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RNA SYNTHESIS DURING SPIROSTOMUM REGENERATION

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PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full, for any other degree or diploma of any University.

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Lastly, I wish to record my gratefulness to the University Grants Commission, for sustaining me through this period by providing a Teacher-Fellowship.

CONTENTS

PROEM

In the event of circumstantial living, occasionally when an adult organism gets injured, loses parts of the body or breaks into pieces, some of them have an inherent response to recover from such a fate by replacing the lost parts or regrowing from the existing body fragments. This faculty of regrowth is expressed significantly in some of the lower animals and gradually becomes restricted along the forward evolutionary scale. On such an occasion of wound healing or regrowth, at first the differentiated and specialized cellular units surrounding the wound retreat to the basic embryonic level through dedifferentiation and then a repetition of the developmental history recurs to an extent of full restitution. The process of regeneration in an organism appears in all intense and purposes similar to a developing system as far as the cellular differentiation is concerned.

From its multipotent status the cell, while differentiating, becomes progressively restricted to a specific configuration through continuous synthesis of the 'differentiation-specific' structural and functional proteins. The spectrum of protein synthesised during differentiation and development owes its initiation

to the transcription of messenger (Monod and Jacob, 1961) and its progress to the polyribosomal site (Goodman and Rich, 1963; Nonomura, et al, 1971) in the cell. It seems clear from a recent model by Maclean and Hilder (1977) that the genomic control of cellular differentiation essentially manifests at the transcriptional and post-transcriptional level.

Attempt to correlate the ribonucleic acid (RNA) metabolism to the development historically stems from the work of Brachet (1941) on amphibian embryo and this has been further substantiated later by Ycas and Vincet (1960), Nemer (1962), Brown (1964) and Denis (1968). After the discovery (Kirk, 1960) and confirmation (Goldberg and Robinowitz, 1962; Hechter and Halkerston, 1965; Sobell, et al, 1971) of actinomycin-D as a transcription inhibitor, it has been exhaustively used for delineating the participation of messenger RNA (mRNA) in translation of the genomic information to protein synthesis in many developing systems like insects (Laufer, et al, 1964; Berry, et al, 1964), sea-urchin embryo (Gross and Cousineau, 1964; Mackintosh and Bell, 1967), chick-embryo, amphibian, polychaeta worms and various tissues of mammals (Scott and Bell, 1964;

Craig, et al, 1971). It is observed that the messenger RNA offers at least two possible synthetic pathways for specific proteins in the differentiating cell. The first way of translation is through continued synthesis of short-lived mRNA and the second is through repeated use of limited amounts of long-lived specific messenger RNA.

The existence of stable, long-lived or masked mRNA has been demonstrated in the studies on globin mRNA (Marks, et al, 1962; Arnstein, et al, 1964; Booyce and Rafelson, 1967), calf lens crystalline mRNA (Stewart and Papaconstantinou, 1967), sea-urchin eggs (Maggio and Catalano, 1968; Farquar and McCarthy, 1973; Gross, et al, 1973; Skoultchi and Gross, 1973) and myosin mRNA (Heywood, et al, 1975). Characteristically the masked mRNA is a preformed template and remains dormant (Marcus and Feeley, 1964, 1965; Dure and Watters, 1965) over a period until it is activated and utilised during either early embryonic development and differentiation (Nemer, 1962; Gross, et al, 1964; Scott and Bell, 1964) or failure of transcription mechanism due to some reason in the cell (Klein and Pierro, 1963; Gross and Cousineau, 1964; Brown and Gurdon, 1966; Denis, 1966). The long half-life and stability of this mRNA is explained in terms of its post-transcriptional polyadenylation at 3' terminal (Hadji-

vassilliou and Brawerman, 1966; Darnell, et al, 1971; Perry, et al, 1973; Greenberg, 1975; Darnell, 1976) and association with protein during translocation from nucleus to cytoplasm as ribonucleoprotein complex or 'informosome' (Nemer and Infante, 1965; Buckingham, et al, 1974) which is believed to have a translational control during development and differentiation (Huez, et al, 1974; Barrieux, et al, 1975; Dworkin, et al, 1977). Due to the long life span, about 40 per cent of mRNA survives more than one cell generation in proliferating eukaryotic cells (Steward, et al, 1968; Hodge, et al, 1969) and thereby suggests a translational control of protein synthesis during development.

In the light of contemporary relevance, it appears that RNA plays a critical and key role during differentiation of eukaryotic cells and an understanding of the sequences of synthesis and metabolism of RNA in such systems is essential to know some of the underlying principles of eukaryotic differentiation. Since also a regenerating system is nearly similar to a developing and differentiating one, it can be advantageously utilised to look into the phenomenon of differentiation. As a matter of fact, many regenerating systems from lower vertebrates to protozoans have been used for studies on development and

differentiation (Berrill, 1957; Singer, 1958; Flickinger, 1967; Giese, 1973; Chandebois, 1976). Because of their intriguing faculty to regrow, the protozoans, however, have been highly favoured for regeneration studies.

A heterokaryotic ciliate protozoan practically becomes indestructible in its intact environment, because each of its multiple fragments produced mechanically regenerates to become a new organism. The organelle responsible for such a behaviour of the ciliate is identified to be its macronucleus, whose tiny node in a fragment helps the fragment regenerate to an adult organism. The importance and indispensability of macronucleus to initiate and maintain the regeneration has been extensively elucidated from studies on Stentor (Tartar, 1961, 1962, 1967), Epistylis articulata (Seshachar and Dass, 1953) and Blepharisma (Suzuki, 1957). Also from autoradiographic studies on Blepharisma (Giese and McCaw, 1963; Giese, et al, 1963; Giese, 1970, 1971, 1973; Gavurin and Hirshfield, 1974) and photometric studies on Epistylis articulata, Blepharisma (Seshachar and Dass, 1954, 1960) the positive role of macromolecular events and the pattern formation in regeneration have been brought to light.

Recently, in our laboratory regeneration experiments with Spirostomum ambiguum, one of the heterokaryotic ciliates, have indicated the involvement of intracellular organelles in the process of restitution. Microspectrophotometric and autoradiographic methods have shown that the micronuclear component in the half-fragment of a Spirostomum is endowed with the morphogenetic ability to resynthesise the amount of deoxyribonucleic acid (DNA) lost by amputation (Jeymohan, 1975).

In context with the above propositions regarding macromolecular synthesis and organelle participation in regeneration, it seems worthwhile to look into the biochemical aspect of cytoregeneration using an apt viable system like Spirostomum particularly because of its remarkable body which is a long cylindrical worm-like cell containing a beaded chain of macronucleus as long as itself. Such a study can also yield informations on the subcellular and molecular mechanism of cytodifferentiation and development during the process of regeneration.

In the present investigation with Spirostomum the mechanism of regeneration has been followed from a level of its half-fragment, obtained by transecting

the organism into two halves, and a time sequence survey of the profile of macromolecular synthetic pattern during *in situ* growth and regeneration has been obtained using radio-precursor incorporation and extraction methods. After the basic informations of RNA and protein metabolism has been obtained the radio-precursor is coupled with pretreatment of actinomycin-D, the transcriptional inhibitor, to determine whether the latter blocks these macromolecular syntheses. In particular, the fate of protein synthesis has been observed beyond the inhibition of transcription in the regenerating system to assess the role of masked mRNA. The study provides the basic data of RNA-protein synthetic profile as vital subcellular entities during the process of regeneration.

MATERIAL AND METHODS

1. SPIROSTOMUM CULTURE

Spirostomum ambiguum, a heterotrichous ciliate, is elongated, roughly cylindrical, of body length 1 to 3 mm and length to width ratio of about 10 to 1 in its uncontracted freely swimming state, highly contractile and distinguishable with unaided eye. The unicellular worm-shaped organism has uniform ciliation in longitudinal row, peristome half of body length, short membranelles closely lined with body, a long and beaded macronucleus, many scattered micronuclei and terminally a large contractile vacuole with long canal close to the dorsal side (Fig. 1).

