DISCUSSION

INHIBITION STUDIES WITH ACTINOMYCIN D

The present experiment has shown that the regeneration of hypostome in hydra can be inhibited with actinomycin D at a concentration of 60 μg/ml of the medium after 12 hours of preamputational treatment, whereas a better differentiation of the basal disc is achieved in the same concentration of the drug. Datta (1968) and Clarkson (1969b) have suggested that there exists a stable mRNA or masked template material for initial hypostome determination. The failure of major cellular events typical for regeneration, which could be seen from 4 hours onwards in our experiment, suggests that the masked template material is switched on active for a limited time. It is assumed that the stable mRNA for hypostome differentiation is having a quicker turnover and that after 4 hours of regeneration, the determination is essentially associated with new messenger RNA synthesis. Conversely, the basal disc differentiation proceeded well to a large extent, even though actinomycin D could effectively suppress the RNA synthesis. It is speculated that the main protein synthetic activities associated with the basal disc regeneration are accomplished through a stable variety of mRNA. Activation of this stable template may occur when the normal state of stability and maintenance of the
basal disc is disturbed. The lack of formation of mesoglea at the regenerating site and absence of development of fibrous materials in the basal disc suggest that essential transcription should take place for their attenuation to a fullfledged state.

The remarkable feature in our experiment is that the major cellular activities involved in the process of regeneration at both the sectors of hydra were found quite contrasting. It has been established that, for the regulation and regeneration in hydra, there exists an interaction of two diffusible gradients (Burnett, 1966; Wolpert, Hornbruch and Clarke, 1974), which runs from oral to aboral tip in an order of unipotential area (Mookerjee and Sinha, 1967). It is assumed here that there may also exist a gradient along the axis of hydra with respect to the distribution of informational templates, the succession of which might be related to stability.

INHIBITION STUDIES ON BUD MORPHOGENESIS

The existence of a stable mRNA or a masked template material for initial hypostome determination in hydra has been suggested by Datta (1968) and Clarkson (1969b). The failure of the bud rudiments to differentiate upon a substantial inhibition of RNA synthesis as noted in our experiments
indicates that essential transcription should take place for their attaining a full-fledged stage. It is suggested that the main protein synthetic activities involved in the bud morphogenesis are accomplished through new DNA-dependent RNA synthesis. The development of nematocysts and morphogenetic activities of gland cells and interstitial cells are also found to be associated with the new DNA-dependent RNA synthesis. The transcription phenomenon involved in the differentiation of these cells may probably be inhibited with a lesser concentration of actinomycin D. However, it is concluded that the inhibition of RNA synthesis affects the gland cells which are then deprived of capacity to proliferate and giving rise to interstitial cells and nematocysts. The delayed but normal differentiation of the buds obtained in spite of treatment with 15 µg/ml actinomycin D suggests that this concentration of the drug is not adequate to inhibit the necessary RNA synthesis required for bud morphogenesis.

INHIBITION STUDIES WITH CORDYCEPIN

The striking resemblance in the cellular activities between cordycepin treated and actinomycin D treated hydras suggest that basically both the drugs have caused a similar type of effect on the differentiating cells lines in the organism.
It could be noted that the differentiation of hypostome failed to occur while the basal disc continued to differentiate inspite of treatment with the drug. Thus, the inhibition studies with cordycepin has further emphasized the fact that differentiation of hypostome is essentially dependent on new DNA-dependant RNA synthesis while the restitution of basal disc is accomplished through activation of stable messenger RNA. The experiment provides further clarification that the developmental activities of gland cells, differentiation of interstitial cells and synthesis of nematoblasts and other types of cells from interstitial cells are primarily dependant on protein synthesis accomplished through fresh templates of mRNA.

The possibility of a positive molecular mechanism operative in bringing out a secondary basal disc in the axis of cordycepin treated hydars has been discussed elsewhere in this thesis. However, the cordycepin induced changes did not appear to last long. Conversely, the organisms disintegrated in the medium, possibly due to the primary effect of the drug. Therefore, it was not possible to rare animals which had a secondary basal disc. Similarly, due to the death of the organisms it was also not possible to see whether the proposed molecular mechanism was operative in the creation of further more basal discs in the hydra.
EFFECTS OF CYCLOHEXIMIDE ON REGENERATION

Analysis on regeneration subsequent to treatment with cycloheximide in hydra revealed two important features; one depicting the response of the differentiating cells to the inhibitory effect of the drug and the other elucidating the mechanism in which the interstitial cells take their origin and further differentiate into the adult state of that cell in the organism. It could be seen that even during the time immediately after amputations, cells at the active site of regeneration had lost their capacity of integration and failed to consolidate themselves in initiating cellular activity so much so that the entire restitution process was found to suffer a set back. Ultimately we can observe that both hypostome and basal disc differentiation do not take place to a full extent in cycloheximide treated hydias and the organisms finally disintegrate in the medium by about 18 hours after the removal of parts. Thus, the failure of the animals to reconstitute the lost parts and their subsequent disintegration must essentially be attributable to the inhibitory effect of cycloheximide on protein synthesis. Biochemical studies (describes elsewhere in this thesis) have shown that when cycloheximide is used at the same concentration i.e. 75 µg/ml of culture medium, the incorporation of $^{14}$C-isoleucine is suppressed by about 95%. 


