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STOLON VS. HYDRANTH DETERMINATION IN PENNARIA TIARELLA PLANULAE:

A ROLE FOR DNA SYNTHESIS

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Larvae of the gymnoblastic hydroid Pennaria tiarella develop and metamorphose in a synchronous and predictable manner. They progress from ovoid pre-planulae 12 hours after fertilization to definitive, filiform planulae by 24 hours of development. Planulae are free living for a defined period of time (approximately 5 days at 21°C) and then metamorphose with the anterior regions of larvae becoming stolons, the posterior regions forming hydranths. The uniformity of this developmental pattern allows the utilization of Pennaria as an experimental system to investigate the principal morphogenetic determination occurring in hydroid larvae, that of stolon versus hydranth.

Previous autoradiographic studies (Haynes et al., 1974) revealed regional patterns of ³H-thymidine incorporation during larval development in Pennaria. Early planulae exhibited concentrations of precursor incorporation in the epidermis and posterior portions of the larvae. In later planulae, however, the greatest level of incorporation was reported in the gastrodermis and at the anterior regions of the larvae. Because the autoradiographic observations are consistent with a possible involvement of DNA synthesis and/or mitotic activity in the regional commitment to stolon and hydranth development that occurs in planula larvae, attention was focused on the role of DNA synthesis in this morphogenetic determination.

Initially, the kinetics of ³H-thymidine incorporation into DNA in P. tiarella had to be examined to verify the efficacy of this probe to analyze synthetic activity in this organism. Once linearity of incorporation was established, morphological and biochemical events of larval development were compared in unmanipulated

planulae, half planulae and planulae exposed to an inhibitor of DNA synthesis (hydroxyurea) or a mitotic blocking agent (colchicine).

MATERIALS AND METHODS

Sexually mature colonies of *Pennaria* were collected at Flatts Islet, Bermuda. After collection all colonies and subsequent larvae were maintained at $21 \pm 1^\circ\text{C}$ in Millipore-strep-sea water (MSSW). MSSW is Bermuda Biological Station sea water that has been passed through both a glass fiber filter and Millipore filter (0.45μ pore size) and to which streptomycin sulfate ($10 \mu\text{g/ml}$) and sulfadiazine ($10 \mu\text{g/ml}$) have been added. To stimulate and synchronize spawning 8 inch fingerbowls containing 1 male and 5 female fronds were placed in light tight containers. Cleaving embryos were harvested 5 hours later, washed to remove excess sperm and placed in fresh MSSW. Developing larvae were transferred to fresh MSSW daily.

Surgical Manipulation

To evaluate the capacity of half planulae to incorporate ^3H -thymidine into DNA and to complete larval development, planulae were sectioned transversely at 12 hour intervals from 12-96 hours of development. The cut was made so that anterior and posterior halves each contained similar amounts of tissue, as measured by protein content. Anterior and posterior half planulae were maintained separately in MSSW which was changed daily.

Labeling Procedures

Incorporation of ^3H -thymidine (methyl-labeled, specific activity 6.7 Ci/mM , New England Nuclear) was selected as a marker for DNA synthesis. Larvae were exposed to specific concentrations of ^3H -thymidine as a component of the culture solution for a defined time interval. After removal from the radioactive solution, planulae were washed 3x over a 5 min period in non-radioactive thymidine ($400 \mu\text{M}$) and fixed in fresh Carnoy's fixative.

DNA analysis was performed according to the method of Schneider (1945). Fixed planulae were uniformly homogenized in 5% ice cold trichloroacetic acid (TCA) and refrigerated at least 4 hours at 2°C . The TCA insoluble material was washed twice with ice cold 5% TCA, extracted once with 95% ethanol and once with 95% ethanol:ether (3:1). The nucleic acids were hydrolyzed in 5% TCA for 20 min at 90°C . After cooling to 4°C the remaining macromolecular components were removed by centrifugation and the supernatant divided into 2 aliquots, one for DNA determination and one for measurement of

radioactivity. Although the Schneider method does not separate DNA from RNA, evidence that, in hydroids, the labeled material isolated by this procedure is DNA has been presented by Lesh-Laurie *et al.* (1976). Using DNase digestion, it was demonstrated that >90% of the labeled material was enzyme sensitive.