Being a fresh water organism it is cosmopolitan in distribution, available in lentic zones of streams, rivers and still waters, prefers decaying plant material as food, and a characteristic inhabitant of forest pools and lakes rich in litter in the condition of

Temperature (^{o}C)	0 - 25
pH	6.0 - 7.8
Dissolved O_2 (mg/1)	0.1 - 7.4
NH_4^+ (mg/1)	0 - 17
H_2S (mg/1)	0 - 1.3

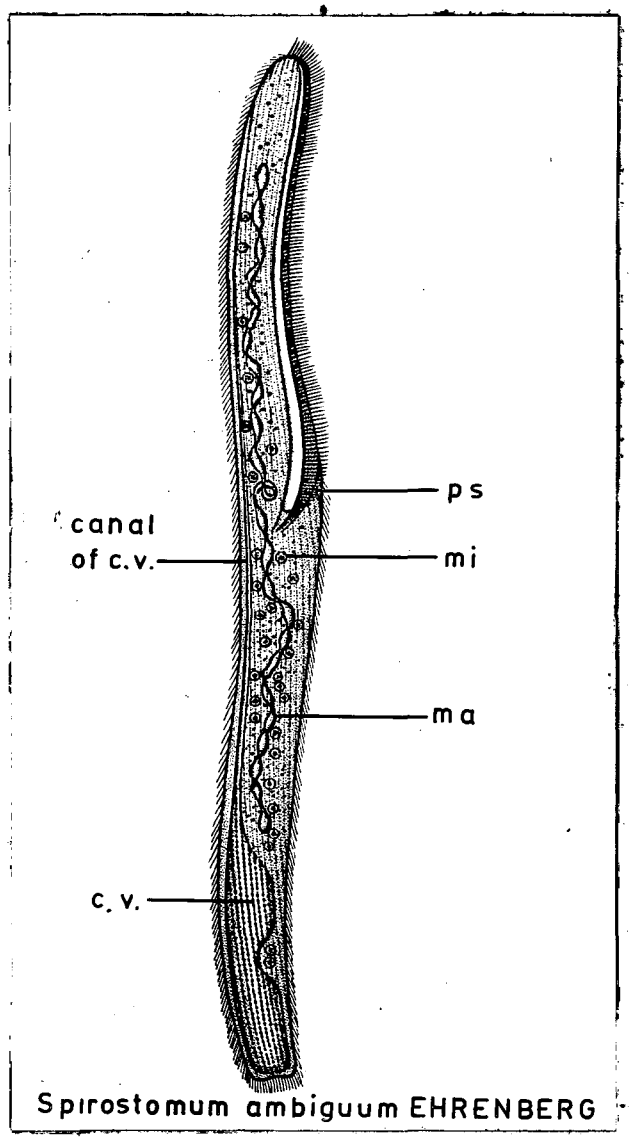

FIG.1: General view of Spirostomum ambiguum in situ showing the presence of beaded macronucleus (ma), many scattered dot like micronuclei (mi), contractile vacuole (c.v) and peristome (ps).

Attempts to culture Spirostomum by the method according to Yagiu and Shigenaka (1963) were unsatisfactory since yields were low and organisms die in week old hay infusion with a few faeces of mouse at 22 ± 1°C. Originally obtained from Zoology Department, Bangalore University and maintained in Prescott medium at 22 ± 1°C in this laboratory, the organism was excellently grown in culture in the same manner and used for experiments. A balanced salt solution of the Prescott medium was prepared with the following composition.

Solution A

$CaCl_2$	3.27 gm
KCl	1.62 gm

Dissolved in 1000 ml distilled water (sterile)

Solution B

K_2HPO_4	5.12 gm

Dissolved in 1000 ml distilled water (sterile)

Solution C

$MgSO_4.7H_2O$	2.80 gm

Dissolved in 1000 ml distilled water (sterile)

1 ml each of solution A, B and C was added into 1000 ml distilled water (sterile).

At 22 ± 1°C the organisms were transferred into the Prescott medium in Petridishes and boiled wheat grains were added as food. The medium and grains were renewed periodically.

2. RADIO-INCORPORATION AND EXTRACTION

RNA and protein synthesis during different conditions of growth of Spirostomum ambiguum were conveniently measured by radio-isotopic neucleotide and amino acid incorporation, extraction and scintillation counting following a suitably modified method in our laboratory as described by Venugopal (1978). Since the organism is small and disintegrates immediately, if taken out of the liquid medium, a group of twenty organisms were used in each experiment by carefully transferring them under microscope using a narrow Pasteur pipette. Pulse hour and continuous label were done with ^{3}H-uridine and ^{14}C-isoleucine at 22 ± 1°C and the incorporated macromolecules were collected in TCA precipitable material at 0°C.

3. RNA SYNTHESIS IN SITU

A few hundreds of Spirostomum ambiguum were transferred from a Petridish culture to fresh Prescott medium, then separated into a small volume of the medium by centrifugation (3000 rpm of Remi T8) and kept unfed in the centrifuge tube for 24 hours. This lot of one day starved organisms was transferred to a watch glass from which for each experiment twenty of them were pipetted under the microscope onto a grooved

slide where after exact counting adjustment the animals were transferred into a clean fifteen ml calibrated centrifuge tube. Fresh Prescott medium was added to make the volume upto 0.4 ml in the centrifuge tube. The isotope concentration was maintained at 5 ul/ml by adding 2 ul of ^{3}H-uridine to the organisms in the centrifuge tube and the time of this labelling was noted. At the end of appropriate hour (upto 36 hours) of continuous labelling the unused isotope was washed out by thirtyfold dilution with Prescott medium and removal of supernatant after centrifugation (4000 rpm of Remi T8). Immediately, the medium volume was made 1 ml and an equal volume of 2% solution of Sodium dodecyl sulphate (SDS) was added to the centrifuge tube in order to lyse the Spirostomum cells. Five minutes after lysing the sample 100 ug of unlabelled RNA was added as carrier for RNA precipitation and 2 ml of chilled 10% TCA was added making the whole volume 4 ml and kept at 0^{o}C for 24 hours for RNA precipitation. The precipitate was collected on Millipore Filter and washed several times with cold 5% TCA under water suction pump. The filter was dried at 60^{o}C for 1 hour and kept in a scintillation vial. The radioactivities of the filters of a set of experiments were counted by a Packard Tri

Carb Liquid Scintillation Spectrometer using a toluene based liquid scintillation fluid containing 4 gm per litre PPO and 50 mg per litre POPOP and expressed in counts per minute (cpm) per twenty Spirostomum.

4. RNA SYNTHESIS DURING REGENERATION

As described in the previous procedure for RNA synthesis in situ, a few hundreds of Spirostomum were collected from the culture to fresh Prescott medium, centrifuged and collected into a small volume of the medium, kept starved for a day and then transferred to a watch glass. Under microscope, for each experiment, ten organisms were pipetted onto a grooved slide where after exact counting adjustment each animal in its extended condition was transversely cut into two half-fragments with single stroke of a sharp, pointed and sterilized steel needle. The twenty half-fragments, thus made, were transferred quickly into a clean fifteen ml calibrated centrifuge tube in which further addition of Prescott medium made the volume upto 0.4 ml.

In a set of experiments for continuous RNA synthesis during regeneration the above half-fragment samples were labelled each with ^{3}H-uridine (5 ul/ml) immediately and kept for growth. At the end of appropriate

hour (upto 36 hours) of labelling, the removal of unused isotope, lysing of cells, extraction of RNA and radioactivity countings were done as described previously. But in a set of experiments for pulse RNA synthesis during regeneration, the half-fragment samples were labelled at different specific hours (upto 40 hours) for 1 hour each with ^{3}H-uridine (5 µl/ml) and as above the RNA extractions and radioactivity countings were done.

5. PROTEIN SYNTHESIS DURING REGENERATION

As in the experimental procedure for RNA synthesis during regeneration, for each of this experiment ten organisms transected into twenty half-fragments were pipetted into a clean fifteen ml calibrated centrifuge tube and the volume of Prescott medium was made 1 ml in it. Quickly 1 µl of ^{14}C-isoleucine was added to the sample and time of this labelling was noted. At the end of appropriate hour (upto 32 hours) of labelling the unused isotope was removed by fifteen fold dilution and cells were lysed as in previous experiments. The sample solution was added with 100 µg of bovine serum albumin (BSA) as carrier for protein precipitation and equal volume of chilled 20% TCA and was kept at 0^{o}C for 24 hours. The precipitate was collected on Millipore filter and washed several times with cold 10% TCA. The

filter was dried at 60°C for 1 hour and kept in a scintillation vial. The radioactivities of a set of filters, thus obtained for continuous protein synthesis during regeneration, were counted as described previously.

