadenylic acid [Poly (A)] sequences at the 3'OH end of messenger RNA and heterogenous nuclear RNA (HnRNA) has been reported from various organisms (Edmonds et.al. 1971; Darnell et.al 1973; Greenberg, 1975; Nakzato et.al. 1975; Srinivasan et.al. 1975). Further, in the eggs and embryos of certain organisms it has been shown that fertilization leads to polyadenylation of the conserved mRNA (Slater et.al. 1972; 1973; 1974; Slater and Slater 1974; Wilt, 1973). Although various possible functions have been attributed to the Poly (A) sequences, the exact role played by it is not yet clear. However, the presence of Poly (A) serves as a useful criteria during isolation and characterization of mRNA and HnRNA from various systems.

I.C. Resume of Pertinent Literature

Reproduction and life cycle of Rotifers: Rotifers are a small group of microscopic, aquatic organisms of the protostome phylum Aschelmenthes. These organisms are wide spread and exhibit certain very interesting features with regard to their reproduction and development. Some of these

striking characteristics are cyclomorphosis (Edmondson and Hutchinson, 1934; Buchner et.al. 1957; Nayar, 1965; Hutchinson, 1967; Gilbert, 1967), sexual transition (Gilbert, 1968; Gilbert and Thomson, 1968; Birky & Gilbert, 1971) and cell constancy (Eutely) (Birky and Field 1966; Birky et.al. 1967; Birky and Power, 1968; Gopinath, 1972). These interesting features combined with the rapid rate of reproduction and the ease of laboratory culturing, make them an interesting system for study of several biological phenomena. Notwithstanding, rotifers have received comparitively very little attention of biologists.

The best studied genus with regard to reproduction and life cycle in rotifers is Asplanchna (Birky & Gilbert, 1971; Gilbert, 1974, Gilbert, 1968). The rotifers usually reproduce asexually by diploid parthenogenesis. The eggs are diploid (2n) and develop into an adult without any fertilization. These diploid parthenogenetic organisms are termed as 'Amictic females'. Although parthenogenesis is the usual mode of reproduction, this amictic life cycle is known to be intervened by sexual or 'mictic' life cycle. In the mictic cycle the diploid eggs give rise to organisms which produce haploid eggs (n). These eggs are termed mictic eggs and these develop parthenogenetically into males which are haploid. Fertilized mictic eggs give rise to resting eggs (2n), which upon germination develop into

FIG. 2 Life cycle of Asplanchna (from Birky 1967).

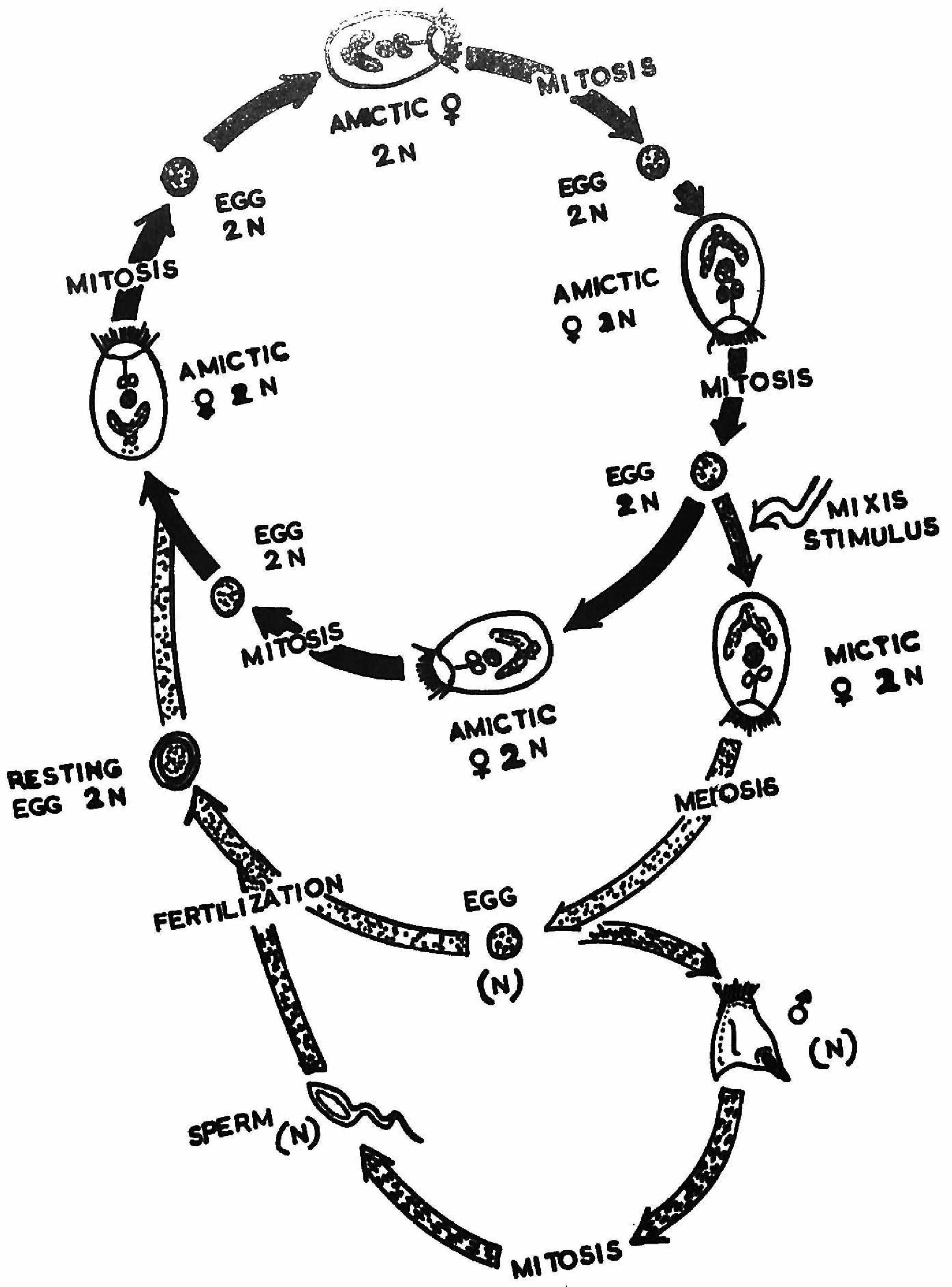

FIG. 2. LIFE CYCLE OF ASPLANCHNA

of Asplanchna. In the amictic organisms the diploid eggs are produced mitotically, on the other hand in the micitic organisms meiosis takes place and results in the production of haploid eggs. The exact mechanism of the vitamin E induced meiosis or of the mixis induction in general, is not clear.

Embryology of Rotifers: There is very little information on the embryology of rotifers and most of these informations is limited to studies on the genus Asplanchna. The best account of the embryology of Asplanchna is by Nachtway (1925), Tannreuther (1920) and Gopinath (1972) have also studied the embryology of Asplanchna. During the development of Asplanchna the early cleavages are of the spiral and determinate type but the later cleavages are bilaterally symmetrical. Once the organ primordia are laid down, cell division caeses and most tissues stop the synthesis of DNA (Birky et.al. 1967; Gopinath, 1972). Thus the embryonic development is divided into distinct (i) gametogenic (ii) mitotic (iii) post mitotic phases (Birky and Gilbert, 1971; Gopinath, 1972). The embryonic development is very rapid and there is only a brief immature stage before the adult emerges. The complete development proceeds inside the body of the parent (in utero) and takes about 22-24 hours at 25°C.

The embryology exhibits many peculiarities and a similarity with the Acanthocephalan embryology has been

emphasized by Mayer (cf Hyman, 1951). Further, Hyman (1951) has pointed out that the embryology of Asplanchna shows a general similarity with that of the flat worms. The spiral and determinate nature of the early cleavage, the formation of the D quadrant, are characteristic features of the protostome development pattern.

Molecular Embryology of the Protostomes*: The protostomes comprise of the lower invertebrate organisms like the worms, insects and molluscs. The name 'protostome' is derived from the fact that during the development of these organisms, the blastopore of the embryo gives rise to the mouth of the adult organism. Besides, the development of these organisms is characterised by spiral and determinate type of cleavage. Since the present study deals with the RNA and Protein synthesis during the development of a protostome i.e. the rotifer A. brightwelli, it was felt appropriate to review the available literature on the RNA and Protein synthesis

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*Protostomes "include flat worms, annelids, molluscs, arthropods and smaller groups. The mouth forms from the blastopore of the embryo. Determinate cleavage, in which fate of cells in the developing embryo is fixed very early; spiral cleavage in which cells (blastomeres) are arranged in a spiral and the origin of the mesoderm is from a single blastomere. In contrast Deuterostomes (echinoderms, hemichordates and chordates) have indeterminate radial cleavage and the mesoderm arises from the wall of the gastrocoel". c.f. Storer T.F. and Usinger R.L. (1965) in General Zoology, P. 272, Mag Grawhill Co.

during the development of the other protostomes.

Molecular embryological studies on the protostomes are not as detailed and extensive as in the case of the Deuterostomes (Eg. Echinoderms and Amphibians).

The worms are the lowest of the protostomes in which RNA and protein synthesis has been studied during embryogenesis. In the case of Ascaris lumbricoides, the embryonic RNA synthesis has been studied by Kaulenas and Fairburn (1966; 1968). Kaulenas and Fairburn (1966) have shown that informational RNA synthesis begins before first cleavage and increases as development proceeds. Further studies by Kaulenas and Fairburn (1968) indicate that the synthesis of ribosomal RNA begins as early as 4-cell stage in the embryogeny. These workers have inferred as to the ribosomal nature of the RNA on the basis of the appearance of nucleoli, base composition and sedimentation analysis of the RNA.

Recently Devries (1976) has studied gene activity during the development of the earthworm Eisemia foetida. He has reported early RNA synthesis during segmentation and further reports mRNA synthesis in the young embryos. Appearance of nucleoli and rRNA synthesis seems to be predominant after blastulation.

Gould (1969, 1969a) has carried out detailed studies on the marine echiuran worm Urechis caupo. She has reported that the unfertilized eggs synthesise RNA and protein over long periods, with the RNA synthesis being mostly nucleolar. Thus the RNA synthesis in the unfertilized eggs is mostly ribosomal but heterogenous RNA and tRNA are also detected in significant amounts. Synthesis of ribosomal RNA ceases prior to fertilization and is resumed at gastrulation. During the cleavage stages a low level of mRNA synthesis goes on. Schawartz (1970) has fractionated the RNA of the oocytes of Urechis caupo and the postfertilization stages by MAK coloumn chromatography and has found 90% of the RNA to be of the high molecular wt. type.

Among the studies on insects the autoradiographic studies of Lockshin (1966) on the coleopteran eggs is well known. Lockshin (1966) has studied RNA and Protein synthesis by microinjecting appropriate labelled precursor into the eggs under special conditions. The cleavage stage embryos show no RNA synthesis, and the synthesis of RNA starts only when the nuclei migrate to the periphery of the egg. The onset of RNA synthesis, however, takes place before the formation of cell membranes and the blastoderm. Experiments with puromycin have shown that cleavage and blastulation require protein synthesis and further, Actinomycin D does not inhibit pregastrular morphogenesis.

Harris and Forest (1967) studied the RNA synthesis in the milkweed bug *Oncopeltus*. They have reported that RNA synthesis attains a peak during gastrulation and then declines. Harris and Forest (1970) have further shown that the template activity of the chromatin declines during development and the RNA polymerase activity varies in the nuclei at different stages of development. They have further shown that the ribosomal RNA is of the 27 S and 16 S species in the *oncopeltus*.

Recently Zalokar (1976) has reported certain very elegant autoradiographic studies of the RNA and Protein formation during the early development of *Drosophila*. He has shown (Zalokar, 1976) that most of the early synthesis of RNA is mitochondrial and, the embryo genomic RNA synthesis starts only at the blastoderm stage. Further, fertilization does not markedly stimulate protein synthesis in the *Drosophila* eggs and both fertilized and unfertilized eggs make almost same amount of proteins. This finding on the RNA synthesis by Zalokar (1976) is not in agreement with the studies of Fausto-Sterling et.al. (1974) on *Drosophila*. Fausto-Sterling et.al. (1974) have reported that the RNA synthesis increases until gastrulation and then declines. They suggest that the RNA synthesis in the embryo begins very early during the development of *Drosophila*.

The crustacean Artemia salina has received special attention of the molecular embryologists due to its interesting embryology. These organisms form 'cyst' like objects which are dormant gastrulae. Urbani (1962) has reviewed the classical biochemical embryology of these cysts. These cysts have been shown to contain a number of enzymes and the activities of some of these increase before hatching. Ribosomes and polyribosomes have been isolated from the cysts of Artemia salina (Hultin & Morris, 1968; Clegg & Golub 1969; Hultin et.al. 1969). Maclean and Warner (1971) have studied the pattern of RNA synthesis during hatching of these cysts and have found that the newly hatched Artemia embryos synthesise 5 different RNA fractions, of which tRNA and heavy RNA are predominant. In Artemia there is a rapid increase in polysome content and protein synthesis following hatching. Recently, Zasloff and Ochoa (1973) have isolated protein synthesis initiation factors from the Artemia cysts. The conserved mRNA from the cysts has been isolated and successfully translated in vitro (Grosteld & Littauer, 1975). Jose et.al (1976) have studied mRNA activity in the dormant and developing Artemia embryos. They have (Jose et.al. 1976) fractionated the mRNA into poly (A+) and poly (A-) classes and report that the poly (A+) mRNA activity increases during development. Christy and Jayaraman (1976) have studied mRNA utilization during development of the

Artemia cysts and have proposed a scheme for the activation of the sequestered messengers during development.

Gene activity during the development of the molluscs has been studied in more detail than any other protostome. Collier (1966) has reviewed the molecular embryology of molluscs based on his and other studies on the gastropod *Ilyanassa*. Collier and Schwartz (1969) studied the rate of protein synthesis and the size of the amino acid pool during *Ilyanassa* development. Protein synthesis is low during cleavage and increases during gastrulation reaching a peak during organogenesis. The amino acid pool remains constant during the first 3 days of development and has no effect on the rapid changes in protein synthesis. Mirkes (1970) studied protein synthesis before and after fertilization and has found that in the unfertilized eggs protein synthesis goes on at low but significant levels. Further, fertilization results in the stimulation of the protein synthesis. Actinomycin D at concentrations of 5-50 μg/ml when administered within 30 minutes after fertilization had no effect on cleavage. Clement and Tyler (1967) have studied protein synthesis in the isolated polar lobe of the *Ilyanassa* embryos. They have found that the polar lobe which has no nucleus carries on protein synthesis over long periods, presumably by the use of stable messengers. Geuskens (1969) has employed electron microscopy and radio-

Another mollusc for which considerable amount of data on the gene activity during development is available is Limnaea. Bramachary and his coworkers have studied the mollusc in detail and Brahmachary has reviewed the molecular embryology of Limnaea (Brahmachary 1968; 1973). In an earlier study they have shown that the incorporation of ^{32}P into RNA increases during development. Van denBiggelaar (1971) has demonstrated the presence of nucleoli and RNA synthesis from morula stage onwards. Brahmachary et al (1971) have reported a rhythmic incorporation of uracil ^{32}P and ^{35}S. Further by sucrose gradient centrifugation Brahmachary et al. (1971a) have studied the transcription pattern during the development of Limnaea. The presence of RNA of the ribosomal region (23S and 16S) in quite marked in the uncleaved eggs and the morula embryos. Further, RNA heavier than 28S has been detected in early embryos by agarose gel electrophoresis. A 10S RNA fraction (presumably mRNA) has been detected in the morula, trocophore and Veliger stages (Brahmachary, 1973). Protein synthesis is very low in the early stages but increases after trocophore stage (Bramachary, 1973).

Thus from the literature survey presented here it is evident that the protostomes do not show any generalized pattern in the onset of gene activity during development. In many protostomes (worms, molluscs etc.) the informational

stages of the organism. Attempts have also been made to study the nature of the RNA stored in the resting eggs (dormant embryos). Efforts have been made here to study the vitellogenic transfer of proteins during oogenesis. A resume of available literature pertinent to the life cycle and embryology of rotifers and the molecular embryology of the protostome invertebrates has been presented (vide supra).

II. A. Introduction

Molecular embryological studies involving both the qualitative and quantitative aspects of RNA and protein synthesis have been carried out in a wide variety of organisms (see review Gross 1967; Nemer 1967; Brahmachary, 1968; 1973). These studies have shown that protein synthesis is essential for development and early embryonic protein synthesis involves utilization of stored maternal templates. Studies have also shown that the various organisms differ in the timing of the onset of informational RNA and rRNA synthesis during development. Such detailed studies are lacking in the case of rotifer embryology. The present study deals with RNA and protein synthesis during certain embryonic stages of the rotifer *A. brightwelli*.