Concentrations were determined using the Burton (1968) modification of the diphenylamine reaction. Standard curves were plotted using calf thymus DNA (Type I sodium salt highly polymerized, Sigma) and were linear to concentrations as low as 2.0 µg/ml. Liquid scintillation counting was executed in Bray's solution (Bray, 1960). Although data from only a single experiment will be presented each biochemical experiment was performed at least once during each of 2 summers. If the results from these experiments were equivocal, additional experiments were executed with the data presented representing neither the high nor the low extreme.

Inhibition Studies

Clarification of any relationship existing between DNA synthesis and/or mitotic activity and stolon or hydranth determination in *P. tiarella* planulae requires observation of the latter processes in the absence of DNA synthesis and/or mitosis. Hydroxyurea (HU) was selected as an inhibitory agent for DNA synthesis. Hydroxyurea reportedly interferes with the reduction of ribonucleotides to deoxyribonucleotides (Young and Hodas, 1964). This effect is temporary and is overcome when the agent is removed. In addition HU has been effective in inhibiting DNA synthesis in hydroids (Clarkson, 1969; Lesh-Laurie *et al.*, 1976).

Planulae were pulsed with 1 mg/ml HU for 96 hours at 12 hour intervals from 12-96 hours of development. Following exposure to the inhibitory compound larvae were washed several times with MSSW and allowed to continue their development in MSSW. The 1 mg/ml concentration was chosen following preliminary biochemical investigations (see Results) which revealed it to be a dosage capable of severely restricting DNA synthesis, yet allowing 100% survival.

Colchicine was selected as a mitotic inhibitor. It binds reversibly to microtubular protein (Borisy and Taylor, 1967a, 1967b), interfering with mitosis through its effect on spindle microtubules. In addition, it reportedly has been employed successfully to induce mitotic arrest in hydroid cells (Ham and Eakin, 1958; Corff and Burnett, 1969). Larvae were pulsed with 0.2 mg/ml colchicine for 24 hours at 12 hour intervals from 12-96 hours of development. After exposure to the alkaloid, planulae were washed several times in MSSW and allowed to continue their development in MSSW. This effective dosage was determined following preliminary experiments

which revealed it to be one capable of arresting mitosis, and ultimately restricting ^3H -thymidine incorporation into DNA.

RESULTS

Kinetics of ^3H -thymidine Incorporation

To establish the kinetics of ^3H -thymidine incorporation in *Pennaria* planulae groups of 20 12 hour and 24 hour planulae were incubated in $10\ \mu\text{Ci/ml}$ ^3H -thymidine for 10 min intervals from 10-90 min. Specific activities (cpm/ μg DNA) were computed for each 10 min interval and a representative experiment for each developmental stage is plotted graphically in Figure 1. Regression lines were calculated and at both developmental stages the relationship between exposure time and specific activity was linear with the x and y intercepts not significantly different from zero. Therefore, *Pennaria* planulae incorporate ^3H -thymidine from the culture medium at a constant rate over a 90 min period.

To determine whether the rate of ^3H -thymidine incorporation varied with the concentration of externally supplied precursor, 20 planulae were incubated 1 hour in varying concentrations of a ^3H -thymidine solution of constant specific activity. The results from one experiment are shown in Figure 2, with the calculated regression line. Although the rate of ^3H -thymidine incorporation was a linear function of external precursor concentration over the range of concentrations examined, the y intercept was not zero. This departure from the predicted result is probably correlated with the mechanism of initial thymidine uptake by the organism; however, no data are presently available that address this problem.