6. INHIBITION WITH ACTINOMYCIN-D

The inhibiting effects of Actinomycin-D on the RNA and protein synthesis during different conditions of growth of Spirostomum ambiguum were determined by setting up different experimental conditions with pretreatment of various contentrations of the drug and then measurement of ^{3}H-uridine and ^{14}C-isoleucine incorporation into the system. To avoid inactivation of actinomycin-D, the experiments were done in dark with minimal exposure to light.

The organisms were collected in a small volume of Prescott medium as in the previous description. Actinomycin-D solution prepared with Prescott medium was calculately added to the sample volume so as to make the final concentration (40 µg/ml or 100 µg/ml) of the drug required for the specific experiment and the time of the drug treatment was noted. After the pretreatment hours (6 hours or 12 hours) as described early, a group of twenty normal organisms or of transected half-fragments in drug medium were labelled with ^{3}H-uridine

(5 µl/ml) or ^{14}C-isoleucine (1 µl/ml) as the case may be, in experiments for RNA and protein synthesis, extraction and radioactivity counting.

7. ISOTOPES AND CHEMICALS

The radioactive precursors, uridine-T(G) (specific activity 9700 mCi/mmole) and L-isoleucine-C-14(U) (specific activity 180 mCi/m mole) were purchased from Bhabha Atomic Research Centre, Bombay. PPO, POPOP, SDS and BSA were obtained from Sigma Chemical Co., MO., USA. Millipore filters (type HAWP, 0.45 µm) were purchased from Millipore Intertech, Inc., MA, USA. RNA (unlabelled) was obtained from Patel Chest Institute, Delhi and Actinomycin-D was purchased from Calbiochem, La Jolla, CA, USA.

EXPERIMENTAL RESULTS

1. PATTERN OF RNA SYNTHESIS IN SITU

A survey of 36 hours of continuous incorporation with ^{3}H-uridine in one day starved normal non-regenerating Spirostomum showed a positive uptake of radioactive precursor indicating RNA synthesis as a normal feature in the system. The pattern of RNA synthesis, as obtained in this experiment, consisted of a two stage build up followed by a two stage fall in 8 hourly sequences (Fig. 2). The incorporation being very rapid in the first hour continued a gradual increase till 8 hours after which it steadily stepped up and reached its maximum by 16 hours. Further onwards the synthesis slowed down significantly till a constant rate occurred between 20 to 24 hours. Another sharp decline of the incorporation beyond 24 hours made its magnitude equal in level with that of initial hours. From 28 hours, once again a small and gradual rise of synthesis was observed till 32 hours, after which it decreased slowly. In the course of increasing phase the maximum incorporation, observed at the 16th hour, was approximately 2.5 fold of the first hour incorporation. Further analysis revealed that the rate of rise was almost double the rate of fall for RNA synthesis in the system.

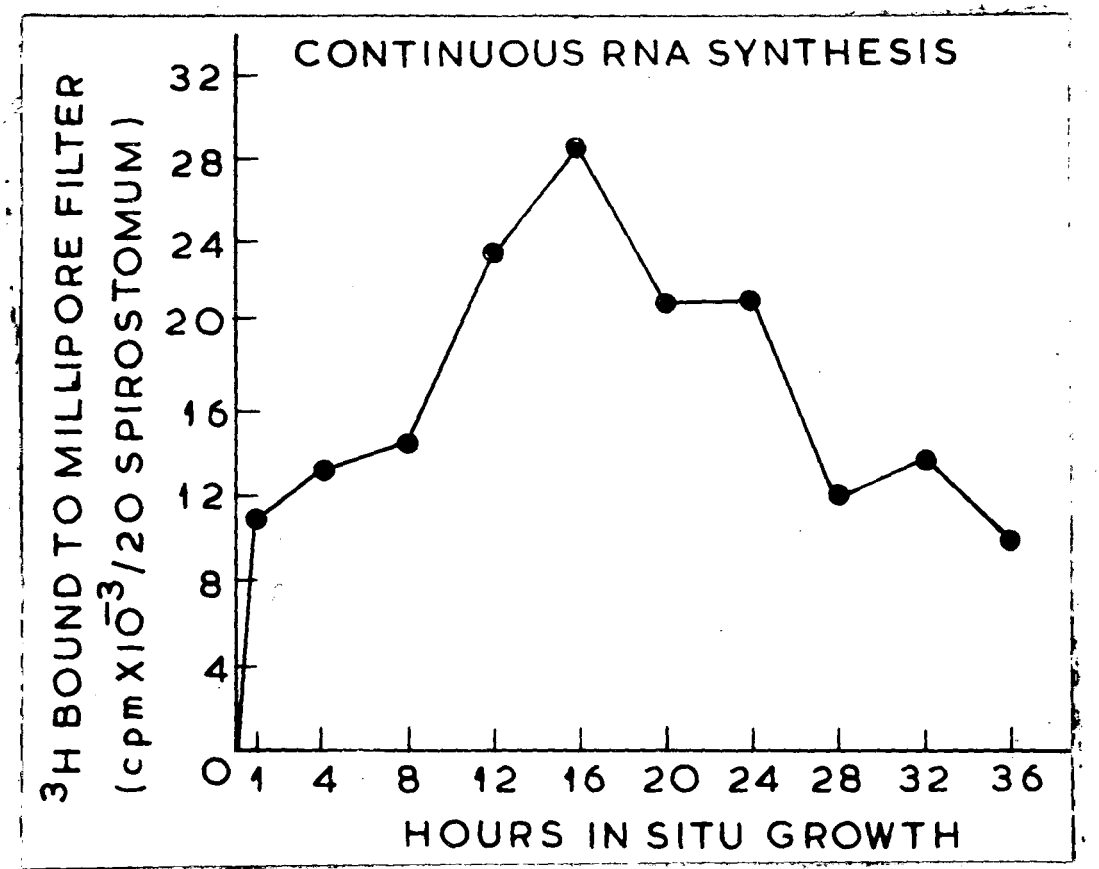

FIG.2: Pattern of continuous incorporation of ^{3}H-uridine (5 µl/ml) into RNA in one day starved non-regenerating Spirostomum.

2. PATTERN OF RNA SYNTHESIS DURING REGENERATION

One day starved Spirostomum while undergoing regeneration from its half-fragment after amputation showed significant uptake of radioactive precursor for RNA synthesis during a period of 36 hours (Fig. 3). Immediately after amputation, the organisms continuously incorporated labelled ^{3}H-uridine at a high rate till 8 hours of regeneration. However, the RNA synthesis slowed down during the period from 8 to 12 hours, after which a sprouting and maximum synthesis was obtained by 16 hours of regeneration. After 16 hours the synthesis decreased and reached a constant phase between 20 and 24 hours. Onwards the radio-incorporation decreased further with rapid rate till 28 hours beyond which the magnitude of RNA synthesis remained approximately constant and appreciable. In this case of continuous incorporation studies, the RNA synthesis presented a course of a two-tier increase followed by a two-tier decrease with an interval of 8 hours between the tiers. The peak values of tiers were not very much different excepting the one around 32 hours of regeneration. The rate of increase was generally observed to be higher than the rate of decrease of RNA synthesis.