In the present investigation attempts have been made to study

i) The nature of RNA synthesis during the embryonic stages of *A. brightwelli*.

ii) Protein synthesis during certain embryonic stages.

iii) The effect of Actinomycin D on the embryonic protein synthesis.

II. B. Materials and Methods

Test Organism: All the experiments have been performed using laboratory cultures of *Asplanchna brightwelli* de Beauchamp.

Materials : The following chemicals were purchased from the sources indicated in the parenthesis: ^{3}H-Uridine Sp. activity 5.3 Ci/m.mole; ^{3}H-Lysine, Sp. activity 250 mCi/ m.mole (BARC, Trombay, India), Acrylamide; yeast RNA (V.P. Chest Institute, New Delhi), Acrylamide was recrystallized in ethanol before use, N'N'N' bis Acrylamide and Diphenyl oxazole-PPO (Eastman Organic Chemicals, U.S.A.), Tetra ethylmethylene diamine TEMED (Fluka AG, Swiss). Ammonium per sulphate (Lab. Chem., India). Sodium dodecyl sulphate-SDS (BDH, India). Bovine Serum Albumin-BSA (Aldrich Co., U.S.A.). Deoxyribonuclease (RNAse free), Bentonite, Poly-U Sepharose, Uridine, Actinomycin D were received as gifts from various laboratories. All other chemicals used were of reagent grade quality.

Culture of Experimental Animals: The method of Birky (1967) was employed throughout the work. Stock cultures of *A. brightwelli* were maintained in 60 ml corning glass culture tubes fitted with screw caps. Mass cultures were raised from these stock cultures in 500 ml corning conical flasks. The culture media has been Hay infusion throughout and the

animals were fed with paramecia (vide infra). The cultures were kept at 25 ±3°C in a Sew BOD incubator. The cultures were maintained such that the concentration of animals was never more than 10/ml. Benzyll penicillin and Streptomycin (200-300 µg/ml) were included in the culture media to minimize bacterial contamination.

Preparation of Hay infusion : 10-15 gms of Hay was boiled in 2-3 litres of triple distilled water for 15-20 minutes. The hot extract was filtered through 4-folds of cheese cloth, cooled and used as the hay infusion media.

Paramecium aurelia Cultures : *Paramecium aurelia* was grown at 25 ±3°C in 2 litre culture bottles on *Aerobacter aerogens*. Hay infusion media was enriched with malted Horlicks (2-3%) and was asceptically inoculated with *Aerobacter aerogens*. The bacteria were allowed to multiply overnight at 37°C. About 2-3 ml of *paramecium aurelia* (conc. 2×10^3/ml) was inoculated into the *Aerobacter* culture and kept at 25 ±3°C. Rich cultures of paramecia were obtained within a week's time. The same was used to feed *A. brightwelli*.

Feeding : Hay infusion cultures of *Paramecia* was filtered through 2 folds of cheese cloth and centrifuged in 10 ml corning tubes at 2000x g for 2-3 minutes in a table top centrifuge. The pellet was washed twice with triple distilled

water and suspended in 1 ml of the same. The suspensions of paramecia were directly transferred into *A. brightwelli* cultures. The organisms were fed once in 24 hours.

Isolation of Embryos : Birky's (1967) method of handling *Asplanchna* embryos has been used in the present work to isolate embryos of the required stages of development. Amictic females with the embryos of the required stage were singled out and transferred onto a single concavity microslide (Blue Star Co.). A glass micro pipette fitted with a rubber dropper was used for this purpose. On each slide a single organism was taken and was washed with distilled water containing Benzyl penicillin and streptomycin (conc. 200-300 µg/ml). The water and the media were completely removed with a Whatman No. 1 filter paper and the organism was covered with a drop of sterile paraffin oil. In this condition the animal could be kept alive and immobile for fairly long periods of time.

With the help of two sterile micro needles the uterine cavity of the organism was ripped open to let the uterine fluid flow out on the slide. The embryo was carefully brought out into the fluid. In this condition the embryo could be kept alive and ~~the~~ *in vitro* development could proceed up to about 10-12 hours. The whole operation was carried out under an Olympus binocular dissecting microscope. A magnification of 60x was employed during

the micro dissection. The embryos were handled using fine sterilized micro pipettes drawn out of corning glass tubes and fitted with rubber droppers.

Embryonic Stages : The experiments were carried out on mitotic and post mitotic embryonic stages. The mitotic stages studied were (i) 2-cell, (ii) 4-cell, and (iii) 10-cell embryos. The post-mitotic stages were 6 hours, 8 hour and 10 hour embryos. These stages denote the time of development after the first cleavage.

Protein synthesis was studied by following the incorporation of Tritiated (^{3}H) Lysine into Trichloroacetic acid (TCA) insoluble material of the embryos. *Asplanchna* embryos of the appropriate stages were dissected out into the uterine fluid on a concavity micro slide as described above. 0.2 µci of ^{3}H-Lysine (0.2 µCi/embryo : 10 µCi/ml) was introduced into the paraffin oil drop containing the embryos using a corning micropipette. The drop of the isotope was brought in contact with the uterine fluid, so as to mix with it. The slide was covered with a cover glass supported by glass wool. The embryos in uterine fluid were incubated for 10 minutes at 25 ± 3°C. The labelling was stopped by transferring the embryos into 100 fold excess of cold Lysine (0.2% soln. kept at 0-4°C). 10-20 embryos of the same embryonic stages were pooled together and homogenised in 0.1 ml of 20% TCA at 0-4°C.

The protein was precipitated using 0.1% BSA as carrier. The precipitate was washed successively 5 times each with cold and hot 5% TCA containing 0.2% Lysine. This was followed by 5 washings with each of the following in succession, (a) mixture of chloroform:ethanol:ether (1:1:1 V/V/V), (b) ether, (c) Acetone. The air dried precipitate was dissolved in 0.1 ml formic acid and an aliquot (20 μl) was loaded on to a Whatman No.1 filter paper disc (25 mm diameter). The filter paper disc was dried under an IR lamp. The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter at an efficiency of 25-30%. 0.5% PPO in Toluene base was used as the scintillation mixture.

To account for the permeability fluctuations of the embryonic cells, the homogenates of the embryos were incubated with the precursors. 10-20 embryos of a specific stage were homogenised in Tris buffer (0.01M, Tris-HCl, pH 7.6) and the ^{3}H-Lysine incorporation into the TCA insoluble material of these homogenates was followed as above.

<u>Effect of Actinomycin D</u> : ^{3}H-Lysine incorporation into TCA insoluble material was measured in the presence of 1 μg/ml of Actinomycin D. Embryos of the appropriate stages were dissected out and treated with 1 μg/ml of Actinomycin D. The rest of the procedure for labelling

with ^{3}H-Lysine and measuring incorporation to TCA insoluble material is same as above. The conc. of the Actinomycin D employed in the above experiments was sufficient to inhibit 80-90% of the RNA synthesis in the embryos (vide table 2a).

RNA Synthesis : RNA synthesis was studied by following the incorporation of tritiated (^{3}H) Uridine into TCA insoluble and Alkali labile material of the embryos.

Embryos of the required stages were dissected out and labelled with 1 μci/embryo (40 uCi/ml) of ^{3}H-Uridine (Spec. Act. 5.3 Ci/mmole). The labelling was carried out for 10 minutes at 25 $\pm 3^{\circ}C$. The labelling was stopped by transferring the embryos into 100 fold excess of cold Uridine at 0-4°C. The embryos were pooled and homogenised as before. The TCA insoluble material was precipitated out using 0.1% yeast RNA as carrier. The precipitate was washed with cold 5% TCA containing 0.1% unlabelled uridine. The processing of the precipitate and the measurement of the radio activity was carried out as described above (vide supra).

Alkali Hydrolysis : The ^{3}H-Uridine radio activity incorporated into TCA insoluble material was tested for lability to alkali hydrolysis. The precipitate was hydrolysed in 0.3N KOH for 16 hours at 37°C and neutralised with Perchloric

acid. The TCA insoluble radio activity was determined as described previously (page 27). 90-95% of the radioactivity in the TCA insoluble material was lost after alkali treatment of the precipitate.

<u>Extraction of ^{3}H-Uridine labelled RNA from Embryos:</u> Embryos were dissected out and labelled with ^{3}H-Uridine as described above. The embryos of the required stages were pooled together and used for RNA extraction. The RNA extraction procedure of Perry et al. (1972) was followed. 50-100 embryos were homogenised in 1 ml of Tris buffer (Tris-HCl 0.1M; pH 7.6; EDTA 0.005M; SDS 1%; Bentonite 1%) along with 200 fold excess of unlabelled Uridine. A precooled all glass homogeniser was used for this purpose. The total RNA was extracted by mixing the homogenate with an equal volume of phenol:chloroform mixture (1:1 V/V). The extraction was done at 20°C for 2-3 minutes. The aqueous phase was collected by centrifugation and the RNA was precipitated with 3 volumes of cold ethanol at -20°C and kept overnight. 10 μgms of purified yeast RNA was used as carrier. The RNA precipitate was pelleted out the next morning by centrifugation at 5000 x g for 15 minutes in a Janetzki refrigerated centrifuge. The precipitate was washed 5 times each, with cold ethanol; chloroform:ethanol:ether mixture (1:1:1 V/V/V), ether and, finally with cold acetone and air dried.

Bentonite was used to inhibit Ribonuclease and was prepared following the method of Brown Hill et. al. (1959). All the glasswares employed were heated to 200°C for 2-3 hours, before use, to minimise RNAse contamination. The RNA preparations were treated with Deoxyribonuclease (DNAse) as below to remove the contaminating DNA. The radio activity in the RNA preparations was determined by loading aliquots on to Whatman No. 1 discs and scintillation counting as described before (page 27).

<u>DNAse Treatment</u> : All the RNA preparations were treated with pure DNAse (RNAse free) to remove any contamination by DNA. The RNA precipitate was dissolved in Tris buffer (0.1M Tris-HCl, pH 7.6) and treated with 10 µgms/ml pure DNAse (RNAse free : Sigma Ch. Co.) at 37°C for 2 hours. After the treatment the RNA was re-extracted and purified as above.

<u>Poly-U Sepharose Chromatography</u> : The ^{3}H-Uridine labelled RNA extracted as above, was fractionated into Poly (A+) and Poly (A-) classes by hybridizing the labelled RNA on to Poly-U Sepharose columns.

<u>Preparation of Columns</u> : Poly-U Sepharose was suspended in equilibrating buffer and packed into columns of the size 6 x 0.6 cm. Pasteur pipettes of the proper size were used for this purpose. The columns were kept equilibrated with the equilibrating buffer.

The RNA sample was dissolved in the equilibrating buffer and about 0.1 to 0.2 ml of the RNA sample (of known radio activity) was applied to the column. After a few minutes the column was excessively washed with the equilibrating buffer (using 5 to 10 ml). This was done to remove the unbound portions of RNA [Poly (A-)] . The bound fraction [Poly (A+)] was eluted out with the eluting buffer. The radio activity from the bound and unbound fractions was determined as before (page 27). The per cent input radio activity bound was expressed as the % poly (A+) RNA. The RNA from the two fractions was precipitated with ethanol as above (vide supra) and subjected SDS-polyacrylamide gel electrophoresis. The composition of two buffers was as follows:

Equilibrating buffer:	0.4M NaCl 2 mM EDTA, 0.01M Tris HCl 0.2% SDS
Eluting buffer :	0.01M pot. phosphate (pH 7.4) 90% formamide, 0.1% SDS.

<u>SDS-Polyacrylamide Gel Electrophoresis of RNA</u> : The ^{3}H-Uridine pulse labelled RNA extracted and purified as above was fractionated on SDS-polyacrylamide disc gels. The RNA electrophoresis system of Loening (1969) was used.

<u>Preparation of Gels</u> : The following stock solutions were used to prepare the <u>Bis</u> crosslinked polyacrylamide gels:

A: 0.4M Tris, 0.2M sodium acetate, 20 mM, EDTA; pH to 7.8 with acetic acid.

B: 20% acrylamide (20 gms of Acrylamide and 1 gm bisacrylamide in 100 ml distilled water).

C: 10% (V/V) TEMED in distilled water.

D: 10% (W/V) Ammonium persulfate in distilled water.

A 2.6% gel was prepared by mixing 0.6 ml of (A); 0.76 ml of (B); 0.05 ml of (C); 0.05 ml of (D) and 4.52 ml of distilled water. About 1.3 ml of the mixture was poured into glass tubes (6.5 x 0.6 cms) with their bottom end closed with rubber corks. The gel solution was layered with distilled water and allowed to polymerise at room temperature for about 2 hours.

Sample Preparation : The RNA sample to be electrophoresed was dissolved in 10 times diluted stock solution (A) and made up to 0.2% with SDS. The RNA sample was made 0.01% with bromophenol blue and 10% with respect to glycerol. 25-50 µl of the RNA sample of known radio activity was loaded on to the bis crosslinked gels. The remaining space in the tube was filled by layering the electrophoresis buffer over the sample.

Electrophoresis : The stock solution (A) was diluted 10 times and made 0.2% with SDS. This was used as the electro-

phoresis buffer. The gels were electrophoresed at room temperature for 90 minutes at 5 mAmps/gel.

Gel removal and Processing : After the electrophoresis the gels were extruded out with the help of a 5 ml glass syringe fitted with a 3" long needle (22 gauge). The gels were removed by lubricating the gel and glass interface with distilled water. After removal the gels were pressed between the folds of a Whatman No. 1 filter paper. The gels were dried in an oven at 80°C for 1-2 hours, and cut into 1 mm slices along with the paper. The slices were immersed in 5 ml of 0.5% PPO in Toluene base. The radio activity was measured in a Packard Tricarb Liquid Scintillation Counter at an efficiency of 18-20%. E.Coli RNA was used as internal markers to mark the position of 23S and 16S.

II.C. Results

Protein Synthesis : The synthesis of protein in the mitotic and the post-mitotic embryos has been studied. Studies in mitotic phase have been made with 2,4 and 10 cell embryos, while the post-mitotic embryos are 6,8 and 10 hr old. In these embryos the synthesis of protein appears to be maximum in the 10 celled stage (fig.3,4) and the synthesis apparently declines with the progress of the embryonic development. The uptake of ^{3}H-Lysine at the 10 hr stage comes to be what it was at the 2 cell stage, which is the minimum.

The permeability fluctuation has been accounted by looking at the incorporation into TCA insoluble material of homogenates of the embryos. The results are presented in Table (1). The pattern of incorporation does not differ very much from the incorporation in the whole embryo. A peak incorporation has been seen in the 10 cell embryo homogenate and thus corresponds with the peak incorporation in the whole embryos. Thus the experiments performed with the whole embryos of different stages of development as well as homogenates of corresponding stages of development are in conformity. In both the cases the incorporation is highest in the 10 cell embryos. It is, however, true that when the CPM are compared in the two cases namely, whole embryos and the homogenates, there is less count in the homogenates (Table 1).

Actinomycin D was used at concentration sufficient to inhibit 80-90% of ^{3}H-Uridine incorporation into RNA (vide table 2a). Figure 5 shows ^{3}H-Lysine incorporation in the presence of Actinomycin D. Further, figure 6 presents the fraction of Actinomycin D resistant ^{3}H-Lysine incorporation at each embryonic stage. During the early mitotic stages 60-80% of the protein synthesis goes on in the presence of Actinomycin D as compared to the controls. Protein synthesis in the Actinomycin D treated post-mitotic embryos is reduced to a low level of 20-30% of the control.

Thus the following are the main results: (1) Protein synthesis is detected at all stages, (2) most of the early stage protein synthesis is insensitive to inhibition of RNA synthesis by Actinomycin D, (3) the protein synthesis during the post-mitotic stages is sensitive to the action of Actinomycin D.

Effect of Actinomycin D on Cleavage : 15-20 embryos of each mitotic stage were examined for cleavage in the presence of Actinomycin D. Actinomycin D at conc. (1 μg/ml) showed a non-specific effect on development. The results are given in Table 2.

RNA Synthesis : ^{3}H-Uridine incorporation into TCA insoluble and KOH labile material (RNA) was studied at different stages of development of Asplanchna. The results are presented in figure 7. Further, figure 8 gives a schematic diagram of the same indicating the general pattern of incorporation. All the stages show incorporation of the precursor into RNA. The incorporation is maximum at the 4 cell stage and after this there is a gradual decline. The embryo of 10 hour stage shows an increase in contrast with the other post-mitotic stages. RNA synthesis is detected at all the stages studied beginning with the 2 cell stage.