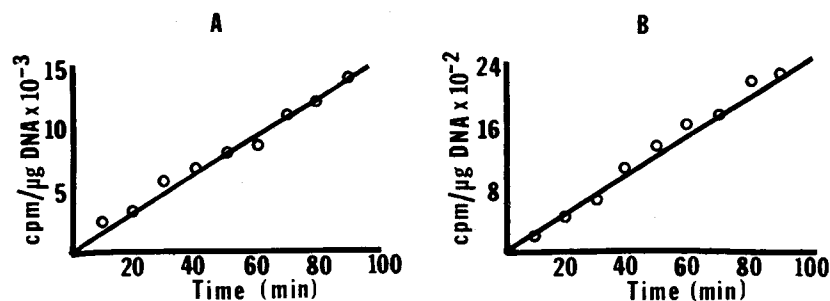


Figure 1. Kinetics of ^3H -thymidine incorporation into DNA. (A). Incorporation into 12 hour planulae, (B). Incorporation into 24 hour planulae.

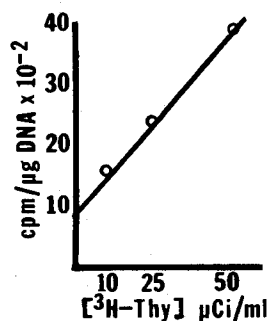


Figure 2. Influence of [³H-thy] on its incorporation into DNA in 24 hour planulae.

To confirm the autoradiographic data which suggested considerable and regional incorporation of ³H-thymidine into DNA during larval development in *Pennaria*, the pattern of ³H-thymidine incorporation was examined at 12 hour intervals from the formation of the definitive planula (24 hours of development) until the initiation of metamorphosis (96 hours of development). Embryos from a single spawn were pulsed in groups of 50 with 50 µCi/ml ³H-thymidine for 1 hour immediately preceeding each 12 hour interval of development. After the exposure interval the larvae were washed in non-radioactive thymidine, fixed immediately in Carnoy's fixative and their DNA subsequently isolated. The resulting pattern of incorporation is shown in Figure 3, where it may be compared with data obtained from half planulae.

Normal, unmanipulated larvae always exhibited a bimodal pattern of ³H-thymidine incorporation into DNA. Incorporation was highest early in larval development (24 hours) and then dropped precipitously over the next 2 days. A second but smaller burst of incorporation occurred late in larval life (84 hours) immediately preceding metamorphosis. The relative height of the latter elevation in incorporation varied from experiment to experiment but it was always < ½ the incorporation level observed at 24 hours of development.

Organizational and Synthetic Capacities of Half Planulae

To qualitatively determine whether the regional pattern of ³H-thymidine incorporation observed autoradiographically was reflected in the developmental potential of specific portions of the larva, planulae were sectioned transversely at 12 hour intervals from 12-84 hours of development in the manner described previously. From the data (Table I) it was evident that the anterior halves of

planulae from any developmental stage possessed the ability to reorganize entire planulae, capable of undergoing a normal, but temporally delayed, metamorphosis. Alternatively, posterior half planulae were incapable of reconstituting metamorphosing individuals and all eventually succumbed. Both types of half planulae remained fully motile throughout the experiment and evidenced no sloughing of cells.

If one examined the capacity of half planulae to incorporate ^3H -thymidine into DNA, a picture similar to the morphological results emerged (Fig. 3). To facilitate both developmental synchrony and accuracy in sectioning the number of animals required to perform the biochemical experiment, a single developmental stage, the 24 hour planula, was selected for this analysis. Three hundred 24 hour planulae were sectioned transversely and allowed to heal. Fifty anterior and 50 posterior half animals were then exposed to $50\mu\text{Ci/ml}$ ^3H -thymidine for 1 hour, fixed in Carnoy's fixative and their DNA extracted at each 12 hour interval from 36-96 hours of development. Specific activity values (Fig. 3) revealed that the anterior halves of 24 hour planulae maintained the capacity to synthesize DNA and exhibited a pattern of synthesis virtually identical to unmanipulated planulae within 24 hours of being excised (48 hours of development). The posterior halves of 24 hour planulae however, continued to synthesize significant amounts of DNA for 12 hours following amputation, but then their capacity to incorporate ^3H -thymidine into DNA declined until barely perceptible amounts of incorporation were detected 3 days after isolation (96 hours of development).