Failure of hypostome and also developing bud (Venugopal and Mookerjee, 1977) to differentiate upon inhibition of protein synthesis at transcriptional level has been shown to occur previously. Attempts are underway to characterize the proteins required for the morphogenesis in hydra but it is tempting to assume that these proteins which inhibited formation are basically required for the cellular integration in the reconstitution processes and also to continue morphogenesis. Similarly, whether the developmental changes noted in cycloheximide treated hydas are also caused by some other effects of the drug other than inhibition of translation is yet to be looked into.

As mentioned previously is could be seen that treatment with cycloheximide in regenerating hydas had greatly helped in understanding the mechanism of proliferation of interstitial cells and their further differentiation. This phenomenon might be taking place in a non-treated hydra ordinarily. Since cycloheximide had caused an inhibitory effect on this process only at a later stage, the initial stages were more predominantly seen. In this regard, it is noteworthy to consider that the gland cells in the endoderm of the hydra are the progenitors of the small interstitial cells which in future are capable of proliferation and differentiating into other somatic cells types. Soon after the amputations are made, activities
in gland cells can be noted in treated hydras and further analysis would reveal that these activities mainly account for the transformation of the nucleus of the gland cells contained within them. In normal hydras such type of activities are not noted vigourously and thus it would carry home the idea that in the organism loss of any parts or organ is chiefly responsible for bringing about this type transformation. Similarly, such activities in gland cells are found uniform all through out the axis of the organism at this stage irrespective of the nature of the cut made. This suggests that during the early stages of regenerating, activation of gland cells as the first prerequisite in the restitution, is alike in the entire axis of the organism and as such no zone-specific activation occurs. During subsequent hours one can notice such changes taking place in cells at the periphery of the endoderm ultimately lead them towards transformation into small round cells occupying a place at the proximal part of endoderm close to the mesoglea. In many cases, it was noted that the gland cells are capable of giving rise to nests of cells amounting as many as 20 to 30 numbers. Activation of gland cells subsequent to amputation of parts in the organism has been shown to take place by Mookerjee and Bhattacharjee (1966) and occurrence of nests of cells close to the gland cells in regenerating hydras has been
noticed by Davis (1970). It is not known why such changes take place in the gland cells leading towards the origin of small cells but the probable reason is that such a mechanism of cell proliferation is required in order to replenish the cells which are lost from the organism due to the mechanical amputation or due to any other reasons. Moreover, many specialized cells in hydra are exhausted regularly (Mookerjee, 1972; Campbell, 1974) by their activities and therefore cells must be replaced periodically if the animal is to retain its size and integrity. One other significant change noticed was that while dedifferentiating and giving rise to interstitial cells, the gland cells lose most of its morphological and physiological characteristics. However, these changes are noted to take place due to the transformation of in the cytoplasm of the gland cells in which they were found to be more basophilic while at the periphery of the endoderm and gradually they acquire a granular nature upon transforming into small cells. It is yet to be analysed how this mechanisms are precisely taking place; however, it becomes fully conceivable that the gland cells in the endoderm remain as the seats of massive cellular reorganization. The structural accomplishment of interstitial cells takes place in the endoderm even though their occurrence is noted only in the ectodermal layer of the organism. Regarding structural changes it is
notable that after the gland cells give rise to a substantial amount of interstitial cells, membranes of the gland cells get atrophied and as such erosion take place in them. Davis (1970) has shown that gland cells which have completely dedifferentiated into interstitial cells and some which have started redifferentiation contained extremely low density and intact, empty evaginated plasma membranes. The mechanism by which the highly organized plasma membrane and probably other parts of the endoplasmic reticulum also become degraded remain equally as obscure. Redifferentiation of gland cells are not much noted in cycloheximide treated hydras and the reason must be that this particular process of redifferentiation requires structural or functional proteins and treatment with cycloheximide inhibited the translation of proteins as a result of which no redifferentiation took place and cell death followed after about 18 hours of amputation.