Results of SDS : Results of SDS-Polyacrylamide gel electrophoresis of the ^{3}H-Uridine pulse labelled RNA from the

different embryonic stages are presented in figures 9—14 The Poly (A+) RNA from the mitotic stages show peaks in the region $>$ 23S and also in the region $<$ 16S. The Poly (A-) RNA from the mitotic stages shows peaks mostly in the low molecular wt. region ($<$16S). The heavier fractions ($>$23S) in the Poly (A+) RNA is presumably heterogenous nuclear RNA (Hn RNA), since Hn RNA is known to contain Poly (A) and it has a heterogenous molecular size. The low molecular wt. peaks ($<$16S) in both the Poly (A+) and Poly (A-) RNA is probably due to mRNA. On the other hand in the post-mitotic stages the poly (A+) RNA is again heterogenous in size and predominantly shows heavy RNA. The Poly (A-) RNA from the post-mitotic stages shows peaks in the riboromal RNA region (approximately 28S and 18S).

Appearance of Nucleoli : The mitotic stages do not show nucleoli, where as the post-mitotic stages have prominent nucleoli.

Poly (A+) RNA Content : The Poly (A+) RNA fraction was determined by Poly-U Sepharose chromatography. The results of the same are presented in figure 15 ~~and table~~ ____. The mitotic stages show 30-34% Poly (A+) RNA, whereas the post-mitotic stages show 40-65% Poly (A+) RNA. This is out of the total RNA extracted after 10 minute ^{3}H-Uridine pulse. The Poly (A+) RNA shows a gradual increase with the development (Fig. 15). The synthesis of the same beginning as early as 2 cell stage.

TABLE 1

^{3}H-Lysine incorporation into TCA insoluble material of the homogenates of A. brightwelli embryos.

Embryonic Stage	^{3}H-Lysine incorporation (CPM/embryo)
2 cell	156 ± 15
4 cell	226 ± 25
10 cell	245 ± 20
6 hr.	214 ± 20
8 hr.	200 ± 30
10 hr.	180 ± 30

Embryos of the different stages were isolated & homogenised in Tris buffer (0·01M) and the homogenates were incubated with ^{3}H-Lysine. Incorporation into TCA insoluble material was studied as described under material and methods (II.B). Values are exprexed as mean ± S.D.

TABLE 2

Effect of Actinomycin D on the cleavage stage embryos of *A. brightwelli*.

Embryonic Stage	Actinomycin D conc. (g/ml)	Effect
Mature egg	1 µg/ml	No cleavage divisions
2 cell stage	--do--	Embryo divided upto the 10 cell stage & then the development was arrested.
4 cell stage	--do--	-do-
10 cell stage	--do--	No cleavage divisions.

15-20 embryos of each stage were examined for cleavage in the presence of Actinomycin D. Embryos were isolated and treated with Actinomycin D as described under methods (II B).

TABLE 2a

^{3}H-Uridine incorporation in the Actinomycin D treated embryos of A. brightwelli

Embryonic Stage	Control	^{3}H CPM/ embryo Actinomycin treated
2 cell	3400	310
4 cell	3700	320
10 cell	3250	400
6 hour	3100	300
8 hour	2750	200
10 hour	3250	320

Embryos were treated with 1 μg/ml of Actinomycin D and incubated with 1 μCi/embryo of ^{3}H-Uridine for 10 minutes.

FIG. 3 ^{3}H-Lysine incorporation into TCA insoluble material of *A. brightwelli* embryos. Values expressed as mean ± S.D.

FIG. 4 Schematic diagram of the pattern of ^{3}H-Lysine incorporation by *A. brightwelli* embryos.

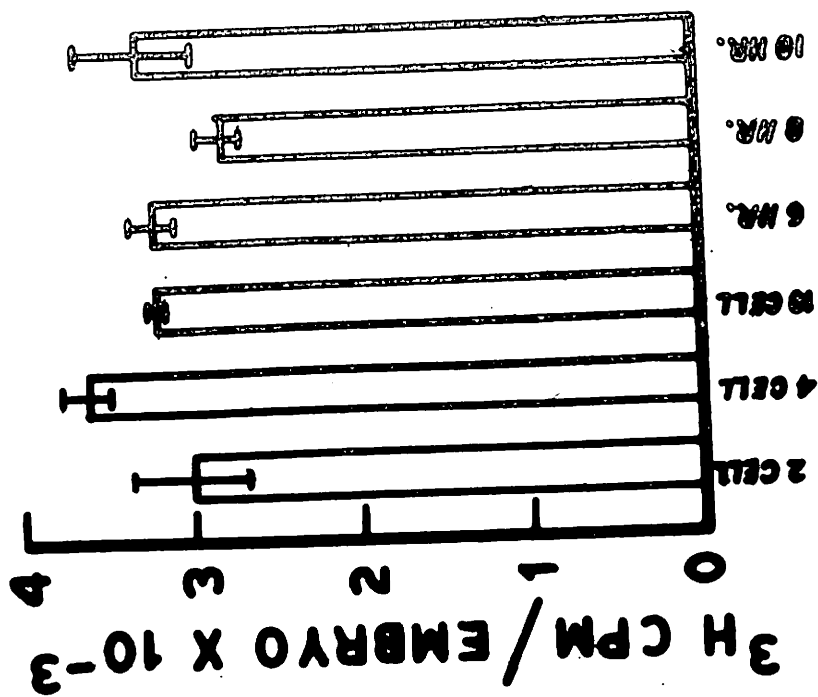


FIG. 3

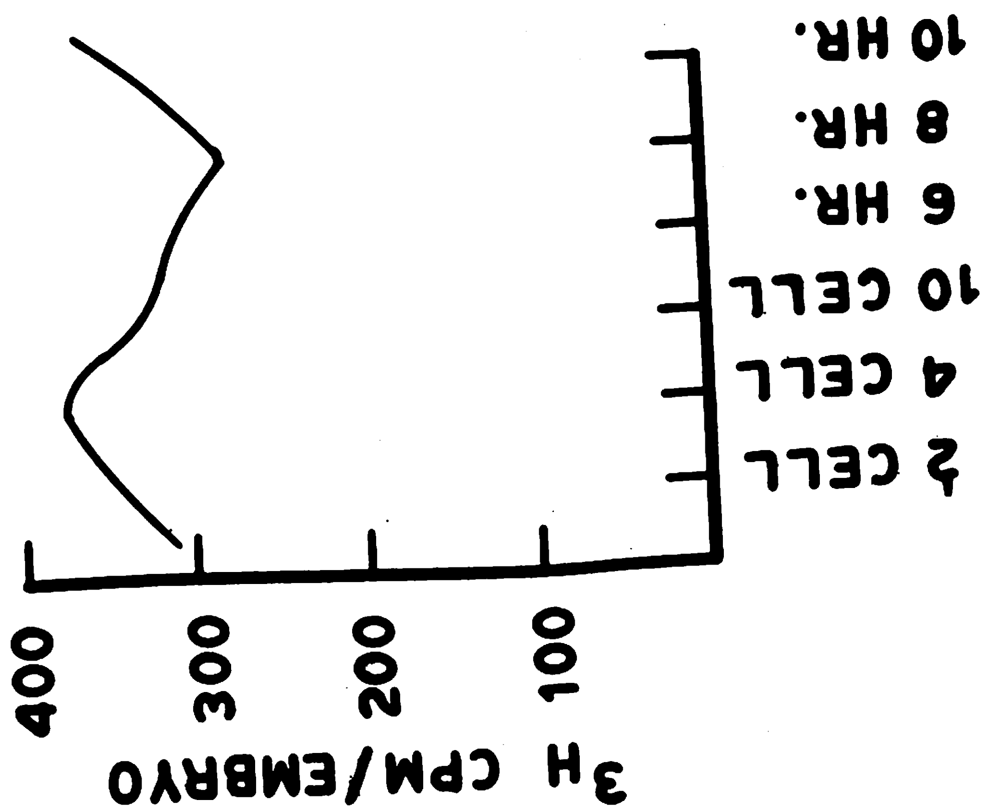


FIG. 4.

FIG. 5 Effect of Actinomycin D on ^{3}H-Lysine incorporation by the embryos of *A. brightwelli*. Embryos were treated with 1 $\mu g/ml$ Actinomycin D and the incorporation of ^{3}H-Lysine into TCA insoluble material was determined. Values expressed as mean ± S.D.

FIG. 6 Fraction of Actinomycin D resistant ^{3}H-Lysine incorporation in the embryos of *A. brightwelli*.

10 Hr
8 Hr
6 Hr
10 CELL
4 CELL
2 CELL
0
10
20
30
40
50
60
70
80
^{3}H LYS INCORPORATION % CONTROL

FIG. 6.

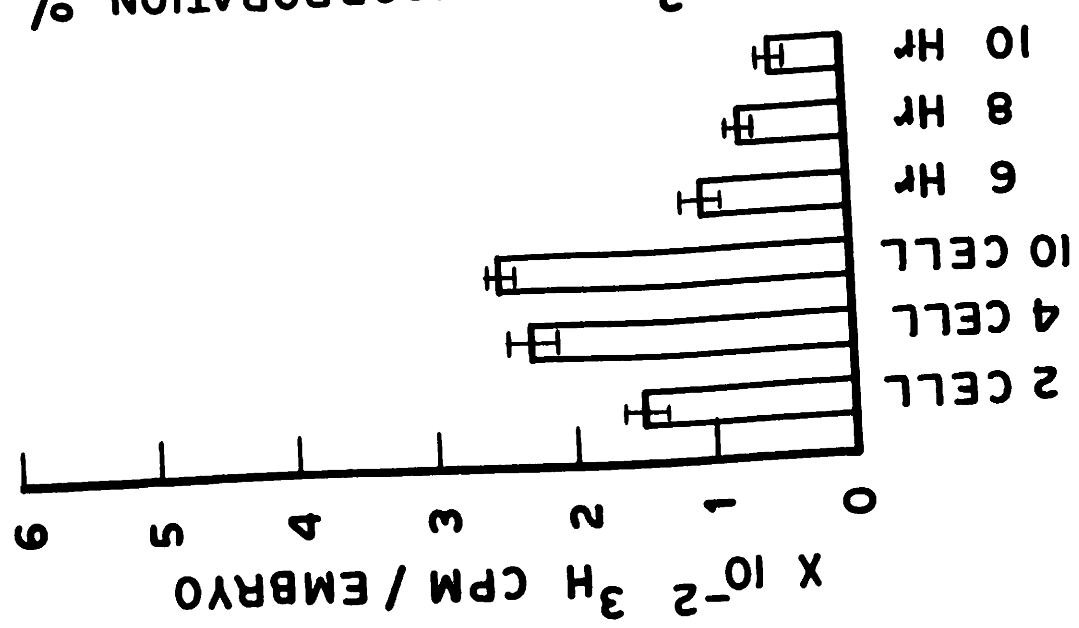


FIG. 5.

<u>FIG. 7</u> ^{3}H-Uridine incorporation into TCA insoluble and alkali labile material (RNA) of the embryos of *A. brightwelli*. Values expressed as mean ± S.D.

<u>FIG. 8</u> A schematic diagram of the pattern of ^{3}H-Uridine incorporation by *A brightwelli* embryos.

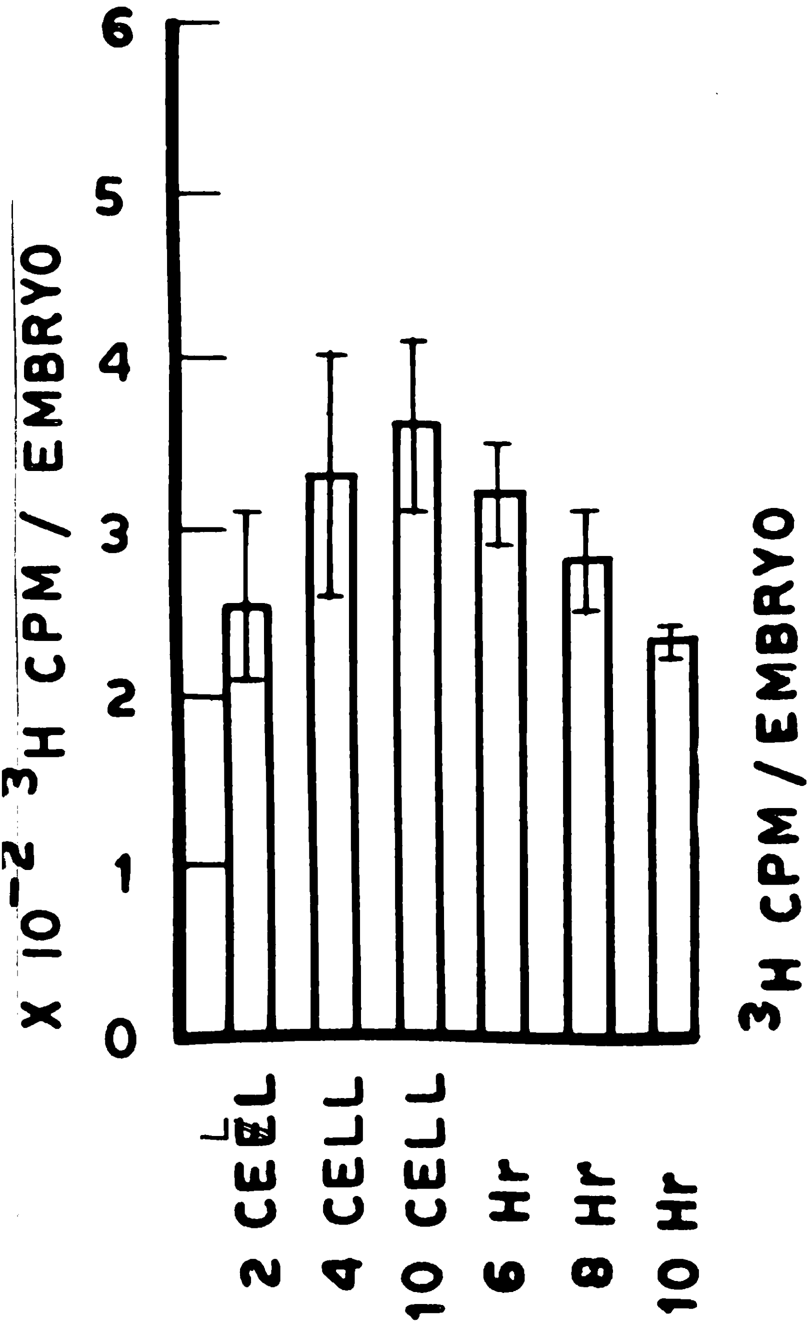


FIG. 7.

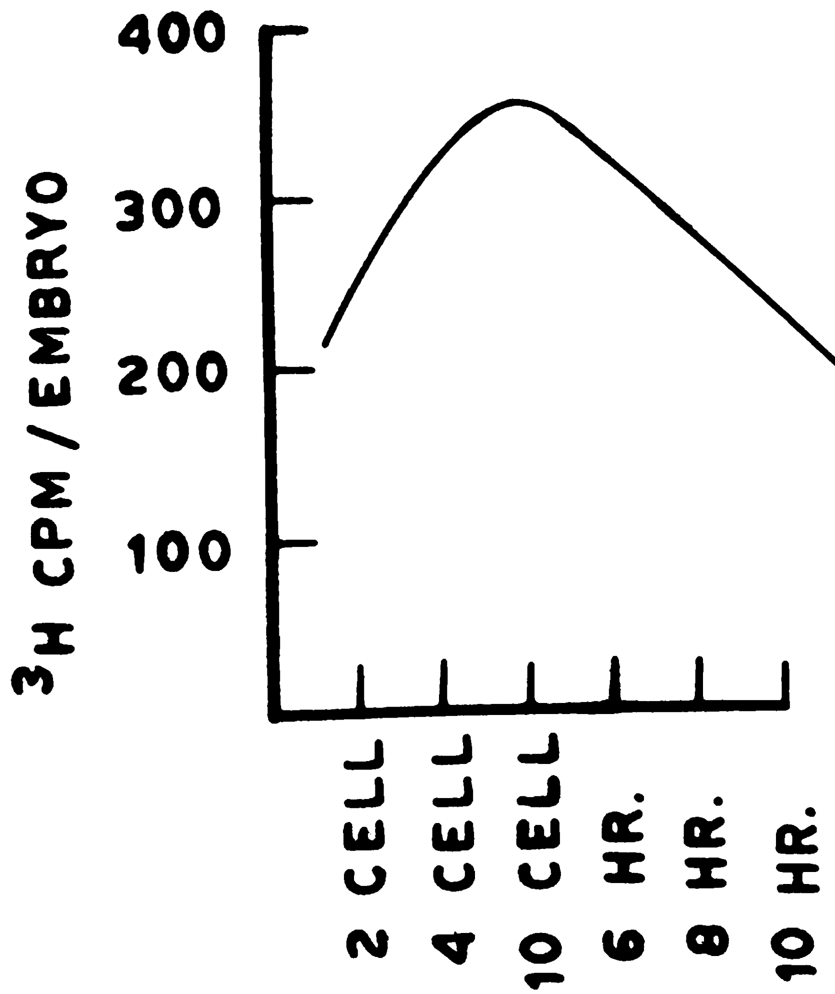


FIG. 8.