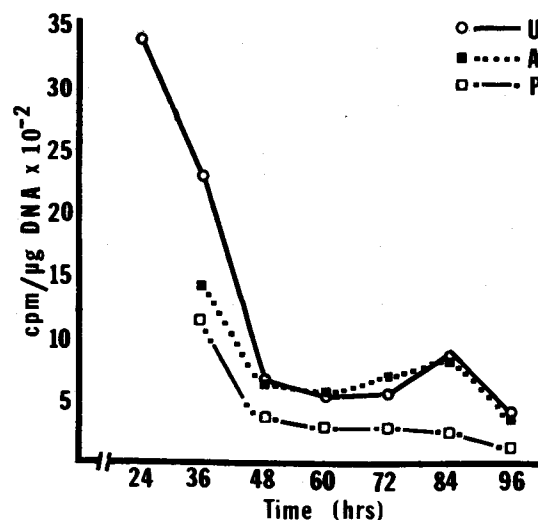


Figure 3. ^3H -thymidine uptake during larval development in unmanipulated planulae, anterior and posterior half planulae.

TABLE I

Survival of half planulae

Developmental stage	No. animals	No. animals completing metamorphosis within 2 weeks
Unmanipulated	20	20
12 hour anterior	22	20
12 hour posterior	25	0
24 hour anterior	25	22
24 hour posterior	25	0
36 hour anterior	10	6
36 hour posterior	10	1
48 hour anterior	10	3
48 hour posterior	10	0
60 hour anterior	10	7
60 hour posterior	10	0
72 hour anterior	10	7
72 hour posterior	10	0
84 hour anterior	7	5
84 hour posterior	7	0

³H-thymidine Incorporation and Developmental Potential
in Planulae Exposed to HU or Colchicine

It is apparent therefore, from previous results, that despite the occurrence of regional stolon and hydranth determination and a regional pattern of ³H-thymidine incorporation during larval development in *P. tiarella*, DNA synthesis is not sufficient to allow the expression of stolon or hydranth morphogenesis. If it were, posterior half planulae would predictably be able to form some definitive hydranth parts. The question of whether DNA synthetic activity is even necessary for these morphogenetic events can be approached by examining the potential for stolon and/or hydranth determination when DNA synthesis is severely restricted.

The ability of planulae exposed to 1 mg/ml HU to incorporate ³H-thymidine into DNA fell quickly and markedly, so that by 4 hours of incubation in the inhibitor the level of incorporation into 12 hour planulae had dropped to <1/9 of that observed in unmanipulated larvae (Fig. 4). Longer incubation periods caused no significant further reduction in incorporation. The developmental potential of HU-treated planulae exhibited a clear and distinct limitation;

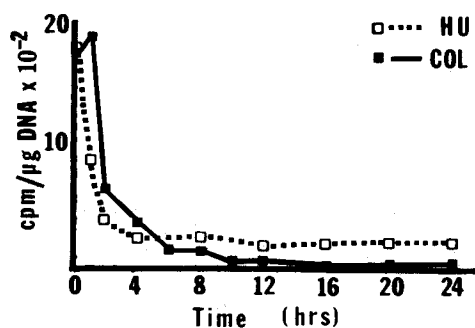


Figure 4. Influence of HU and colchicine on ^3H -thymidine incorporation into DNA.

TABLE II

Developmental potential of planulae exposed to HU
n = 20

Developmental stage when placed in HU	Developmental potential expressed 10 days after removal from HU	
	Stolon and upright, no hydranth	Normal metamorphosis
12 hours	16	0
24 hours	20	0
36 hours	13	0
60 hours	10	0
72 hours	16	0
84 hours	11	0

hydranths failed to form (Table II). Groups of 20 planulae incubated 4 days in 1 mg/ml HU at developmental stages from 12-84 hours of development revealed a complete absence of hydranth formation 10 days after removal from HU. These planulae attached normally to the substratum, elaborated stolons and uprights, yet definitive hydranths did not develop. On occasion abortive blisters appeared at the apex of the upright, but neither tentacles nor hydranth morphology ever became evident.