Secondarily, it was noted that following treatment with cycloheximide, a higher amount of cellular activity occurs in the ectoderm of the hypostome as well as basal disc regenerating hydras. This peculiarity has been resulted due to the presence of extra-number of interstitial cells in the ectoderm. It would become clear that proliferation of interstitial cells and their migration from endoderm
Towards ectoderm are not affected by treatment with cycloheximide and at the same time the formation of cnidoblasts and other types of cells in the ectoderm are inhibited. This must be the reason for the presence of large number of interstitial cells in the ectoderm and thus giving the ectoderm an appearance of an area of high cellular activity. Primarily, it could be said that the cells which are found in the ectoderm close to the mesoglea are the ones which migrate from the endoderm towards the ectoderm and their further differentiation take place as they migrate towards the periphery of the ectoderm. Active migration of interstitial cells has been noted previously in the ectodermal layer (Campbell, 1974). Stray cases of division are also observed among the newly formed interstitial cells which is similar to what observed by Davis (1970). However, it must be assumed that the differentiation of interstitial cells to nematoblasts and other types of cells necessarily involve synthesis of proteins which are required structurally and functionally. The primary differentiation of interstitial cells must be a mechanism which is non-dependant of translation and it could be assumed that this process might be accomplished with the help of stable proteins already synthesized in the system.
REGENERATION IN CYCLIC AMP TREATED HYDRAS

The most significant feature observed in the case of regeneration in cyclic AMP pretreated hydras was that both types of regeneration, the hypostome as well as the basal disc had been accelerated profoundly following the treatment. If both the types of regeneration are analysed, it becomes apparent that an overall reduction of approximately 12 hours of time had been achieved in successfully completing the restitution process. In normal cases, rudiments of tentacles appear only after 36 hours after amputation of hypostome whereas in cyclic AMP pretreated hydras it can be seen that the tentacle rudiments make their appearance at about 24 hours after the removal of hypostome and the entire hypostome together with the tentacles are restituted at about 36 hours after amputation. Similar type of situation prevails in the case of basal disc regeneration also in which an adhesive basal disc is found completely regenerated after 36 hours of amputation of basal disc. It could be seen that this faster attenuation of the morphological features in regenerating organisms had taken place due to the accelerated cell activities associated with restitution process which had occurred as a result of the cyclic AMP treatment.

Accumulating evidences suggest that increase in levels of cyclic AMP can bring out considerable changes in developmental
pattern in sea urchin embryos; progress in differentiation has been accounted for an increase in cAMP content (Nath and Rebhum, 1973a; Yasamasu, Fujiwara and Ishida, 1973) and a similar change has been noted to occur during the development of *Drosophila* larvae (De Reggi and Cailla, 1975) and in *Xenopus* embryo (Lovtrup and Lovtrup, 1975) also. Increase in cAMP levels brings out developmental changes in rat mammary gland (Sapag-Hagar and Greenbaum, 1973), in rat liver (Christofferson et al., 1973; in brain cells and in glial tumour cells of rat (Schroder and Hsie, 1973; Schrier and Shapiro, 1973) and in rat muscle cells (Novak et al., 1972). Evidence of control of differentiation in cell types has been reported more than once. Cyclic AMP causes initiation of cell movement in chick myoblasts (Zalin, 1973) and also in rat muscle cells (Wahrmann, Winand and Lazzati, 1973). An increase in cAMP has been found to occur during chick skeletal muscle development (Zalin and Montage, 1975). Prasad and Hsie (1971) observed cAMP induced differentiation in cultured mouse neuroblastoma cells. These and other observations clearly depict the controlling role of cAMP levels in differentiation. In a similar way, increase in the levels of cAMP which occurred due to the treatment might have resulted in exerting a control mechanism by the drug in the differentiation processes of hydraz also. This may be the cause for the elaboration of gland cells like cells in the ectodermal region.
Changes in cell shape and in overall morphology in cultures by externally added cAMP have been noted in many cases. Johnson, Friedman and Pastan (1971) and Sheppard (1971) found that cultured fibroblasts change their morphology in presence of cyclic AMP. This was followed by similar reports in which shape change of ovarian (Hsie and Puck, 1971), hepatic (Van Wijk, Wicks and Clay, 1972), glial (Macintyre et al., 1972) and neural cells (Prasad and Hsie, 1971) take place. Promotion of circular morphology of cultured schwannoma cells is caused by cAMP analogues (Sheppard, Hudson and Larson, 1975) and in mouse blastomma cells there occurs induced axon formation following treatment with cAMP (Prasad, Bondy and Purdy, 1975). As molecules of cell communication, the role of cAMP has been well presented (Hax et al., 1974) and in many cases cyclic AMP has been reported to cause biochemical changes such as exerting a regulation of many enzyme activities too (Bombik and Burger, 1973; Yasamasu, Fujiwara and Ishida, 1973). Apart from these demonstrations, there are numerous reports suggesting a positive role of cyclic AMP in the regulation of gene expression. It has been shown that cAMP is capable of stimulating gal mRNA synthesis in Escherichia coli (Anderson, Gottesman and Pastan, 1974) and mRNA synthesis in chicken embryonic fibroblasts (Russel and Pastan, 1974). Prasad, Bondy and Purdy (1975) have further demonstrated that
in cAMP induced differentiated neuroblastoma cells, an increase of poly A containing cytoplasmic RNA occurs. They have further suggested that the stability of total RNA in cAMP induced cells is greater than normal suggesting that cAMP can modify protein synthesis also.