FIG. 9 SDS - Polyacrylamide gel electrophoresis of the ^{3}H-Uridine pulse labelled RNA from the 2-cell stage embryos of A. brightwelli.

O——O : Poly (A+) RNA (CPM loaded : 9000)

●——● : Poly (A-) RNA (CPM loaded : 7000)

FIG. 10 SDS - Polyacrylamide gel electrophoresis of ^{3}H-Uridine pulse labelled RNA from 4-cell stage embryos of *A. brightwelli*.

O——O : Poly (A+) RNA (CPM loaded : 12000)

●——● : Poly (A-) RNA (CPM loaded : 5500)

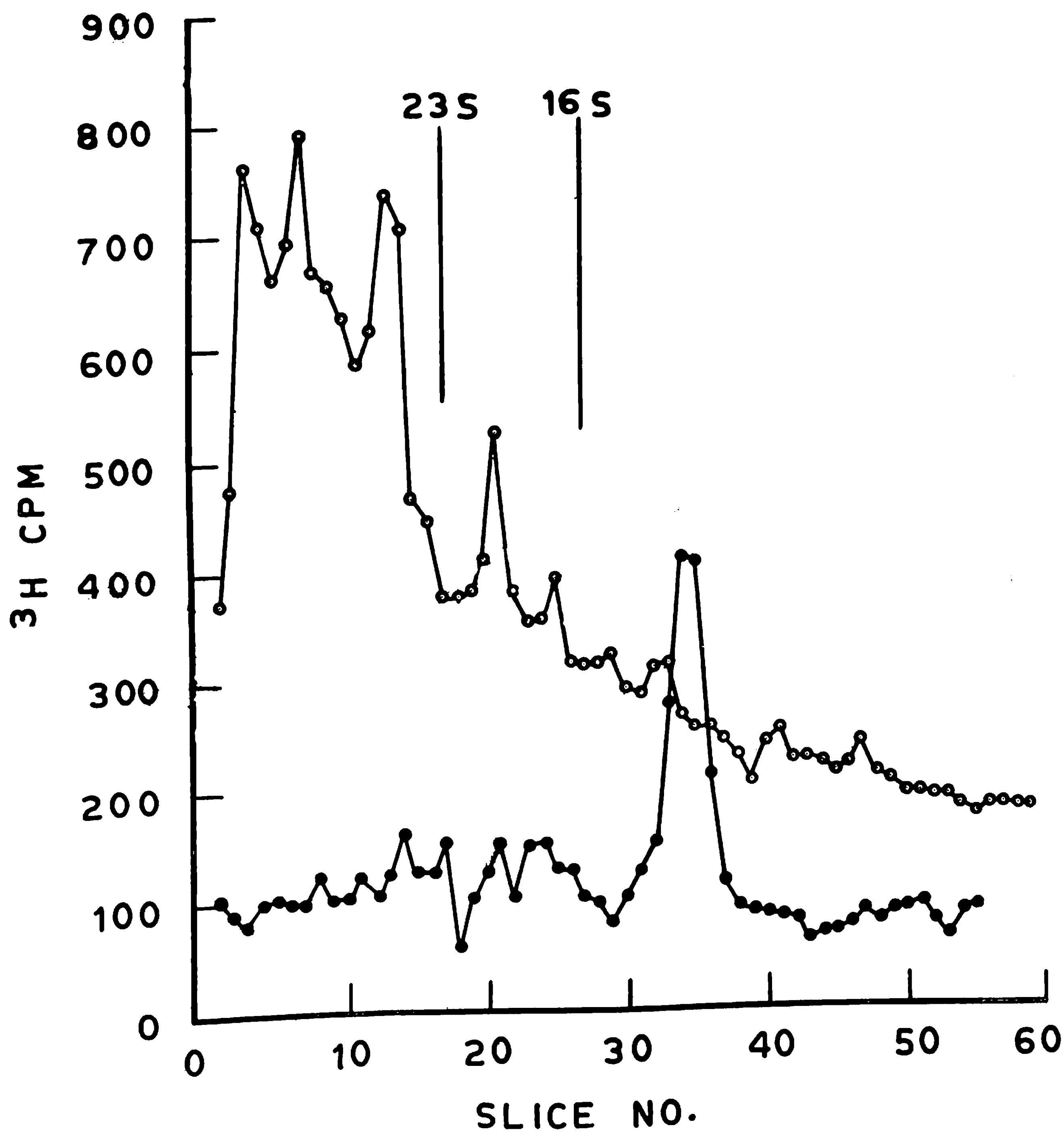


Fig. 10.

FIG. 11 SDS - Poly acrylamide gel electrophoresis of the pulse labelled RNA from the 10-cell stage embryos of *A. brightwelli*.

O——O : Poly (A+) RNA (CPM loaded: 8500).

●——● : Poly (A-) RNA (CPM loaded: 6000).

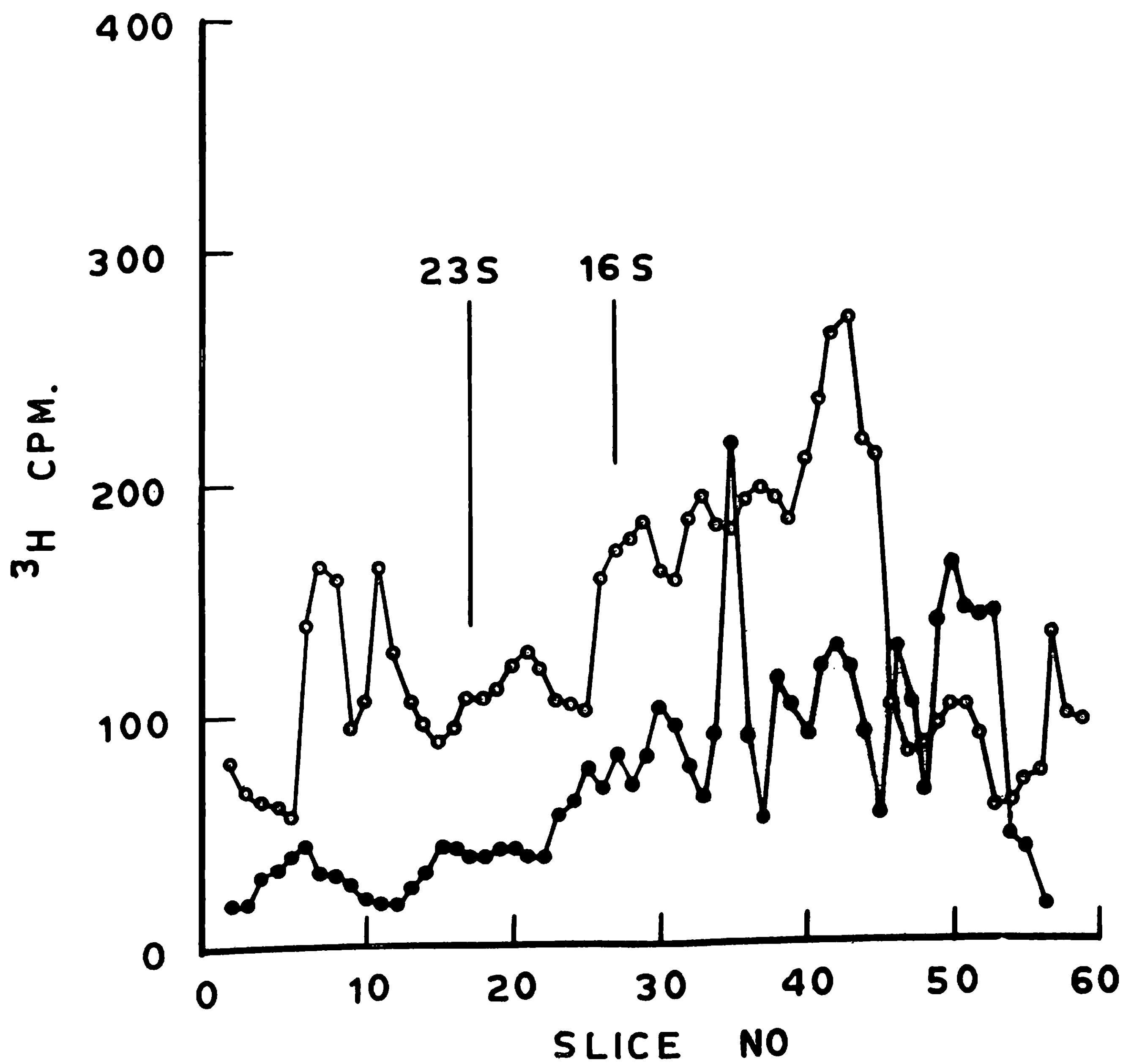


FIG. 11.

FIG. 12 SDS - Polyacrylamide gel electrophoresis of the ^{3}H-Uridine pulse labelled RNA from 6 hr old postmitotic stage embryos of *A. brightwelli*.

Poly (A+) RNA (CPM loaded: 8000).

Poly (A-) RNA (CPM loaded: 7000).

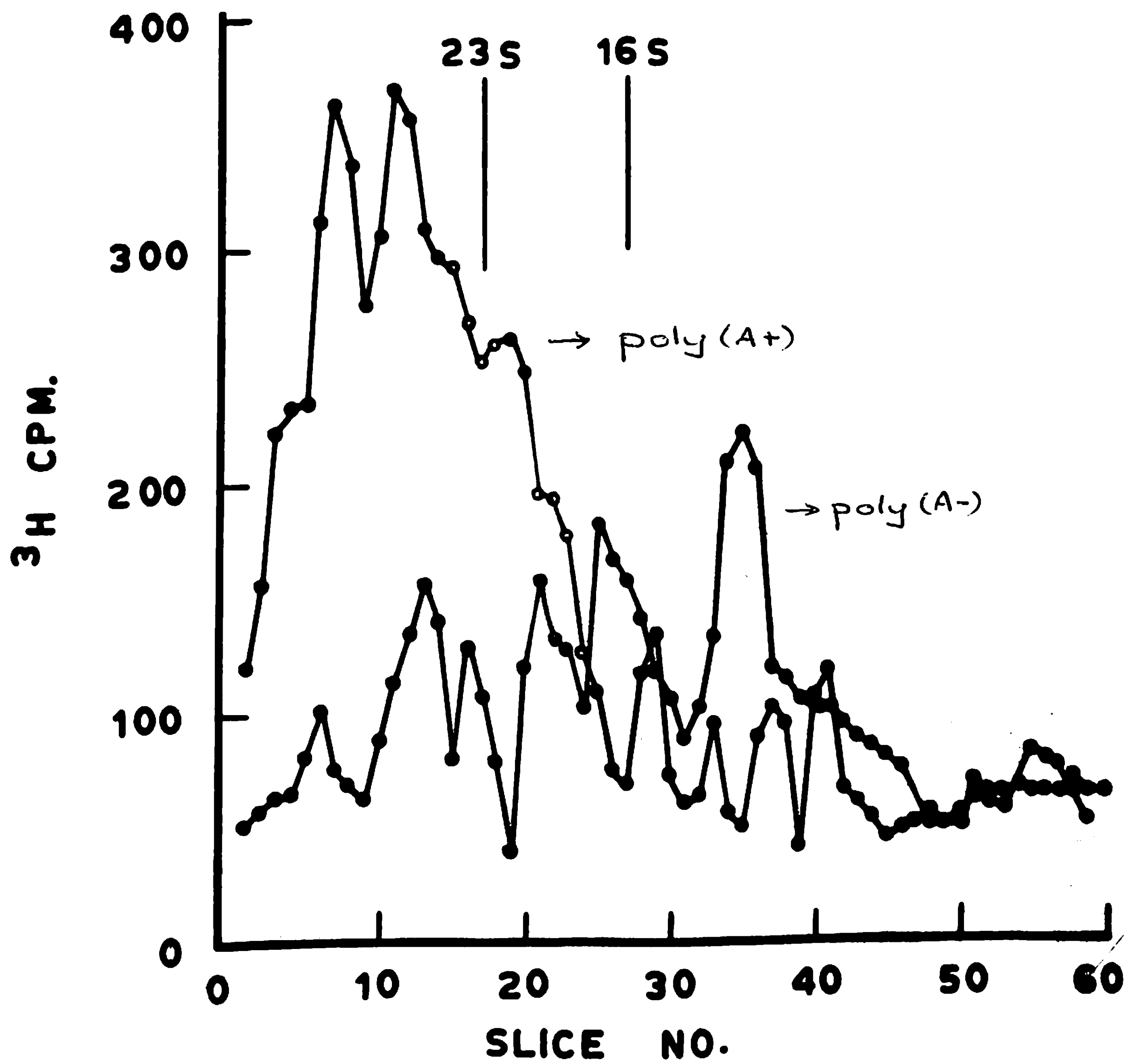


FIG.12.

FIG. 13 SDS - Polyacrylamide gel electrophoresis of the ^{3}H-Uridine pulse labelled RNA from 8 hr postmitotic stage embryos of *A. brightwelli*.

O——O : Poly (A+) RNA (CPM loaded: 8200)

●——● : Poly (A-) RNA (CPM loaded: 6000)

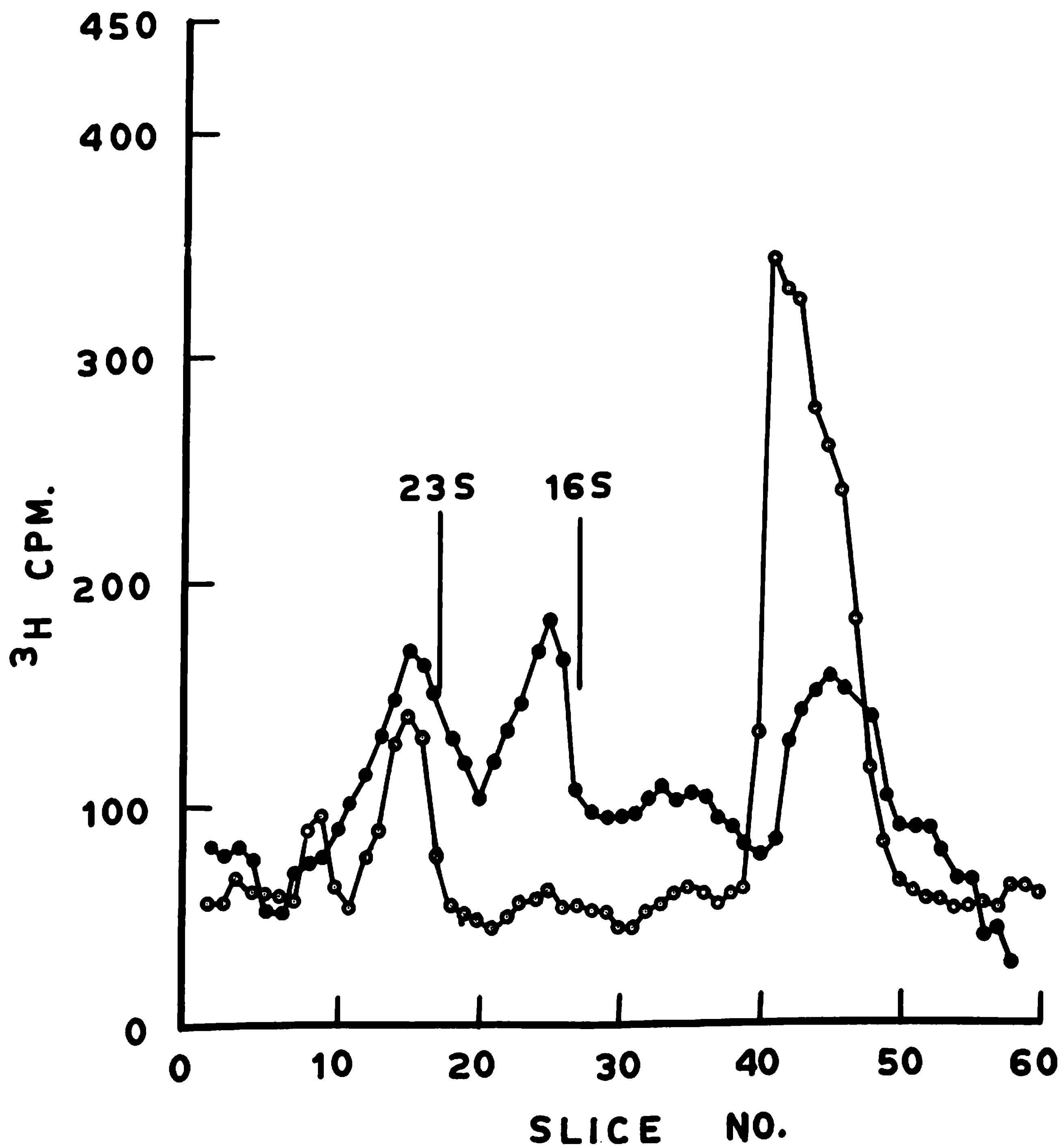


FIG. 13.