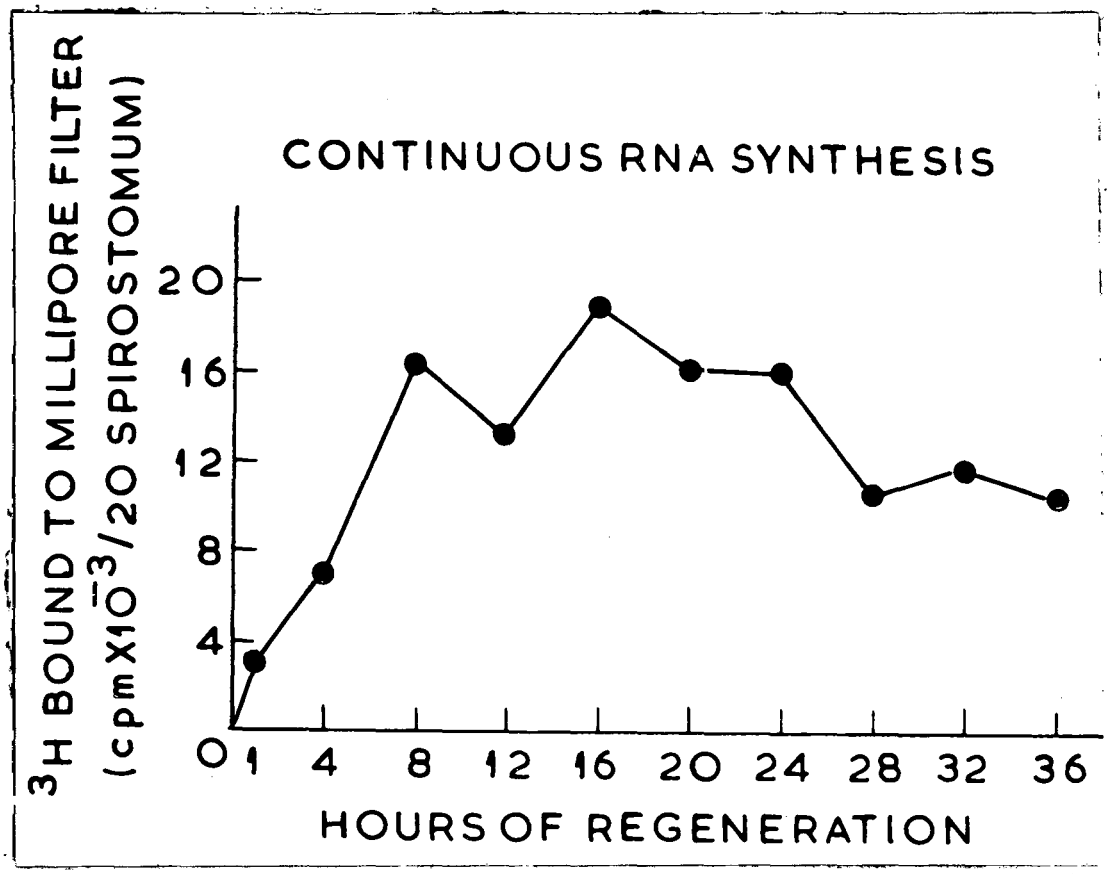

FIG.3: Pattern of continuous incorporation of ^{3}H-uridine (5 µl/ml) into RNA in one day starved regenerating Spirostomum.

In the pulse labelling studies made with the same system as above, one hour duration of radioprecursor incorporation during particular hours of regeneration revealed the capacity of Spirostomum to synthesise RNA during the corresponding periods. Immediately after amputation of the one day starved organisms each into two half-fragments, the pulse label analysis was carried out till 40 hours at 4 hourly intervals. It was noticed that a burst of RNA synthesis had taken place, as evident from the above study, in the first hour after amputation. The pulse hour incorporations at the 4th and 8th hour of regeneration were very low. A progressive increase of the synthetic activity was noticed after 8 hours of regeneration till the 16th hour, when a peak of activity was observed (Fig. 4). However, after this stage, a declining rate of synthesis made the magnitude decreased 50 per cent by 20 hours ahead of which a constant rate was noticed till 24 hours of regeneration. After this period, once again the synthesis started increasing steadily for 8 hours followed by 8 hours of an equally decreasing steady rate. Thus, a second peak of activity occurred at the 32nd hour of regeneration. The significant feature of this pulse hour RNA synthesis during regeneration was the two peaks at 16 hour interval, the magnitude of both being approximately equal. At the

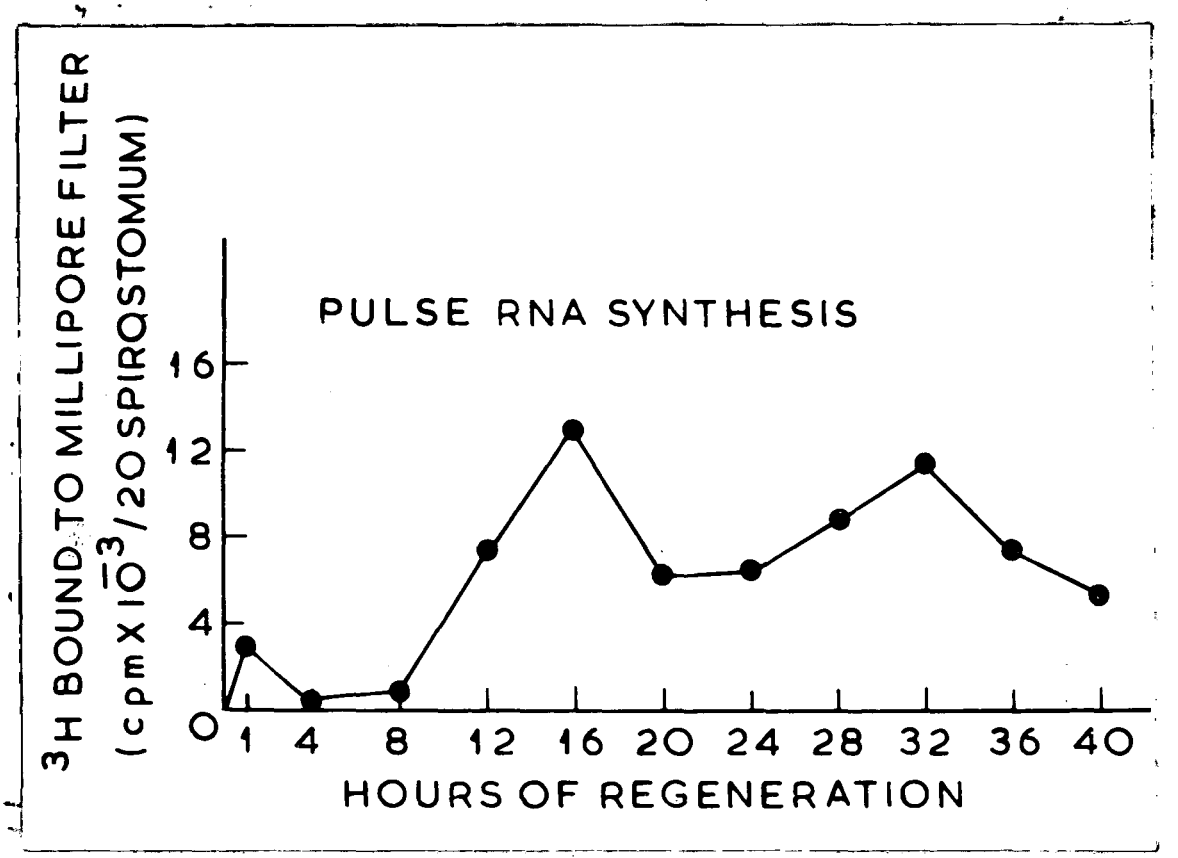

FIG.4: Pattern of hourly pulse incorporation of ^{3}H-uridine (5 µl/ml) into RNA in one day starved regenerating Spirostomum.

later periods of regeneration the synthesis, whatever low, remained far more than the minimum obtained around the early hours.

3. PATTERN OF PROTEIN SYNTHESIS DURING REGENERATION

Trial observations of continuous labelling with ^{14}C-isoleucine suggested a positive uptake of the precursor by the regenerating Spirostomum for its protein synthesis. A course of 32 hours of regeneration in one day starved organisms revealed that the protein synthesis built up from the beginning with accelerating rate and culminated with sprouting incorporation at the 6th hour (Fig. 5). During the next 2 hours of regeneration, a 20 per cent fall of activity was noted and it was followed by a constant phase of incorporation. From 12 hours onwards the system resorted to synthesise further amount of protein rapidly and thus a second peak of activity was noticed at the 16th hour when the incorporation assumed a level equal to the first peak. As happened beyond the first peak, the synthetic expression beyond the second peak also entered a highly declining status for 4 hours after which a variable nature of incorporation was resulted. However, the activity showed its elevating tendency around the 24th and 32nd hours of regeneration and reached its minimum at 28 hours where the level of incorporation was a little higher than the half of the peak value.