Because numerous hydroid cell types reportedly possessed long and variable G₂ periods (David and Campbell, 1972; Campbell and David, 1974) it was possible that DNA synthesis and mitosis represented distinctly separable events in *Pennaria* planulae. Therefore, stolon and hydranth determination were also investigated in the presence of the mitotic inhibitor, colchicine. Interestingly, colchicine proved to be a more potent, although slower, inhibitor of ³H-thymidine incorporation into DNA than HU (Fig. 4). Twelve hour planulae placed in 0.2 mg/ml colchicine maintained normal DNA specific activity levels for at least 1 hour; after that time the level of ³H-thymidine incorporation dropped to <1/20 the value observed in unmanipulated larvae.

The morphological manifestation of the colchicine response was also particularly significant. Several planulae pulsed with colchicine for 24 hours during periods of elevated ³H-thymidine incorporation into DNA (i.e. early or late larvae) formed hydranths without attaching to the substratum or exhibiting demonstrable evidence of stolon growth (Table III).

TABLE III

Developmental potential of planulae exposed to colchicine
n = 15

Developmental stage when placed in colchicine	Developmental potential expressed 10 days after removal from colchicine	
	Normal metamorphosis	Normal hydranth formation, no attachment or stolon development
12 hours	4	5
24 hours	2	2
36 hours	0	0
48 hours	0	1
60 hours	2	0
72 hours	0	1
84 hours	4	1
96 hours	1	2

DISCUSSION

It is evident therefore, that Pennaria planulae exhibit both a regional stolon and hydranth determination and regional patterns of DNA synthesis, as measured by ^3H -thymidine incorporation into DNA. Although DNA synthesis does not appear to be a sufficient process for morphogenetic expression, it is clearly a necessary one, as evidenced by the modifications in the normal elaboration of developmental potential observed in animals in which DNA synthesis was restricted. The morphogenetic significance of DNA synthesis in stolon versus hydranth determination in Pennaria larvae cannot be fairly assessed at this time, however. Autoradiographic and cell population studies corresponding to the biochemical experimentation are necessary to provide a cellular basis for the synthetic data. These studies are in progress and will be presented in subsequent papers.

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REFERENCES

- Borisy, G.G., and E.N. Taylor, 1967a. The mechanism of action of colchicine. Binding of colchicine- ^3H to cellular protein. J. Cell Biol., 34:525-533.
- Borisy, G.G., and E.N. Taylor, 1967b. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus. J. Cell Biol., 34:535-554.
- Bray, G.A., 1960. A single efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279-285.
- Burton, K., 1968. Determination of DNA concentration with diphenylamine. Pages 163-166 in L. Grossman and K. Moldave, Eds., Methods in Enzymology, Volume 12. New York, Academic Press.
- Campbell, R.D., and C.N. David, 1974. Cell cycle kinetics and development of Hydra attenuata. II. Interstitial cells. J. Cell Sci., 16:349-358.

- Clarkson, S.G., 1969. Nucleic acid and protein synthesis and pattern regulation in Hydra. II. Effect of inhibition of nucleic acid and protein synthesis on hypostome formation. J. Embryol. Exp. Morphol., 21:55-70.
- Corff, S.C., and A.L. Burnett, 1969. Morphogenesis in hydra. I. Peduncle and basal disc formation at the distal end of regenerating hydra after exposure to colchicine. J. Embryol. Exp. Morphol., 21:417-443.
- David, C.N., and R.D. Campbell, 1972. Cell cycle kinetics and development of Hydra attenuata. I. Epithelial cells. J. Cell Sci., 11:557-568.
- Ham, R.G., and R.E. Eakin, 1958. Time sequence of certain physiological events during regeneration in hydra. J. Exp. Zool., 139: 35-54.
- Haynes, J.F., R.G. Summers and M.I. Kessler, 1974. The application of autoradiography to developmental studies in the Hydrozoa. Amer. Zool., 14:783-795.
- Lesh-Laurie, G.E., D.C. Brooks and E.R. Kaplan, 1976. Biosynthetic events of hydrid regeneration. I. The role of DNA synthesis during tentacle elaboration. Wilhelm Roux' Archiv., in press.
- Schneider, W.C., 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., 161:293-303.
- Young, C.W., and S. Hodas, 1964. Hydroxyurea: Inhibitory effect on DNA metabolism. Science, 146:1172-1174.

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