FIG. 14 SDS - Polyacrylamide gel electrophoresis of ^{3}H-Uridine pulse labelled RNA from 10 hr postmitotic stage embryos of *A. brightwelli*.

●——● Poly (A+) RNA (CPM loaded: 20,000)

O——O Poly (A-) RNA (CPM loaded: 16,000)

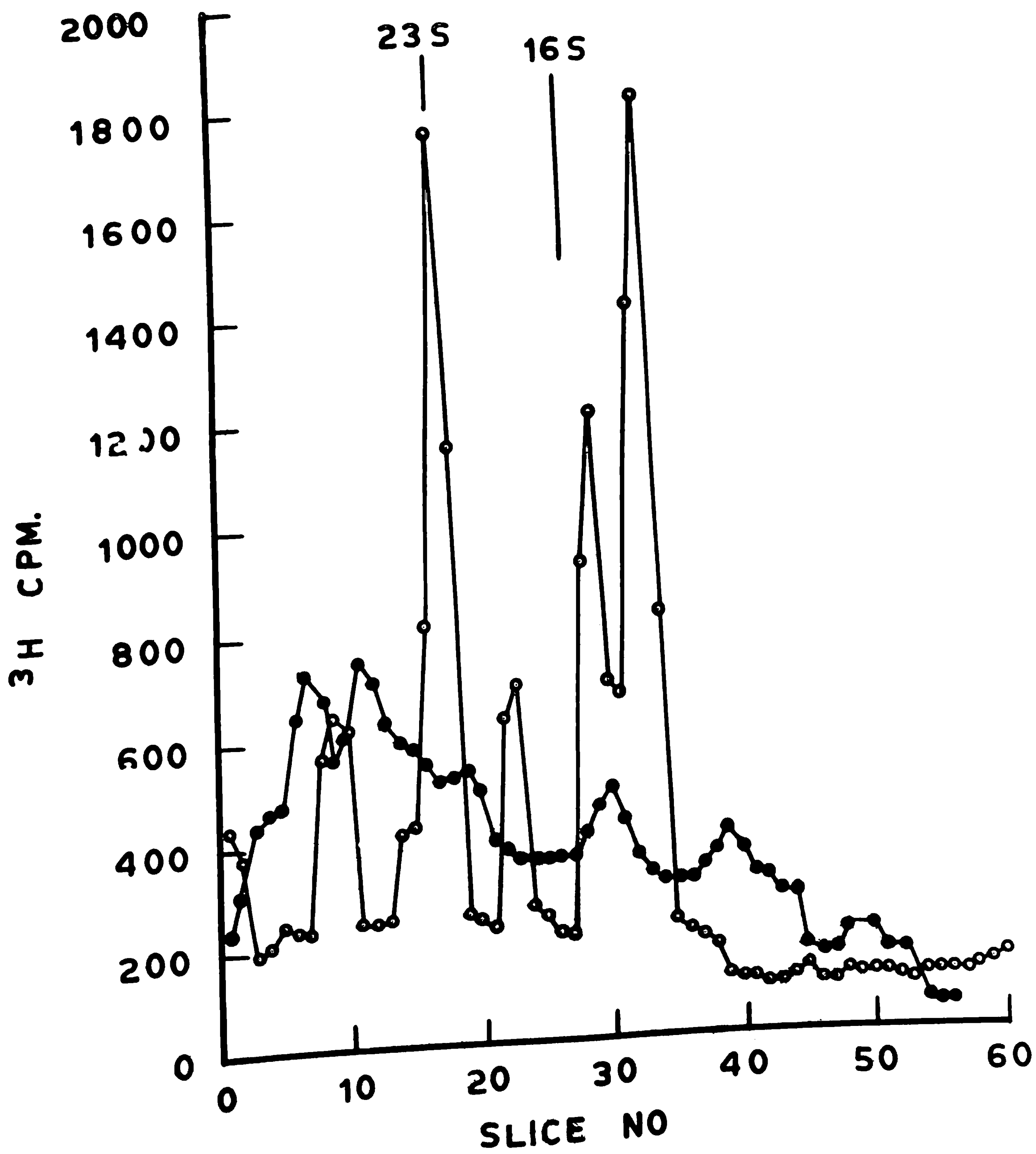


FIG. 14.

FIG. 15 Poly (A+) RNA content of the ^{3}H-Uridine pulse labelled RNA from the embryos of *A. brightwelli*.

Values expressed as mean ± S.D.

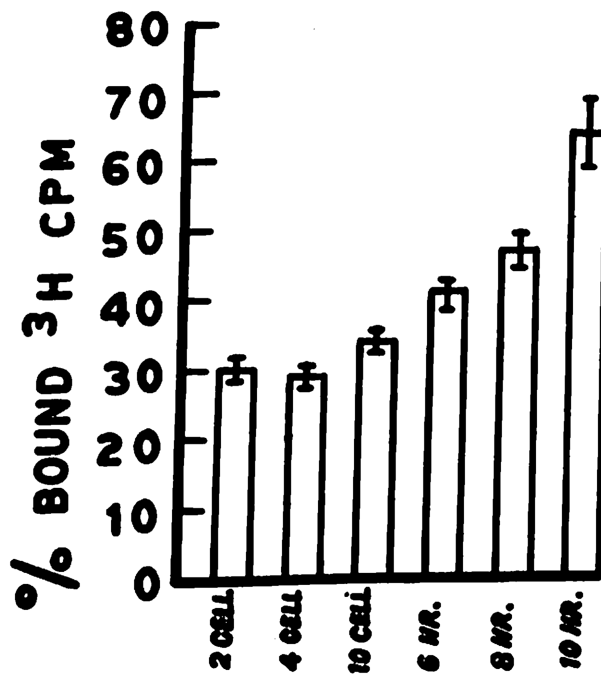


FIG. 15.

II. D. Discussion

Studies on RNA and Protein synthesis serve as useful molecular indices in understanding the pattern of genome function during development and differentiation. The results obtained here present some aspects of RNA and Protein synthesis in the embryonic stages of *Asplanchna brightwelli*.

Protein Synthesis : The results obtained from the experiments on the parthenogenic embryos of *A. brightwelli* indicate thatprotein synthesis is continuous during development, arriving at its maximum in the 10 cell stage. The results of the experiments employing actinomycin D further show that most of the (60-80%) early protein synthesis is insensitive to the blockade of RNA synthesis. On the other hand the post mitotic embryonic protein synthesis is inhibited by the administration of actinomycin D. The results of the experiments involving actinomycin D are not easy to interpret, since it is known that the dosage employed is crucial. At higher doses actinomycin D has been known to have non-specific effects (Collier, 1966). In the present work low doses of actinomycin D (1 µg/ml) have been used, which inhibited 80-90% of the RNA synthesis. The drug shows a non-specific effect on the cleavage and development of *A. brightwelli* (Table 2). However, during the early developmental stages most of the protein synthesis goes on in the presence of actinomycin D. This actinomycin D

resistant protein synthesis goes on probably by the utilization of maternal mRNA templates accumulated during oogenesis. On the other hand the protein synthesis during the post-mitotic stages is evidently linked with immediate RNA synthesis. This particularly is so since most of the protein synthesis during these stages is inhibited in the presence of actinomycin D.

The presence of stable, conserved maternal mRNA and its utilization during early embryogenesis is well known (Gross 1967; Spirin, 1966; Nemer, 1967; Tyler, 1968). Spirin (1966) and Nemer (1967) are of the view that most of the early embryonic protein synthesis is programmed by 'old mRNA' or maternal templates. Birky and Gilbert (1971) and Gopinath (1972) made similar observations from their auto radiogrphic embryological studied of Asplanchna. The present work which is the first attempt at the level of molecular embryology of rotifers provides evidence in favour of use of maternal templates during embryo development.

In A. brightwelli evidently early embryonic protein synthesis takes place on maternal templates to a considerable extent. This presumably accounts for the extremely rapid rate of embryonic development of the rotifer and also the large scale transfer of cytoplasmic compliments during oogenesis.

Thus while the early protein synthesis may be independent of the immediate RNA synthesis, the developmental events namely the eleavage need not necessarily be independent of the RNA synthesis. At least the present observation is not indicative of such independence, especially when actinomycin D shows a very nonspecific action on cleavage.

RNA Synthesis : The problem of RNA synthesis during early embryogeny and its possible role in development has aroused much interest in the recent times. This has resulted in extensive studies in many organisms especially in the echinoderms and amphibians. Brown and Littna (1966, 66a) have studied the RNA synthesis in the cleavage stage xenopus embryos. By carrying out base composition analysis and sucrose gradient centrifugation, they have shown that the pulse labelled RNA is heterogenous in size and has a 'DNA like' (A+U rich) base composition i.e. the RNA synthesised in the cleavage stages is informational in nature. Similar studies have been carried out in the sea Urchin by various workers (Nemer, 1963; Wilt, 1963; Nemer & Infante, 1965; Gross et al. 1965; Glisin et al. 1966; Whitely et al 1966; Kedes and Gross, 1969; Kuing 1974) and they have shown that the synthesis of the informational RNA begins very early during development. Studies with the organisms of other phylogenetic groups have also yielded similar results (Nemer, 1967; Cather, 1971; Brahmachary, 1973). In most of

these studies the conclusion as to the <u>informational</u> nature of the RNA is based on the finding that the pulse labelled RNA from the early stage embryos is heterogenous in size and is DNA like in base composition.

The synthesis of ribosomal RNA (rRNA) has been reported to begin late during gastrulation in echinoderms, amphibians, echiurans (Brown, 1967; Guidice & Mutolo, 1967; Gould, 1969, 69a) where as in certain protostomes rRNA synthesis begins early during cleavage. In these studies the conclusion as to the ribosomal nature of the RNA is based on the criteria that the appearance of nucleoli is indicative of rRNA synthesis and the rRNA has a G+C rich base composition.

<u>RNA Synthesis in A. brightwelli</u> : The studies on the RNA synthesis during the embryonic stages of the rotifer <u>A. brightwelli</u> have been presented here. The results indicate that the RNA synthesis begins as early as 2 cell stage during development. The results of the SDS-Poly acrylamide gel electrophoresis experiments show that the RNA synthesised is heterogenous in size and further Poly-U Sepharose chromatography shows that Poly (A+) RNA is detected as early as the 2-cell stage. The presence of Poly (A) is characteristic of messenger RNA and heterogenous nuclear RNA (Hn RNA), (Brawerman, 1974; Greenberg, 1975), whereas ribosomal RNA lacks Poly (A). The results indicate that the mitotic stages predominantly synthesise informational RNA (mRNA &

Hn RNA), on the other hand the synthesis of rRNA begins during the post-mitotic stages. This is indicated by the appearance of RNA peaks in the ribosomal region on SDS polyacrylamide gels and the appearance of nucleoli.

Thus in A. brightwelli it is presumable that the synthesis of informational RNA begins as early as 2-cell stage and the rRNA synthesis begins during the post-mitotic stages. In the present experiments the fraction of Poly(A+) RNA shows an increase as development proceeds. The post mitotic stages show large amounts of (40-50%) Poly(A+) RNA. Nemer et al (1974) showed a 40-50% Poly(A) RNA in ^{3}H-Uridine labelled sea urchin blastula stage embryos. This is by labelling the embryos for 60 minutes and fractionating the polysomal RNA on oligo-dt cellulose columns and poly-U glass fibre filters.

In the present work the total RNA has been fractionated after a 10 minute pulse labelling. In the data the large amounts of Poly(A+) RNA with short labelling times is probably due to contribution by the HnRNA. Increase in Poly (A) content and of the Poly(A+) RNA fraction upon development has been known in other systems (Slater and Slater, 1974; Slater et al. 1972; 73; 74; Wilt, 1973; Dubroff & Nemer, 1975). Poly (A) has been associated with development and differentiation. However, the exact function of Poly (A) and its role in messenger function is not yet clear (Huez et al. 1974; Sippel et al 1974; Gorski et al 1975; Levy et al 1975; Marbaix et al 1975).

CHAPTER III

The Nature of RNA in the resting eggs of *A. brightwelli*.

III.A. Introduction

The rotifer A. brightwelli enters a phase of dormancy by the formation of thick walled resting eggs. These are formed during the sexual (mictic) phase of the life cycle of the organism. Mictic females produce haploid (n) eggs which develop parthenogenetically into males. Fertilized mictic eggs give rise to resting eggs which characteristically synthesize a thick outer shell and in which further development is arrested. These encysted embryos can withstand unfavourable environmental conditions. After varying periods of dormancy the resting eggs hatch out into amictic females, under favourable conditions. There are no cytochemical or biochemical studies on the resting eggs of rotifers (Gilbert, 1974).

As a part of the studies on the RNA and Protein synthesis during the embryogenesis of Asplanchna it has been explored whether the resting egg has a store of conserved messengers. In the present study attempt has been made to investigate (i) RNA and Protein synthesis in the resting eggs, and (ii) The nature of the RNA in the resting eggs.

III. B. Materials and Methods

Materials: ^{3}H-Lysine (Sp. Act. 250 mCi/m.mole) ^{3}H-Uridine (5.3 Ci/m.mole), ^{32}P-orthophosphate (Carrier free) were

obtained from BARC, Trombay, India. dl-α tocopherol (vitamin E) was a gift from E. Merck, Germany. All the other chemicals and reagents were obtained as described earlier.

Test Organism : Cultures of *A. brightwelli* were maintained in the laboratory as per the methods detailed earlier (page 23).

Mixis Induction: Mixis can be induced by administering Vitamin E (α - tocopherol) into amictic cultures of *A. brightwelli*. The amictic → mictic transformation is indicated by the appearance of males in the culture. In the present study mixis was induced by directly introducing α-tocopherol into mass cultures of amictic *A. brightwelli* A drop of standard α-tocopherol (0.1 ml) was introduced into each culture flask and allowed to float on the surface of the culture media. Within a week males appeared in the culture indicating amictic → mictic transformation.

Collection of Resting Eggs : The resting eggs after they are laid by the mictic females settle to the bottom of the culture flask. Since the eggs could not be directly collected from culture flasks, the cultures were transferred into petri plates. The eggs were sucked into a glass micropipette (fitted with rubber droppers) under a Olympus binocular microscope. The eggs were collected daily, pooled and stored in a refrigerator at 4°C.

Protein and RNA Synthesis : For the protein and RNA synthesis, the uptake of ^{3}H-Lysine and ^{3}H-Uridine into the eggs was studied by incubating batches of eggs with the appropriate precursor. 10-20 eggs were incubated with 10 µCi/ml of either ^{3}H-Lysine or ^{3}H-Uridine in 0.1 ml of the culture media. After the eggs were thus put into incubation samples were drawn at different time intervals. After the incubation the eggs were repeatedly washed with a 0.1% solution of the unlabelled precursor. The eggs were further washed with cold 5% TCA and finally with ether. The washed eggs were transferred on to a Whatman No. 1 filter paper disc and the radioactivity was counted as described earlier (page 27).

Radioactive Labelling of Resting egg RNA : To prepare radioactivity labelled resting eggs, mictic cultures were continuously labelled with the appropriate precursor. The mictic cultures were daily fed with either ^{32}P-orthophosphate or ^{3}H-Uridine labelled paramecia. *Paramecium aurelia* cultures were labelled with 4-5 µCi/ml of ^{32}P-orthophosphate. ^{3}H-Uridine labelling of paramecia was done at a concentration of 8-10 µCi/ml. ^{3}H-Uridine was also directly introduced into *A. brightwelli* culture media. The eggs were collected as described above and used for the extraction of RNA.