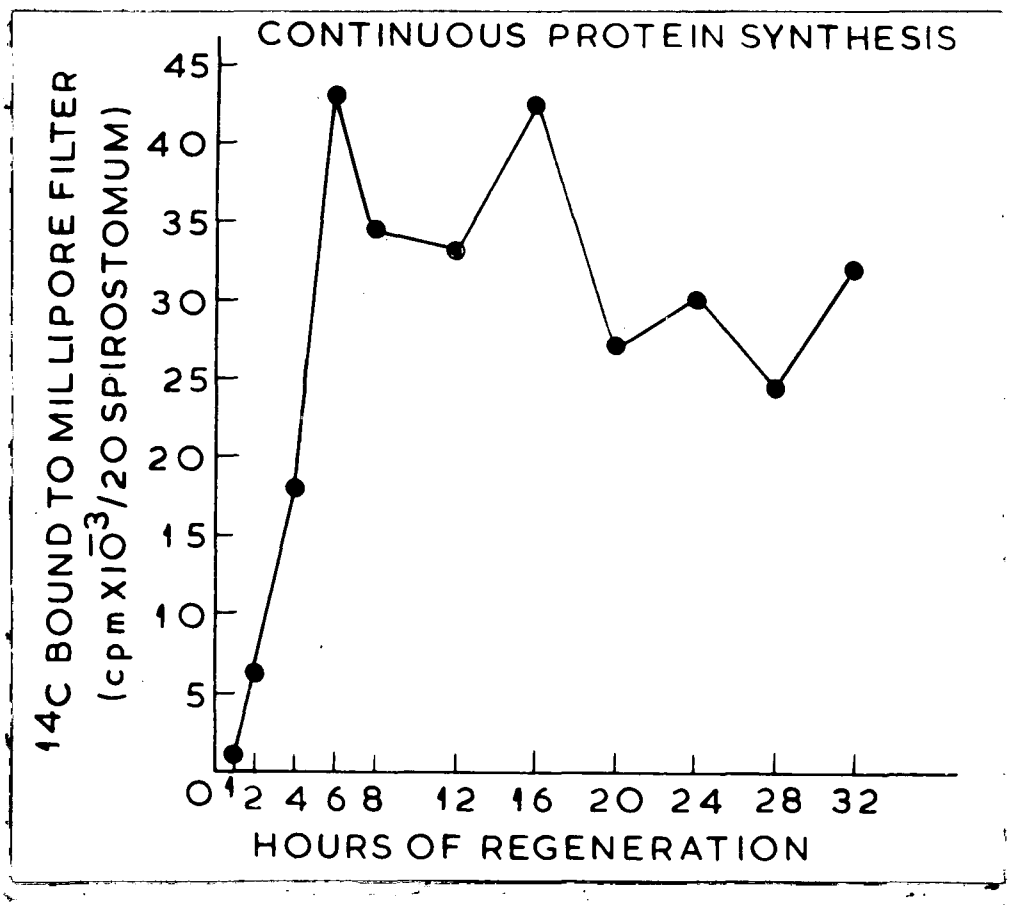

FIG.5: Pattern of continuous incorporation of ^{14}C-isoleucine (1 µl/ml) into protein in one day starved regenerating Spirostomum.

4. INHIBITION WITH ACTINOMYCIN-D

(i) ON RNA SYNTHESIS

Preliminary studies with the inhibitor were performed in two sets of experiments and its effect on the synthetic capability of Spirostomum was found positive. In the first set, a group of non-regenerating organisms pretreated with 40 µg/ml actinomycin-D for 12 hours was used for continuous ^{3}H-uridine incorporation into RNA synthesis, while in the second set a 6 hours pre-treatment with 100 µg/ml drug was made. As is evident from Fig. 6, the higher concentration of the drug started arresting the RNA synthesis around 12 hours beyond pre-treatment.

Likewise, a treatment with 100 µg/ml actinomycin-D for 12 hours prior to regeneration appreciably affected the incorporation of ^{3}H-uridine and suggested a progressive break down of RNA synthesis after 4 hours of regeneration (Fig. 7). It was noticed that 12 hours beyond the pre-treatment with the drug, the regenerating organisms were almost devoid of synthesising fresh RNA. This fact was clear when the results of this experiment were compared with the control system in Fig. 3.

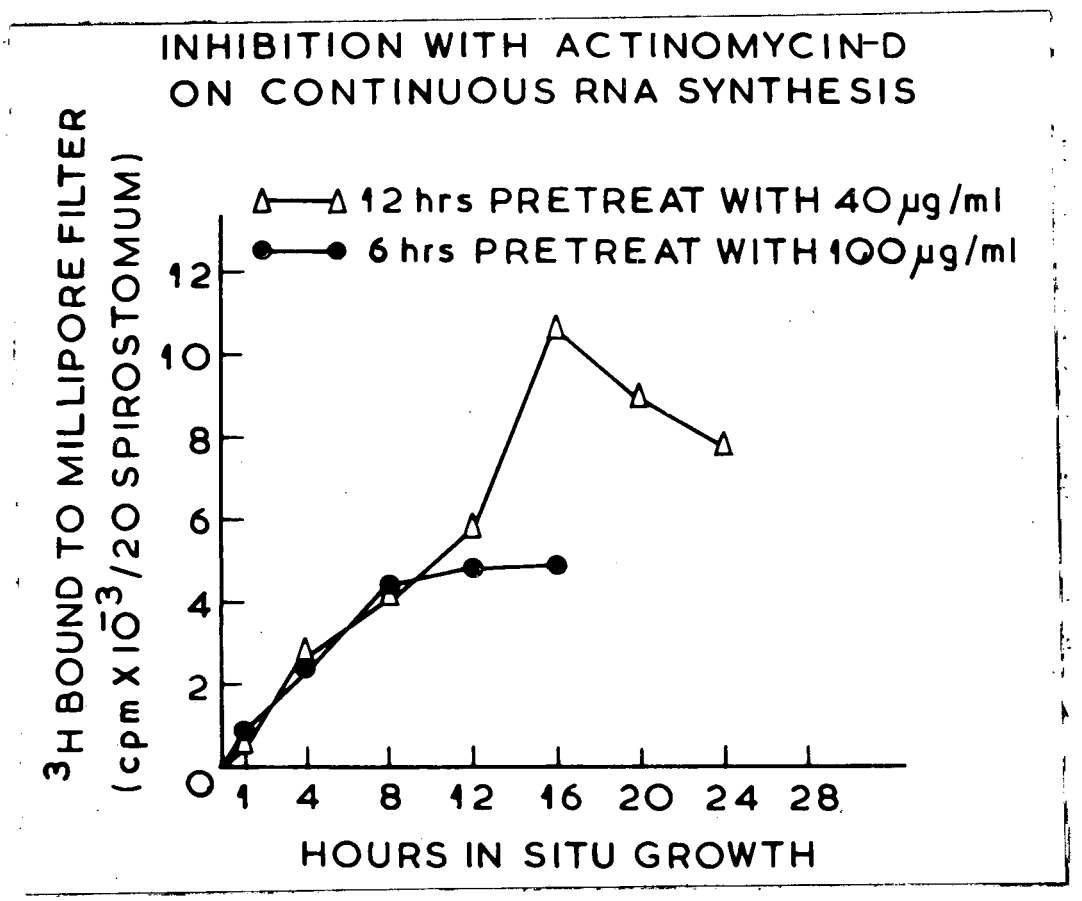

FIG.6: Pattern of continuous incorporation of ^{3}H-uridine (5 µl/ml) into RNA in actinomycin-D pretreated non-regenerating Spirostomum.

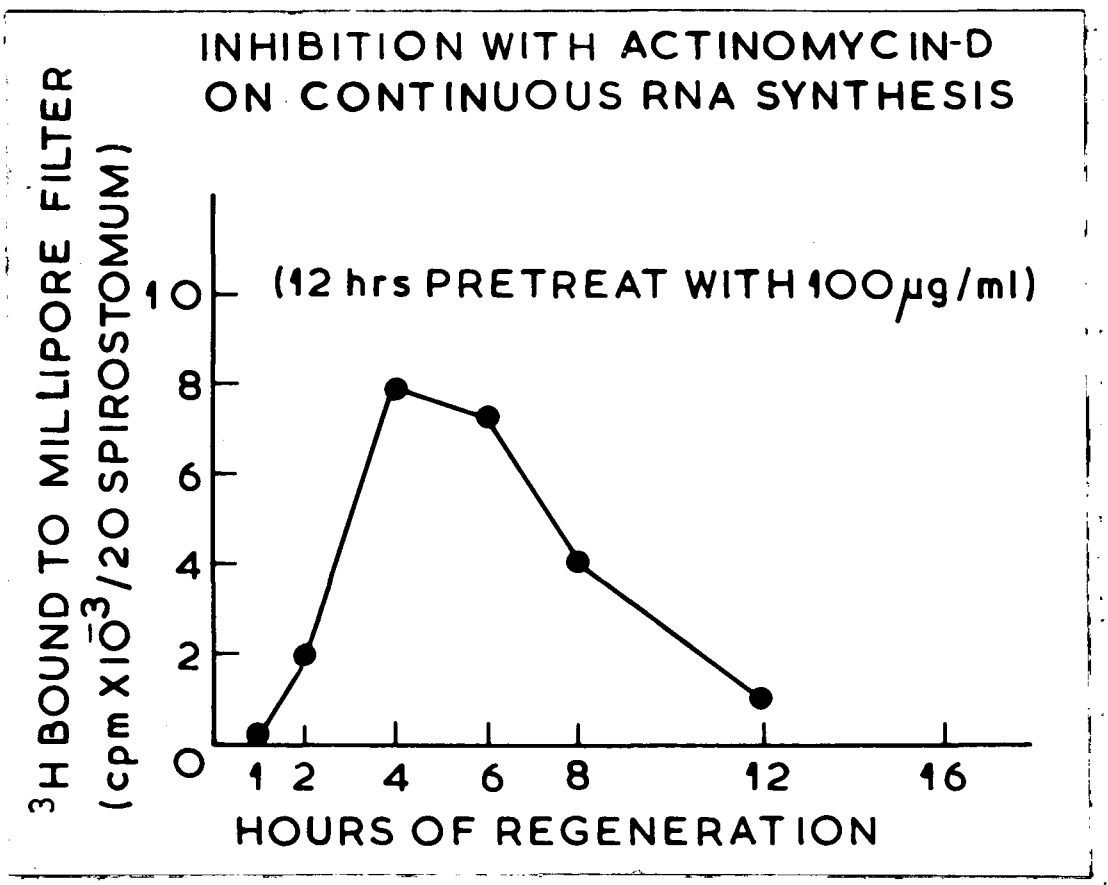

FIG.7: Pattern of continuous incorporation of ^{3}H-uridine (5 µl/ml) into RNA in actinomycin-D pretreated regenerating Spirostomum.

(ii) ON PROTEIN SYNTHESIS

From the knowledge of RNA synthesis profile as gained from the previous experiment, under the influence of the drug on Spirostomum, two specific conditions were pinpointed to scan the protein synthesis during regeneration.

After a 12-hour pre-treatment with 100 µg/ml actinomycin-D the regeneration was started with ^{14}C-isoleucine (1 µg/ml) label. Although the protein synthesis was quite negligible in the first two hours, it accelerated up to reach a peak at the 6th hour of regeneration (Fig. 8). Suddenly a 90 per cent fall of the incorporation occurred in the next two hours. Further onwards a 4-hour rise followed by a 4-hour fall indicated a second peak of activity at the 12th hour of regeneration. Beyond 16 hours, the protein synthesis was negligibly small.

In the other experimental set, however, without radiolabel the regeneration was started after 12 hours of pre-treatment with 100 µg/ml drug and continued. Assuming a complete inhibition of fresh RNA synthesis by 12 hours of regeneration, as noticed in experiments for RNA, the radio-precursor for protein was given at this stage from the 12th hour continuously. It was

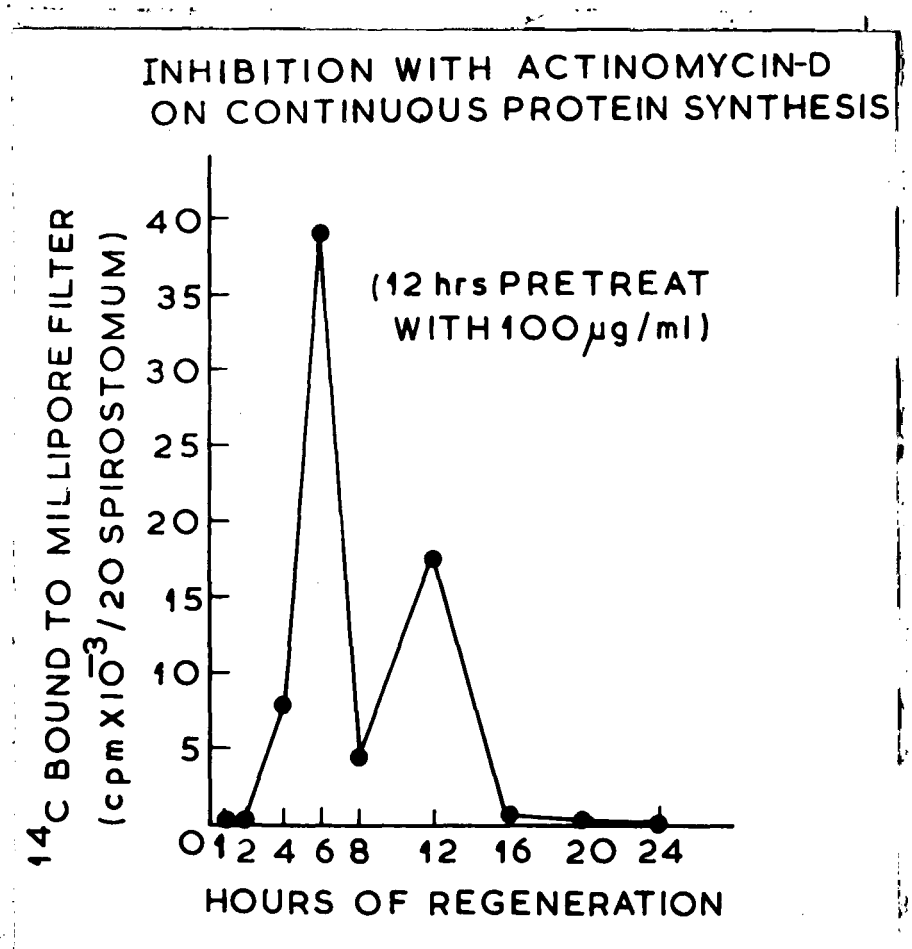

FIG.8: Pattern of continuous incorporation of ^{14}C-isoleucine (1 µl/ml) into protein in actinomycin-D pretreated regenerating Spirostomum.