<u>Extraction of ^{32}P-orthophosphate Labelled RNA from Resting Eggs</u> : Brawerman (1974) has described a procedure for the differential extraction of RNA with and without poly (A). This procedure was used for the extraction of the ^{32}P labelled RNA from the resting eggs.

500-1000 eggs were homogenised in a teflon pestle glass homogeniser in 1 ml of buffer (Tris-HCl 0.1M, pH 7.6; EDTA 0.005M; SDS 1%; Bentonite 1%). The total homogenate was extracted with equal volume of phenol. The aqueous and the organic phases were separated by centrifugation in a Sorvall RC-2B centrifuge (5000x g for 5 minutes). The upper aqueous layer was removed with a pasteur pipette and designated as the pH 7.6 fraction or Poly (A-) RNA fraction. The organic phenol phase was re-extracted with tris buffer pH 9.0 (0.1M Tris-HCl; 0.5% SDS). The aqueous phase was collected as before and marked as the pH 9.0 fraction or (Poly A+) RNA fraction. The RNA from the two aqueous phases was precipitated with 3 volumes of ethanol (-20°C) and kept overnight. On the following day the precipitate was pelleted by centrifugation in a Sorvall RC-2B centrifuge (5000xg for 10 minutes). The RNA was purified and processed as described earlier (page 29).

The pH 9.0 RNA fraction was hybridized to Poly-U sepharose coloumn and the bound RNA was eluted and analysed for base composition. The pH 7.6 RNA (Poly A-) was

fractionated on 2.6% SDS-polyacrylamide gels. The RNA from the 9-11S peak was eluted and analysed for base composition.

<u>^{3}H-Uridine Labelled RNA Extraction</u> : The RNA was extracted from 500-600 eggs as described under Chapter II (page 29). The RNA was subjected to Poly-U sepharose chromatography and separated into Poly (A+) and Poly (A-) RNA fractions. Both the fractions were subjected to SDS-polyacrylamide gel electrophoresis.

<u>Poly-U Sepharose Chromatography</u> : Conditions were the same as described under Chapter II (page 30) and the procedure employed was that of Adesnik et al. (1972).

<u>SDS-Polyacrylamide Gel Electrophoresis</u>: Gel electrophoresis of the different RNA fractions was carried out on 2.6% gels. A bis cross-linked gel system of Loening (1969) was used. The method in detail has been described (page 31).

<u>Base Composition Analysis of RNA</u>

<u>Alkali hydrolysis</u> : The ^{32}P-labelled RNA from the 9-11S peak of the Poly (A-) fraction and the RNA eluted from the Poly-U column was hydrolysed with 0.3N KOH for 16 hours at 37°C. The alkali was neutralized with perchloric acid. The neutralised hydrolysates were subjected to paper electrophoresis to separate the nucleotides.

Electrophoresis of RNA hydrolysates : The RNA hydrolysates were analysed by the paper electrophoresis method of Smith (1967). The samples (0.1 ml. of known radioactivity) were loaded on small Whatman No. 3 filter paper discs (punched out with a paper punch). The discs were intermittently dried with an air blower during the loading of the sample. A mixture of unlabelled nucleotides - AMP, CMP, GMP and UMP (Sigma Co., 10 µgm/ml) was included as internal standards. The air dried discs loaded with the sample, were spotted on a Whatman No. 3 filter paper (40 x 10 cm). The filter paper was previously equilibrated with the electrophoresis buffer. The paper electrophoresis was carried out in ammonium formate buffer (0.05 M,pH 3.5) at 900V (20-30 mAmps current) for 4 hrs. After the electrophoresis the paper was air dried and cut into 1 cm strips and counted in 5 ml of 0.1%. Toluene based PPO. The counting was done in a Beckman LS 100 liquid scintillation counter. The position of the different nucleotides was identified by the fluorescence under U.V. light.

III.C. Results

RNA from the Resting Eggs: The experiments with the ^{3}H-Uridine labelled RNA show about 30% binding to Poly-U sepharose columns, thus indicating a 30% Poly (A+) RNA of the total RNA extracted (vide table No. 5).

TABLE 3

^{3}H-Lysine uptake by the resting eggs of *A. brightwelli*

Time (hours)	Background (CPM)	Uptake (CPM/10 eggs)
4	18	24
8	20	24
12	20	30

Resting eggs were isolated and incubated with ^{3}H-Lysine. The uptake of the precursor was determined as described under methods III. B.

TABLE 4

^{3}H-Uridine uptake by resting eggs of *A. brightwelli*

Time (hours)	Background (CPM)	Uptake (CPM/10 eggs)
4	24	32
8	24	30
12	22	35

Restings were incubated with ^{3}H-Uridine and the uptake of the precursor was determined as described under methods (III. B).

TABLE 5

Poly (A+) RNA content of the resting eggs of *A. brightwelli*

Expt. No.	^{3}H-Uridine labelled RNA loaded (CPM)	Bound (CPM)	Unbound (CPM)	Poly (A) RNA (%)
1	13200	4100	9500	31
2	8000	3200	4500	40
3	15400	8000	10200	32.4

Resting eggs were continuously labelled with ^{3}H-Uridine and the total RNA was extracted. The RNA was purified and subjected to poly-U sepharose chromatography as described under methods (III.B)

TABLE 6

Base composition of the RNA fraction from the resting eggs of *A.brightwelli*

Fraction	A (CPM)	U (CPM)	G (CPM)	C (CPM)	G+C/A+U
9-11S Poly(A-) RNA	240	239	235	130	0.97
Poly(A+) RNA	244	68	45	140	0.57

The ^{32}P-labelled RNA fraction from the resting eggs were analysed for base composition, as described under methods (III.B).

<u>FIG. 16</u> SDS-Polyacrylamide gel electrophoresis of the ^{3}H-Uridine labelled Poly (A+) RNA from the resting eggs of *A. brightwelli*.
(CPM loaded : 3000).

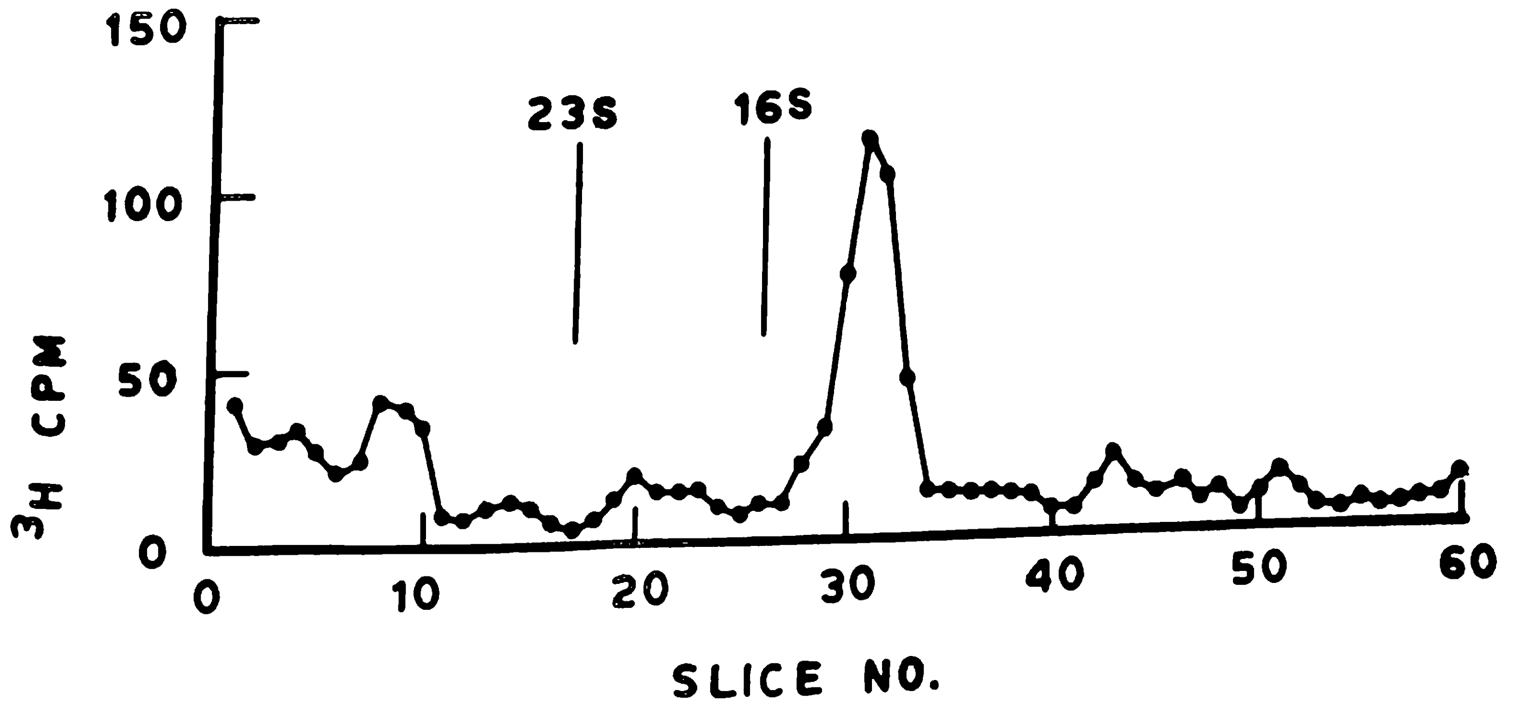


FIG. 16.

FIG. 17 SDS - Polyacrylamide gel electrophoresis of the ^{3}H-Uridine labelled Poly (A-) RNA from the resting eggs of *A. brightwelli*

(CPM loaded : 5000).

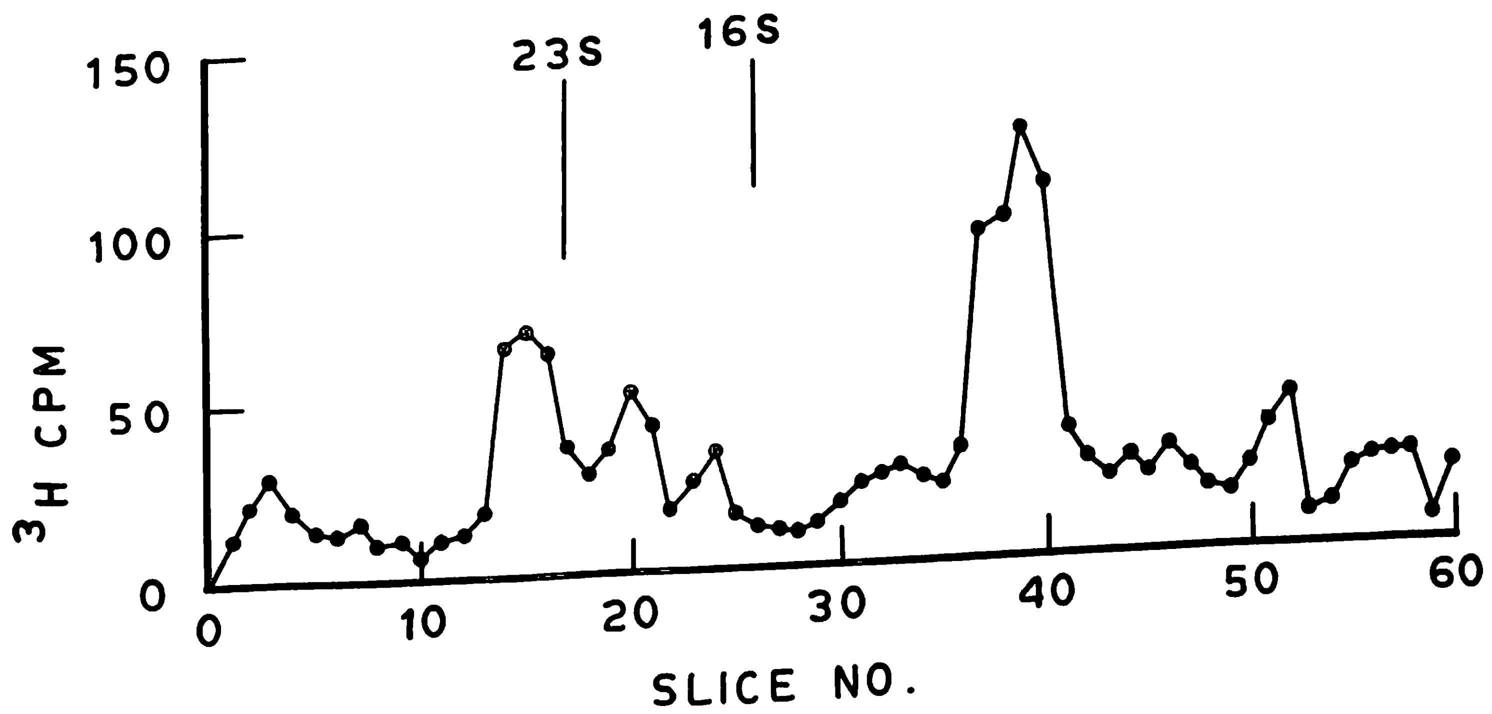


FIG. 17.

FIG. 18 SDS - Polyacrylamide gel electrophoresis of the ^{32}P-labelled Poly (A-) RNA from the resting eggs of *A. brightwelli*.
(CPM loaded : 5000).

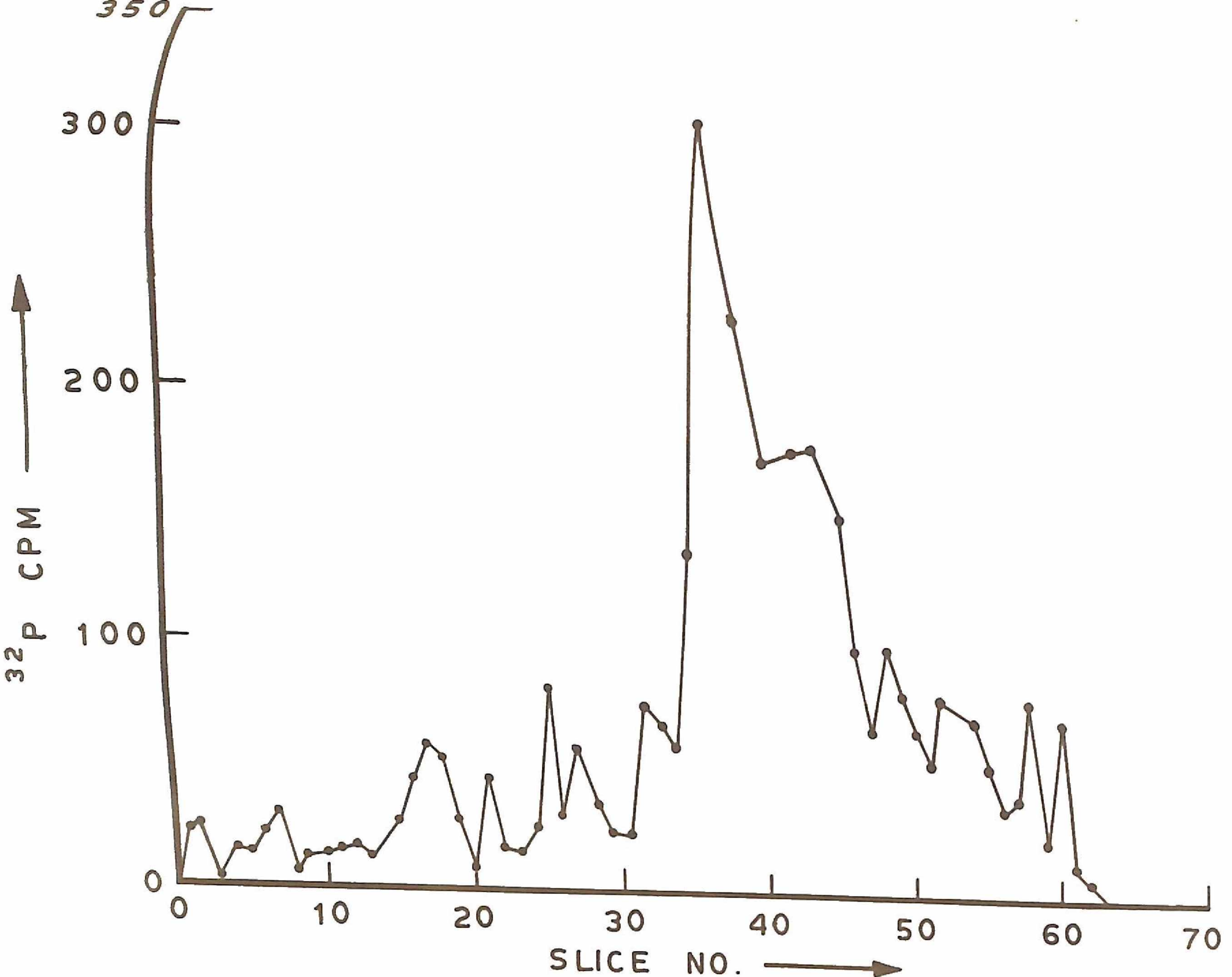


Fig. 18.