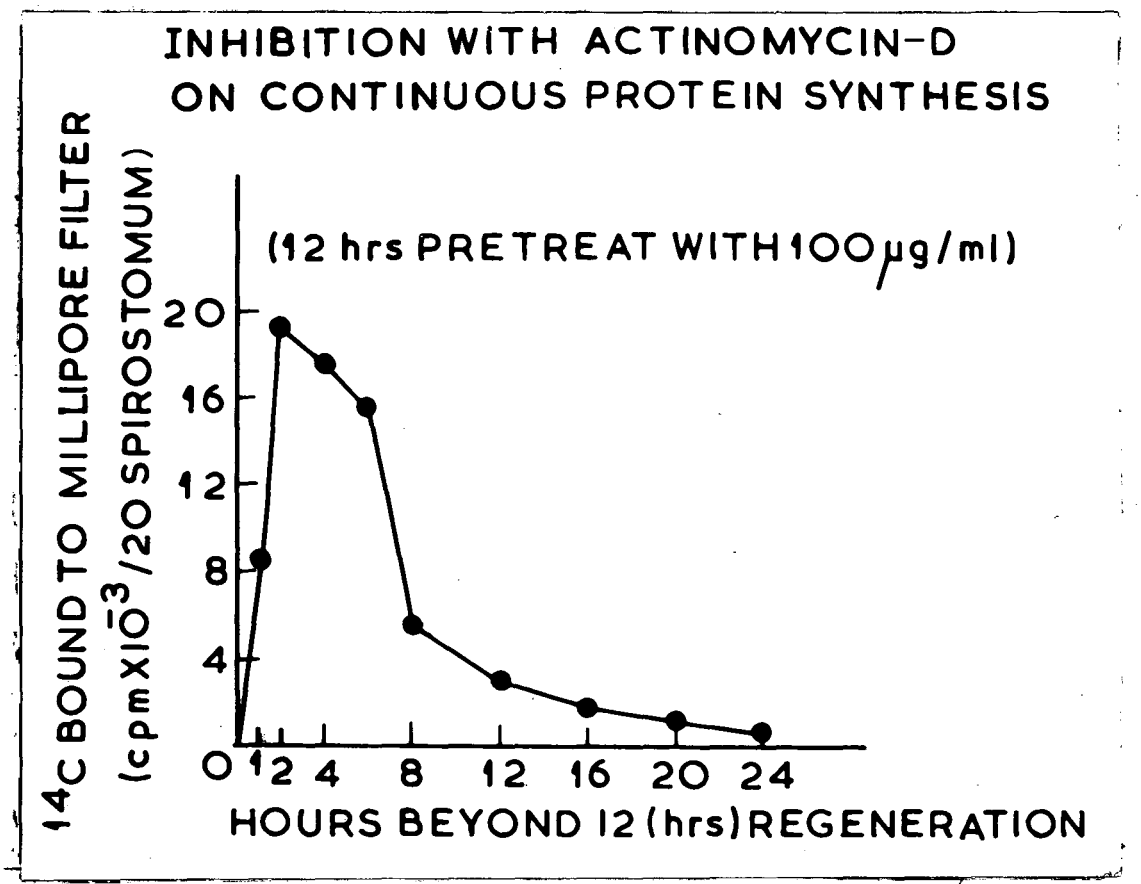

FIG.9: Pattern of continuous incorporation of ^{14}C-isoleucine (1 µl/ml) into protein in RNA synthesis inhibited regenerating Spirostomum.

observed that the amino acid got sufficiently incorporated and showed a peak of activity after two hours of labelling. Beyond this point the activity gradually slowed down for 4 hours followed by 2 hours rapid decrease (Fig. 9). Thereafter a slow declination brought down the protein synthesis to negligible state by 20 hours from the point of RNA inhibition. It was also noted that the occurrence and magnitude were approximately same for the peak of this experiment and the second peak of the previous experiment.

DISCUSSION

The life-style of this cell-born and cell-matured ciliate *Spirostomum ambiguum* has been uased in this study to extend the frontier of cytoregeneration at the level of macromolecular events concerning the transcriptional and translational aspects. Certain biochemical issues in the induction, organization and genomic control of subcellular regeneration indicated from DNA metabolism during the span of its restitution from transected half-fragment have been substantiated in terms of RNA and protein metabolism (Jeymohan, 1975). The molecular mechanism underlying the process of regeneration in ciliate has been observed to have certain range of similarity with the multicellular regenerating systems like *Hydra*, *Planaria*, etc. where the involvement of many a cell is, otherwise, a prerequisite. *Spirostomum* has provided certain clues regarding the genomic control of growth and regeneration at cellular level.

1. RNA SYNTHESIS IN NON-REGENERATING SPIROSTOMUM

The pattern of RNA metabolism observed from the above studies with continuous incorporation of ^{3}H-uridine

in non-regenerating Spirostomum shows the capability of the organism to continue its growth in the face of initial starvation. After undergoing a starved day, it seems able to synthesise fresh RNA to maintain the cell cycle events, but possibly fails to complete the process during further hours of starvation. In this condition the amount of synthesis records essentially two pronounced phases of RNA metabolism over a period of 36 hours. A considerable amount of mRNA is built up during the first phase while in the second phase the degradation of RNA molecules generally occurs. Although in want of nutrients, the organism tends to complete the cell cycle duly and this tendency is reflected in the first phase of RNA synthesis and hence this phase can be referred to as the synthetic or growth phase. However, a clear situation of starvation is noticed in the second phase where the organism loses the synthetic capability progressively and the growth is concomitantly hampered. This situation can be taken as the phase of starvation.

As evident from Fig. 2, the synthetic phase continues over a period of 16 hours beyond the initial 24 hours starvation. This feature suggests that in normal conditions the growth of the organism would have progressed

well till 40 hours beyond which its cell division could have occurred. The maximum incorporation at this 40th hour of starvation indicates a peak phase of its cell cycle events and apparently this period can be considered as the S-phase of the cycle. Another fact of importance in the growth phase is the non-uniform rate of RNA synthesis which initially being very rapid assumes a small increase followed by a second step of rapidity. The two stage growth phase occurring 8 hours apart could be due to certain periodic cellular drive prior to the S-phase. It seems that the organism could not mature for a division and rather the long hours of starvation could reverse the metabolism to a pattern of anabolism just after the peak phase of growth.

Kinetic studies of RNA synthesis during the period of growth in systems like HeLa cells (Ringertz, et al, 1970), Neurospora (Alberghina, et al, 1975), Xenopus (Hallberg and Smith, 1975), Bacillus (Testa and Rudner, 1975), yeast S 288 C spheroplasts (Hynes and Phillips, 1976) and Hydra (Haynes, 1973; Venugopal, 1978) have clearly established that the growth rate requires a proportional increase in the rate of RNA synthesis. Out of these transcriptory products the majority of the RNA population amounts to ribosomal

RNA. Enhanced accumulation of RNA during S-phase of cell cycle has been reported in ciliates (Rao and Prescott, 1967) and yeast (Mitchison, et al, 1969). Likewise, in the growing Spirostomum the RNA synthesis rate must be sufficient to carry on the development. Besides this, it is possible that some of the RNA synthesised during this period may remain stable till a later stage when these templates are activated and utilised towards the stability and maintenance of the organism.

The earlier termed starvation phase of Spirostomum is also not a continuous decceleration in the rate of RNA synthesis. The breakdown occurs in steps of 8 hours and slowly so much so that fresh RNA remains available for about a day more in the starvation. Within four to eight hours beyond the S-phase peak, a constancy of incorporation observable even in this anabolic phase could pertain to another period of growth prior to division. In this condition of long starvation further growth and division are unlikely, but the organism manages to pull on for quite a long time during which the turning over of earlier synthesised RNA pool looks a possibility.

Starvation due to lack of various nutrients and amino acids has been established as a major parameter for the

degradation of RNA molecules in E. coli cells (Gallant and Harada, 1969; Varney, et al, 1970; Kaplan and Apirion, 1975). All fractions of RNA reduce in their contents during the starving period (Midgley and Smith, 1974). The breakdown of the preformed RNA in the starving cells has been reported to be a slower process by Erlich, et al (1975), Hackett, et al, (1977) and Venugopal (1978). In case of starving Spirostomum, a similar situation seems to be prevalent to justify the reduction in the rate of fresh RNA synthesis and consequent turnover of preformed templates for maintaining its stability over an appreciable period of time.

2. RNA SYNTHESIS IN REGENERATING SPIROSTOMUM

Right from the period of amputation the regenerating half-fragment of Spirostomum has progressively shown increase in the RNA content and continued through varying phases of development over a period of 36 hours. This information can be enough to say that there exists a close relation between RNA synthesis and regeneration in this organism. One of the remarkable features observed is its ready response to the mechanical infliction of wound upon its body whose fragment is neither activated nor depressed to synthesise RNA immediately after amputation. This behaviour

of Spirostomum finds close parallelism with the other ciliate Blepharisma (Giese, 1970). But it is known that the postamputational transcription in Hydra (Mookerjee and Sinha, 1967; Clarkson, 1969; Venugopal, 1978; Rattan, 1978) and lower vertebrates (Reyer, 1962; Hay, 1968; Morzlock and Stocum, 1971) gets affected by mechanical operation.

The sequences of increase and turnover in the pattern of RNA metabolism here imply that regeneration involves temporal cellular activities and complementary molecular make-up. The peak of activity around 16 hours after amputation (Fig. 3) can be the expression of some elaborative morphogenetic structuring during regeneration of Spirostomum. By this hour of regeneration, the magnitudes of DNA (Jeymohan, 1975) and protein (Fig. 5) are also recorded to be maxima. While the restitution gets under way, it is likely that the elevation of RNA content can consist of different classes of RNA and that some amount of it can remain as stable template in the system. Beyond this point the organism continues a normal growth in starving situation. Its efforts of maintaining the stability are observable from the stationary phases and gradual breakdown of RNA synthesis. During the declining period the turnover of accumulated RNA is likely to occur and it is more so because the growing

cells characteristically begin to turnover the accumulated RNA only upon attaining confluency (Emerson, 1971).

On the other hand, the hourly pulse labelling studies (Fig. 4) in regenerating Spirostomum shed important light on the stability and turnover of RNA molecules synthesised during that hour. During any hour of regeneration the marginal difference between the rates of RNA synthesis in continuously labelled and pulse labelled organism gives an approximate idea about the amount of stability of the macromolecule at that period and a comparison of the pulse label with its previous hour goes well to indicate the amount of turnover in the period considered (Venugopal, 1978). In this study an exact estimation of the accumulation and turnover of various population of RNA cannot be made but some suggestions can be brought out from comparative evidences in other systems. In this connection, it becomes worthy to note the reports on the hn RNA as a major partner having very short half-life (Darnell, 1968; Rubinstein and Clever, 1972), the age-dependent degradation of RNA (Elicheiri, 1976), the stage-specific RNA synthesis (Britten and Kohne, 1968) and the conformational rearrangement of RNA (Meier and Brownstein, 1976). Further lines of argument can be

brought from the concept of temporal element in gene expression (Planta, et al, 1976) and post-transcriptional modifications of messenger RNA (Sarkar, et al, 1973; Buckingham, et al, 1974; Dolecki, et al, 1977). Spirostomum as a regenerating system appears to carry forward its growth and development with major participation of messenger RNA both at transcriptional and post-transcriptional levels and this fact finds confirmation in the protein synthesis and transcription inhibition studies. At this stage, it can also be said that the regulation of RNA synthesis, its accumulation and the regulation of cell growth and division are highly integrated processes.

3. PROTEIN SYNTHESIS IN REGENERATING SPIROSTOMUM

The well conceived fact that protein synthesis is critically essential to regeneration has been observed in this case of continuously labelled regenerating half-fragments of Spirostomum ambiguum. From the very onset of regeneration in this system, accelerating accumulation of protein takes place and throughout the span the synthesis pattern shows a variable kinetics. Such an extensive pattern of protein synthesis and degradation cannot but be due to an able machinery operating at the molecular level of organization and the presence of the mechanism is indicated in the reports of Benz, et al (1977) and Shiokawa, et al (1977). Hereby,

a feasible translational control on cytoregeneration gains ground.

Another novelty which draws attention is the close parallelism between RNA synthesis (Fig. 3) and protein synthesis (Fig. 5) during the regeneration process in Spirostomum. Peaks, elevations and depressions of synthetic activities for both RNA and protein occur in synchronous phases of restitution and growth. The proportionate syntheses of RNA and protein indicate the existence of a fast operating translational control in the system. It has been shown that the rate limiting agent for protein synthesis is the messenger RNA (Johnson, et al, 1974) and not the ribosomal RNA (Stanners and Becker, 1971). It has also been suggested that neither the rate of transcription (Mauck and Green, 1973) nor the stable pool of mRNA (Abelson, et al, 1974) regulates the protein accumulation in the cell, but apparently the rate of conversion of Poly A(+) nuclear RNA into cytoplasmic messenger RNA does so (Johnson, et al, 1976).

In regenerating Spirostomum the participation of fresh RNA in protein synthesis is amply clear here. During the present range of regeneration the status of stable mRNA is not clear excepting its possible turnover at

long hours of starvation. The profile of protein synthesis gives corroborating informations on important phases of restitution and development in the organism. As far as the stability of the synthesised proteins no information can be generated unless the pulse labelled protein synthesis studies are at hand.

4. INHIBITION STUDIES WITH ACTINOMYCIN-D

The most familiar action of actinomycin-D to inhibit the DNA-dependent RNA synthesis (Kirk, 1960: Goldberg and Robinowitz, 1962; Reich, 1963) has been considered here to specifically estimate the transcriptional control on cytoregeneration. When actinomycin-D treatment is given to Spirostomum, the RNA synthesis is delayed or inhibited in accordance with the concentration of drug and the length of exposure. Consequently its regeneration and growth processes are proportionately retarded. Quite a few reports on inhibited cell division (Lazarus, et al, 1964; Whitson and Padilla, 1964) and inhibited regeneration (Giese, 1970; Venugopal, 1978) gives strength to this argument. As such by a specific condition of the drug treatment the cell can be completely devoid of fresh RNA synthesis and at this stage it is likely that the cell may collapse unless otherwise some alternate possibility for the survival is imminent. Survival beyond such a crucial juncture gives definite informations on the existence and breakdown of masked mRNA in the system.

In the present investigation a pre-amputational 12 hours in continuance with post-amputational 12 hours exposure to 100 µg/ml actinomycin-D has been found to inhibit the transcription process of regenerating Spirostomum (Fig. 7). The survey of protein synthesis in the RNA synthesis inhibited organism reveals the presence of long-lived templates which mainly stand responsible to carry on the protein synthesis as far as 20 hours beyond the inhibition point (Fig. 9). This point of view on the turnover of the preformed stable mRNA also proposes a characteristic half-life of 20 hours for mRNA in Spirostomum regeneration. However, an approximate nature of this half-life is agreeable in the sense that actinomycin-D yields inexact estimates of mRNA half-lives (Stewart, 1975) and that actinomycin-D resistant RNA synthesis occurs in animal cells (Stern, et al, 1973; Sargent and Raff, 1976). In any case, it remains obvious that the metabolic fate of RNA is expressible in terms of a long-lived population besides the freshly synthesised ones. In particular, the stability associated with mRNA can be viewed as its polyadenylation which is known to shorten with age of mRNA till a minimum size when the RNA molecule gets degraded (Sensky, et al, 1975; Levy, et al, 1975).

In view of the foregoing discussion, the importance of RNA synthesis as a subcellular macromolecular event during the regeneration process in Spirostomum ambiguum has been amply established. But the detailed regulation and pathways of the synthetic behaviour of the regenerating organism remains yet elusive. A comprehensive conclusion on cytoregeneration can be derived only through a coordinated analysis of genomic, transcriptional and translational macromolecules from both biochemical and morphogenetic facets of Spirostomum.

SUMMARY

1. RNA and protein have been studied in the ciliate Spirostomum ambiguum following its regeneration from the transected half-fragment level under the influence of starvation and actinomycin-D by using radio labelling techniques.

2. Continuous ^{3}H-uridine incorporation in non-regenerating Spirostomum shows a growth (synthesis) phase followed by a slow starvation (degradation) phase. S-phase of its cell cycle occurs around 40 hours growth, the period preceding and succeeding that showing 8 hourly events of synthesis.

3. With continuous ^{3}H-uridine labelling, the regenerating Spirostomum exhibits its ready response to mechanical amputation and its close association with cellular activities which assume peak by 16 hours after amputation.

4. The pulse labelling survey when compared with continuous labelling indicates the extent of stability and turnover of RNA during various hours of regeneration in Spirostomum.

5. During the cytoregeneration, a fast operating translational control in Spirostomum is in action in relation to RNA dependent protein synthesis.

6. A pre-amputational 12 hours in continuance with post-amputational 12 hours exposure to 100 µg/ml actinomycin-D inhibits the transcription process of regenerating Spirostomum.

7. In the actinomycin-D influenced regenerating Spirostomum, protein synthesis continues to occur for 20 hours beyond the point of inhibition of fresh RNA synthesis and thereby reflects the turnover of preformed stable mRNA whose half-life is recorded to be approximately 20 hours.

8. The relevance of RNA synthesis particularly as a subcellular biochemical event during cytoregeneration in ciliate has been discussed.

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