FIG. 19 Base composition analysis of RNA. Paper electrophoresis of Poly (A+) RNA hydrolysates. (CPM loaded : 2000).

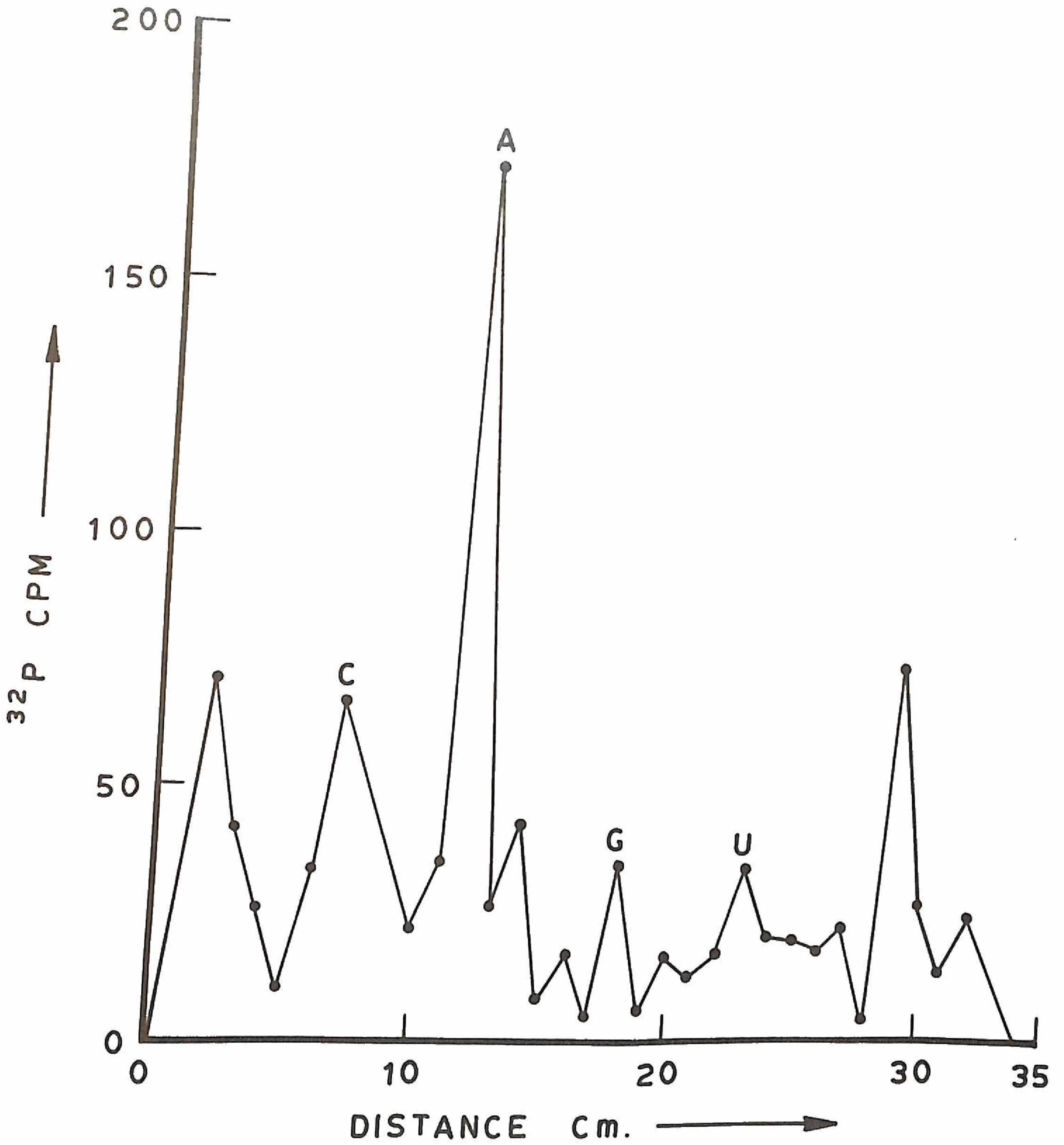


FIG. 19.

FIG. 20 Base composition analysis of Poly (A-) RNA. The 9-11 S RNA peak from the polyacrylamide gel was elected out and subjected to Base analysis. (CPM loaded : 3000).

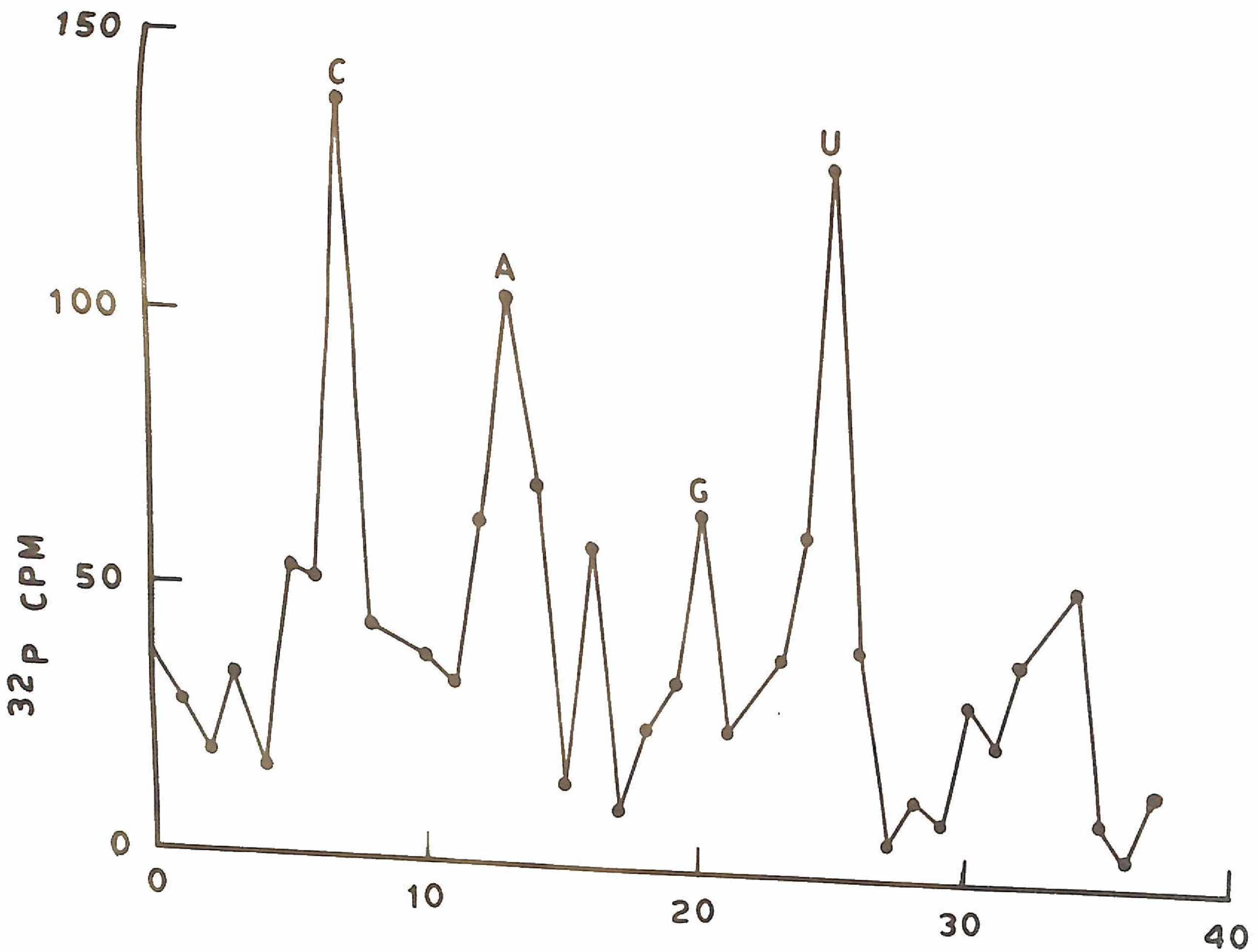


Fig. 20.

The results of SDS-polyacrylamide gel electrophoresis of the various RNA fractions are given in figs. 16 - 18. The poly (A+) RNA fraction eluted from the Poly-U column shows a major peak in the region of 12-15S (Fig 16). The Poly (A-) fraction shows a prominent peak in the 9-11S region and minor peaks in the heavier regions of the gel (16 and 23S) (Figs 17 and 18).

The results of the base composition analysis of the ^{32}P RNA have been presented in Table 6 and Figs 19 & 20. The 12-15S peak of Poly (A+) RNA has a G+C/A+U ratio of 0.57. Similarly the 9-11S peak of the Poly (A-) RNA fraction has a G+C/A+U ratio of 0.97. Both the RNA fractions have an A+U rich base composition i.e. they are DNA like or mRNA like in base composition.

RNA and Protein Synthesis : Tables III and IV show the results of the experiments on the uptake of labelled precursors by the resting eggs. The resting eggs do not show any appreciable intake of the precursors and as such no RNA or protein synthesis could be detected.

III. D. Discussion

The resting egg of *A. brightwelli* offers a very interesting system of study, since it comprises of a group of embryonic cells in a dormant state. These are in a way analogous to the dormant cysts of the crustacean

Artemia salina, which are gastrula stage embryos in an encysted condition. *Artemia salina* embryology has been studied extensively in the recent years (Urbani, 1962; Brahmachary, 1973; Zasloff & Ochoa, 1973; Grosteld and Littauer, 1975). On the other hand there are no cytochemical and biochemical studies reported on the resting eggs of any rotifer.

RNA and Protein Synthesis in Resting Eggs : In the present work experiments on the intake of labelled precursors by the resting eggs show that the resting eggs are not permeable to the external precursors. Consequently no RNA and protein synthesis could be detected by incubating the eggs with either ^{3}H-Uridine or ^{3}H-Lysine. The embryo in the eggs being in a dormant condition may be carrying out very little or no synthesis of macromolecules. Whatever synthesis is being carried out must be by the use of internal precursor pools. In the present experiments no appreciable uptake upto 12 hrs of incubation could be seen. Since it is not known exactly when the development resumes inside the egg, it is possible that longer periods of incubation is needed. The embryo in all probability has a precursor pool of the size, large enough so as to prevent any uptake from outside.

Nature of the RNA Stored : The total RNA from the ^{32}P-orthophosphate and ^{3}H-Uridine labelled resting eggs was

extracted and fractionated into various classes as described in the preceeding sections (III B). Two major fractions which are mRNA like in base composition and molecular wt. were obtained. Further, poly-U sepharose chromatography shows that one of the fractions is polyadenylated.

The Poly (A+) RNA eluted from the Poly-U sepharose columns when subjected to SDS-polyacrylamide gel electrophoresis migrates to the region of 12-15S. This indicates an approximate size of 4-5S to the Poly (A) stretches. The Poly (A+) RNA further shows a typical mRNA like base composition and has a large proportion of Adenine, probably due to the presence of Poly (A). The Poly (A-) RNA fraction which did not bind to the Poly-U column was similarly analysed. Both the ^{32}P and ^{3}H-Uridine labelled Poly (A-) RNA show a major peak in the region of 9-11S on SDS-polyacrylamide gels. This peak upon base analysis was seen to be mRNA like in that it has a G+C/A+U ratio less than one. The Poly (A-) RNA shows minor peaks in the heavier region (16 and 23S) which is presumably ribosomal RNA.

Thus the resting eggs seem to contain two mRNA fractions, one of which is polyadenylated and has a mol. wt. in the region of 12-15S. The second fraction is without Poly (A) with a mol. wt. of 9-11S.

Significance of mRNA in the Resting Eggs : The presence of stable conserved mRNA is well known in the case of plant seeds (Dure & Watters, 1965; Bhat & Padayatty, 1974; Payne 1976) and eggs of many organisms (Spirin, 1966; Gross, 1967; Nemer, 1967; Brahmachary, 1968; Tyler, 1968). It is also known that germination and fertilization involves active utilization of these stored templates. In the case of seeds it has been shown that imbibition of water during germination leads to increase in polysome content and accompanied translation of the conserved mRNA (Marcus & Feely, 1964; Barker and Rieber, 1967; Marre, 1967; Payne, 1976). Similarly in the case of eggs of various other organisms it has been shown that fertilization stimulates protein synthesis and this is programmed by the unmasking of the mRNA in the egg (Davidson et al. 1965; Nemer, 1967; Davidson, 1968; Tyler, 1968; Brahmachary, 1968; Gross & Gross, 1973).

In the case of Asplanchna resting eggs the conditions for the hatching are not well known. However, it is known that fully developed young ones emerge out upon hatching. In the present laboratory conditions (temperature 25 ±3°C) it took anywhere between 1-7 days for the young animal to emerge out from the resting eggs. It is difficult to say when exactly the development resumes after transfer into fresh culture media. Presumably the resumption of development is at least partly

dependent on the utilization of stored rsources. The experiments here show that the eggs are impermeable to external precursor molecules, which may imply that the egg has a pool of the necessary precursor molecules or a high rate of turnover of molecules within the system. It would, however, be very interesting to study the free precursor pool in the resting egg. Further, the present experimental results show that the resting eggs contain messenger like RNA. The stored mRNA must be of importance in the embryogenic processes, especially in the resumption of development from the encysted condition. Thus the developmental events which take place within the egg consist of the utilization of these stored messenger templates.

The presence of both poly (A+) and Poly (A-) mRNA in resting eggs is rather interesting. Nemer et al (1974) has shown that in the sea urchin eggs and embryos Poly (A+) and Poly (A-) mRNA coexist. He has further shown that the Poly (A-) mRNA consisted of both histone and non-histone mRNA fractions. In the present preparations the Poly (A-) mRNA has a mol. wt. in the range of 9-11S. But it cannot be said whether it is histone mRNA or non-histone mRNA. The histone mRNA in all the systems so far studied has been shown to lack the Poly (A) segment at the 3'OH end. The Poly (A) segment at the 3'OH end of the messenger has been widely associated with stability, storage and

transport of mRNA in the cells. However, the exact function of these Poly (A) sequences is not yet clear.

To arrive at more definite conclusions on the developmental significance of the stored messengers in the Asplanchna resting eggs, it should be important to carry out cell-free translation of the stored mRNA as has been performed in certain other systems (Gross et.al. 1973; Gross et.al. 1973a; Verma et.al 1974). The analysis of the nature of the protein products would lend further support. However, some of the experiments on A. brightwelli (not presented here) show that the cell free system of the resting eggs supports amino acid incorporation into protein. When a S23 supernatant (23000 x g) fraction of the resting eggs (Brawerman, 1974) was incubated with ATP generating system and ^{14}C amino acids, the amino acids were incorporated into TCA precipitable material. The results have not been presented in view of the fact that the protein product has not been characterised.

CHAPTER IV

Vitellogenic Transfer of Proteins in *A. brightwelli*

IV.A. Introduction

The mature oocyte is a product of a long course of cell differentiation - the process of oogenesis. Apart from the genetic material, the growing oocyte inherits a large stock pile of cytoplasmic components from the mother. Such a transfer of cellular components from the mother to the growing embryo has been termed as "Heterosynthetic transfer" (Glass, 1971). The accessory cells of the ovarian tissues like the nurse cells and follicle cells have been known to be significantly important in such heterosynthetic transfer. A large amount histological and histochemical data on the accumulation of protein, carbohydrate and lipid reserves by the oocytes has been reviewed by Raven (1961). This transfer of material from the maternal tissue into the growing embryo has been demonstrated in organisms of different phylogenetic groups. Glass (1971) has reviewed the subject of heterosynthetic transfer of proteins and presented a comparitive amount of the studies on different organisms.

The present study is an attempt to demonstrate the vitellogenic transfer of proteins into the growing oocyte of the rotifer A. brightwelli.

IV.B. Materials and Methods

^{3}H-Lysine (Sp. act. 250 mCi/m.mole) was purchased from BARC, Trombay, India. Kodak NTB 3 Nuclear track emulsion and AR 10 autoradiographic stripping film were purchased from Kodak Co. Ltd., London, U.K.

Experiments were performed on the laboratory cultures of *A. brightwelli*. The details of culture methodology and isolation and handling of embryos have been as described previously (p. 23).

^{3}H-Lysine Labelling : Young amictic organisms bearing no oocytes were isolated for experiment and pulse labelled with ^{3}H-lysine (20-30 µCi/ml) in hay infusion for 1-2 hours. After the pulse labelling the organisms were cultured (chased) in hay infusion containing 0.2% cold Lysine. After 3-4 hours the animals with different stages of oocytes were transferred onto microslides and oocytes were dissected out along with the vitellarium. The dissection of the organism was carried out as described earlier (vide supra). The tissues were fixed in Acetic : Alcohol (1:3 v/v) for 2 hours. The fixed tissues were mounted on microslides and squashed by covering with a micro cover glass and tapping with a pencil. The cover glasses were flipped off by freezing under liquid nitrogen. The slides with the material were washed with cold 5% TCA for 1 hour and hot 5% TCA for 1 hour. This wash

was followed by washings in 70% and 90% ethyl alcohol after which the air dried slides were subjected to autoradiography.

Autoradiography : The method of Caro (1964) was followed in general. The air dried slides were either coated with Kodak NTB 3 emulsion or covered with stripping film as described (vide infra).

Emulsion Coating : Kodak NTB3 emulsion was melted at 47°C inside a dark room and diluted 1:1 (v/v) with triple distilled water. The air dried slides were dipped once or twice into the diluted emulsion, so as to give a thin and uniform coating. The excess emulsion was drained off and the slides were air dried. The air dried slides were packed into black bakellite boxes along with activated silica gel as dessicant. The boxes were sealed and kept away for exposure for 3-4 weeks at 4°C.

Strip Film Technique : Kodak strip film AR 10 was cut into small pieces, big enough to cover 2/3rd of the slide and floated on distilled water. The film was kept in water for 2-3 minutes with the emulsion side in contact with the water. After this the slide to be coated was passed below the floating piece of film and lifted such that the film covered the area of the slide containing the tissue. Care was taken to avoid wrinkles on the film over the slide. The excess water was drained off and the slides were air

dried, which were then packed and kept for exposure as described above.

The whole operation both during emulsion coating and strip film technique was carried out inside a photographic dark room, with the minimum use of the safe light only (Ilford safe light, red filter, Wratten No. ?).

Development of Slides: After the exposure time the slides were developed in the dark room using D-76 Kodak fine grain developer and fixed in Kodak Unifix, acid fixing salt solution. The slides were kept in the developer for 7-8 minutes and fixed for 10-12 minutes at 25°C.

Staining : After the development and fixing the slides were washed in running water and the tissues were stained in 0.2% aqueous toludene blue. The excess stain was removed in 70% alcohol and the slides were air dried. Euparol was used to mount coverglasses on the slides and the tissues thus prepared were photographed using an olympus photomicroscope.

IV.C. Observations and Discussion

Observation: The vitellarium of *A. brightwelli* is a syncytial structure with large prominent nuclei (plate 1aA). The ovary is situated at the base of the vitellarium. Oocyte

maturation in Asplanchna takes about 5-6 hours at 25°C. In the amictic organism only one polar body is formed during oocyte maturation and the mature oocyte is diploid. The oogenesis involves a vitellogenic phase of growth during which the oocyte remains attached to the vitellarium. During the vitellogenic phase the vitellarium is in direct connection with the growing oocyte and the cytoplasm of the vitellarium streams into the rapidly growing oocyte (plate 1B). In these experiments the organisms were labelled before the appearance of the oocyte and chased for 2-3 hours to eliminate free radioactivity in the pseudocoel cavity. ^{3}H-Lysine labelled protein is seen passing into the growing oocyte (plate 1D) from the vitellarium and this has been detected by autoradiography. This transfer is evident from the labelled protein in the cytoplasm of the oocyte (plate 1C). Besides, globlule like substances are also seen passing into the growing oocyte and these globules are in all probability yolk material. The fully mature oocyte is separated from the vitellarium and by the oocyte membrane and the mature oocyte sinks into the uterine cavity.

Discussion: In Asplanchna the oocyte during its growth draws most of its nourishment from the vitellarium. The vitellarium thus seems to behave like the nurse cells of the insects. During the vitelline phase of the oocyte growth there is rapid synthesis of macromolecules in the vitellarium.

These macromolecules are transferred into the growing oocyte along with the streaming cytoplasm of the vitellarium. ^{3}H-Uridine autoradiographic studies of Birky and his coworkers (unpublished; cf. Birky and Gilbert, 1971) and Gopinath, (1972) have shown that the vitellarium rapidly takes up the radioactive precursor and actively synthesises RNA. The immature oocyte takes up very little radiocactivity. Gopinath (1972) has shown the transfer of ^{3}H-Uridine labelled RNA from the vitellarium into the growing oocyte. The vitellarium actively synthesises RNA especially rRNA during the vitelline phase of the oocyte maturation. Large lobed nucleoli appear in the vitellarium nuclei during this phase. The rRNA, fully formed ribosomes and presumably other components of the protein synthetic machinery pass into the growing oocyte (Birky and Gilbert, 1971; Gopinath, 1972). Vitellarium in addition acts as the yolk gland and yolk material synthesised in the vitellarium is passed into the oocyte along with the cytoplasmic stream. In the present study ^{3}H-Lysine labelled proteins are inherited by the oocytes from the vitellarium. However, the nature of the protein molecules transferred is not clear from the experiments presented here. Since in the present experiments no specific protein was labelled, it is probable that a large portion of the labelled protein is associated with the yolk material and the ribosomes.

The exact nature of the role played by the heterosynthetically transferred molecules is not still clear

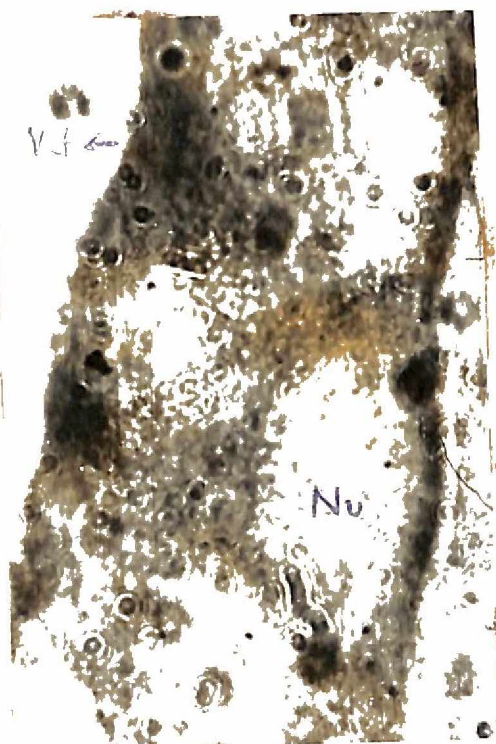
(Glass, 1971), even in other systems. However in the case of other organisms it is known that serum proteins and enzymes are transferred into the growing embryo. The transfer of the serum antibodies is known to play a role in the active immunization of the embryo, in the mammals especially. Among the invertebrates insects offer very interesting examples of the nurse-cell mediated transfer of molecules into the oocytes. The streaming of a basophil material from the nutritive chamber to the oocyte was reported early in the research on insect oogenesis. Later researches showed that labelled RNA and protein pass into the growing oocyte from the nurse cells (Bohhag 1955; ~~1956~~; Bier, 1965; Raven 1961; Sharma, 1968; Roth & Porter, 1968; Anderson, 1964; King & Aggarwal, 1965). In many insects the nurse cells are known to be polyploid and polytenic also (Bier, 1967; Davidson, 1968). In this regard the vitellarium of _Asplanchna_ is comparable with the nurse cells since the vitellarium of _Asplanchna_ is reported to be polyploid (Seth & Dattagupta - in press). This polyploidy of the vitellarium nuclei is probably very essential to ensure the rapid and large scale synthesis of macromolecules.

Plate I. A. Vitellarium (Vit) of A. **brightwelli** showing prominent nuclei (Nu)

Plate I. B. Vitellarium of A. **brightwelli** with the attached maturing oocyte (oc) streaming of the cytoplasm of the vitellarium into the oocyte.

Plate I. C. Autoradiogram of the mature oocyte showing labelled protein (P) transferred from the vitellarium. Yolk bodies (YB) are also seen.

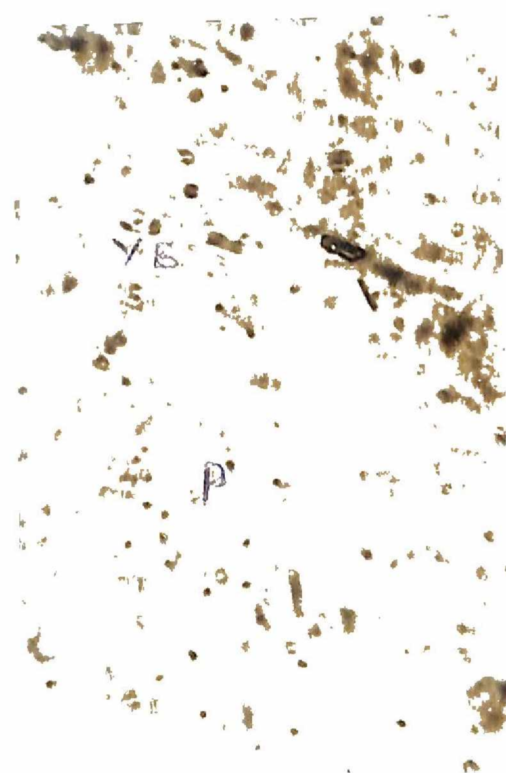
Plate 1 · D Autoradiograph showing the transfer of ^{3}H-Lysine label from the Vitellarium (Vit) into the Oocyte (Oc)



1 A 1500x

1 B 600x

1D 1500x



1 C 1500x

CHAPTER V

General Considerations & Summary

V. A. General Considerations

The rotifer *Asplanchna brightwelli* is a highly specialised form, nevertheless, studies on the reproduction and embrylogy of rotifers have been made mostly in the genus *Asplanchna* of this group. Therefore a generalization on the basis of studies on *Asplanchna* cannot be regarded as typical to the group rotifer (Hyman, 1951), although problems relating to the development of rotifers refer to evidences drawn from the embryology of *Asplanchna*. The development of *Asplanchna* presents many peculiarities which are indeed deviations from the normal protostome pattern. In *Asplanchna* the basic developmental pattern is of the mosaic type with the initial spiral, determinate cleavage. Besides, there is the formation of the D quadrant (vide supra) at the 4 cell stage which gives rise to adult reproductive structures. But the absence of any specific larval stages, the bilateral symmetry of the later cleavages and the alternation of the amictic generations (parthenogenetic) by a single mictic (sexual)generation, are some of the interesting peculiarities of the reproduction and the development of *Asplanchna*.

The present work is an attempt at the molecular embryology of the rotifer *A. brightwelli* and there is hardly any information in this field. The protein and RNA synthesis, as has been observed in the present work, are

continuous throughout the course of development of A. brightwelli. The embryos were treated here with actinomycin D in order to inhibit RNA synthesis. Apparently treatment with actinomycin D does not inhibit the major part of the protein synthesis during the early phase of development. It therefore follows that the early embryonic protein synthesis is a function of the conserved messengers to a considerable extent. These stable messengers are most probably conserved by the growing oocyte through the oogenetic vitellogenic transfer.

The accumulation and utilization of maternal messengers during early development is now well known in many different organisms. In all these different organisms the early embryonic protein synthesis is uninhibited by treatment with actinomycin D. In A. brightwelli as observed in the present work the use of maternal templates during early embryonic development probably facilitates the rapid embryonic development. A similar view was expressed earlier by Birky and Gilbert (1971) and Gopinath (1972).

The present experiments on the RNA synthesis during the embryonic phases of A. brightwelli indicate precocious synthesis of informational RNA, as early as 2 cell stage. The inference with regard to the informational RNA has been drawn by using the criteria that the RNA synthesized contains poly (A).

The presence of poly (A) is characteristic of informational RNA (mRNA and HnRNA; see review Greenburg, 1975) and in A. brightwelli poly (A) RNA appears as early as 2 cell stage. This early informational RNA synthesis during embryogeny is known in other protostomes and deuterostomes. On the other hand the synthesis of ribosomal RNA (rRNA) begins late during the development of A. brightwelli since the nucleoli make their appearance in the post-mitotic phase. In this regard A. brightwelli differs from certain closely related protostomes. In the worms, for instance, the nucleoli appear very early during cleavage. This late rRNA synthesis in A. brightwelli may be explained by the large scale transfer of rRNA during oogenesis and the transferred maternal rRNA and ribosomes assumably serve to function during early embryogenesis.

The results of the present experiments indicate the total synthesis of RNA and protein or the incorporation of their appropriate precursors. But the results do not indicate the actual rates of synthesis since no estimation of the precursor pool size has been made. Similarly, in the studies on the RNA synthesis at different embryonic stages, although the presence of poly (A) is characteristic of informational RNA, the base composition analysis of the RNA fractions would have yielded more conclusive results about the nature of the RNA, as has been determined in the

case of the resting eggs in the present work. This, however, could not be carried out since facilities for the use of ^{32}P were not available in the laboratories where the major part of the present work was carried out.

The presence of conserved mRNA in the resting eggs, as is evident in the present work, is interesting. These messengers probably have a significant role in the resumption of development from the dormancy of the resting eggs.

The occurrence of poly (A) in the RNA of the resting eggs and also in the pulse labelled RNA from the different embryonic stages of A. brightwelli adds the rotifers in the list of organisms which have been found to contain poly (A) in their RNA. Although various functions have been attributed to poly (A) the exact role played by it is not clear. Poly (A) probably has a role in development and differentiation through its association with the messenger RNA functions.

In A. brightwelli the rapid embryonic development and complete in utero development presumably necessitates a large scale transfer of cytoplasmic complements into the growing oocyte. This transferred material includes stable mRNA, ribosomes, fully formed protein molecules, and other components of protein synthesizing machinary in addition to the nutrient material in the form of yolk. These maternal compliments serve to function during the early embryogeny of the rotifer A. brightwelli.

V. B. Summary

Studies have been carried out on some aspects of RNA and Protein synthesis in the embryogenesis of the rotifer *Asplanchna brightwelli*. The embryogenesis of *A. brightwelli* has distinct gametogenic, mitotic and post-mitotic phases.

RNA and protein synthesis were studied in certain mitotic and post-mitotic embryonic stages by following the incorporation of ^{3}H-Uridine and ^{3}H-Lysine, respectively, into TCA insoluble material of the embryos. Incorporation of the radioactive precursors into RNA and protein was detected at all the stages studied.

The embryos were treated with Actinomycin D to inhibit RNA synthesis and protein synthesis was studied in the treated embryos. Major part of the early embryonic protein synthesis was insensitive to inhibition of RNA synthesis by Actinomycin D. On the other hand most of the protein synthesis in the post mitotic embryonic stages was inhibited by the treatment with Actinomycin D. Thus the early embryonic protein synthesis of *A. brightwelli* presumably involves the utilization of conserved messengers, where as the later stage embryonic protein synthesis depends largely on the immediate RNA synthesis.

The development of the early stage embryos did not proceed to completion in the presence of Actinomycin D. Actinomycin D showed a non-specific effect on cleavage.

The nature of the RNA synthesised during the embryonic stages was studied by SDS-polyacrylamide gel electrophoresis and poly-U sepharose chromatography of the RNA. The ^{3}H-Uridine pulse labelled RNA in the early embryos was heterogenous in size and contained poly (A). The early embryonic RNA did not show any peaks in the ribosomal RNA (rRNA) region on SDS-polyacrylamide gels. The rRNA peaks appeared in the post mitotic embryos along with the appearance of nucleoli. In <u>A. brightwelli</u> the synthesis of informational RNA begins early during development and rRNA synthesis seems to begin in the post-mitotic stages.

The fraction of poly (A+) RNA showed an increase with development as seen by the binding of the pulse labelled RNA to poly-U sepharose columns.

The RNA from the resting eggs (dormant embryos) was fractionated after labelling with either ^{32}P-orthophosphate or ^{3}H-Uridine. The resting eggs showed two mRNA like fractions one which was poly (A+). The poly(A+) fraction was 12-15S in size and the poly (A-) fraction was 9-11S in size on SDS-polyacrylamide gels. The base composition of the two RNA fractions showed that both the

RNA fractions were 'DNA like' or mRNA like in base composition, i.e. they were A+U rich. Since the resting eggs are dormant embryos, these stored messengers probably have a role to play in the resumption of development from encystment.

The vitellogenic transfer of ^{3}H-Lysine labelled protein into the oocytes was studied by autoradiography.

B I B L I O G R A P H Y

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