Thus it becomes conceivable that cyclic AMP played a positive role in the differentiation processes of hydra by accelerating developmental processes resulting in an early differentiated state. This has been reflected from the very onset of differentiation, through the processes of wound healing, delamination of ectoderm from endoderm, activation, migration, multiplication and transformation of cells and culmination in an early attenuation of the lost structures. Whether this early differentiated cells have a higher content of poly A containing RNA and whether they contain mRNAs with greater half-lives is yet to be analysed in this organism. However, it is not untenable to assume that treatment with cAMP might have resulted in an earlier translation of proteins essentially involved in the differentiation process due to its suggested positive mechanisms.

AUTORADIOGRAPHY

The autoradiographic studies conducted in regenerating organisms labelled with $^3$H-uridine revealed the characteristics
of cells synthesizing RNA particularly during the restitution processes. Previous studies (Campbell, 1965) have demonstrated that when cells in normal non-regenerating hydras were labelled with tritiated thymidine, the incorporation of the radioactive precursor into DNA was uniform along the entire body column of the organism. Hansman and Burnett (1972) using tritiated proline as the label, showed the origin of mesoglea autoradiographically. Most of these studies were pertaining to aspects other than RNA synthesis and the first attempt in successfully labelling the cells synthesizing RNA during the regeneration processes has been concurrently made here.

It could be seen that as early as 4 hours after amputation, RNA synthesis start to take place in the gland cells of endoderm. This is clearly depicted in the Figure in which the gland cells in the body column of the organism show more or less uniform distribution of grains. This observation is much similar to the studies of Campbell (1965) on DNA synthesis in an entire hydra in which he had obtained a uniform pattern of synthesis along the body column of the hydra. However, it has been tentatively observed that during early periods of regeneration in hydra, RNA synthesis is not confined to ectodermal cells and the maximum activity has been shown by the endoderm gland cells.
Preliminarily it could be envisaged that the RNA synthesis is being localized in the nucleus of the gland cells. The presence of grains in the cytoplasm of the cells after few hours of regeneration reveals positively the transport of the templates in the cytoplasm and also the concentration of the substance there. Further, it could be seen that when the process of delamination takes place, the endoderm cells at the regenerating site synthesize more amount of RNA as revealed by the amount of grains contained by them and it could be due to the fact that the developmental process of delamination, like many other process of development, must essentially be related with an enhancement of RNA synthesis. The activity of the gland cells were so much pronounced that migratory cells found in the enteron also were found synthesizing RNA molecules. However, it could be seen that eventhough RNA synthesis is invariably taking place in the endoderm, the newly constituted endodermal derivative forming an ectoderm layer at the cut end is practically devoid of any RNA synthesis.

Apart from these observations, one other significant feature notable from the autoradiographic studies is the nature of origin of interstitial cells and their relationship with the synthesis of RNA during regeneration processes.
Studies with cycloheximide has shown that the interstitial might take their origin from the gland cells of endoderm and successively transform into fully differentiated cells as they migrate from the interior of the endoderm towards the ectodermal periphery. During the early period of regeneration, it could be seen that the peripheral gland cells alone synthesized RNA and is found to be uniform all throughout the body. This rules out the possibility of any zone-specific activation with regard to RNA synthesis in hydra. During subsequent hours, the transformed cells move towards the proximity of the mesoglea and take up the form as undifferentiated cells which still synthesize RNA. It could be said that the RNA synthesis noted to take place in the nuclei of the gland cells must necessarily be associated with their transformation into interstitial like cells. Similarly, the fact that these cells even after reaching the vicinity of the ectoderm continue to synthesize RNA suggests that the differentiation process is not yet completed. In fact, the cells actively synthesizing RNA in the ectoderm are found to be differentiating interstitial cells and fully differentiated interstitial cells could be observed at the boundary of ectoderm. Fully differentiated cells also synthesize of RNA but it could be seen that their differentiated forms, namely the nematocysts and other
types of cells do not involve with substantial synthesis of RNA. Upon attaining complete differentiation, probably RNA synthesis ceases to take place in these cells. The very feature that the grains are detectable only in the transforming and differentiating interstitial cells tend to assume that in the axis of hydra, the interstitial cells are mostly endowed with the capacity for synthesizing RNA molecules and not other types of cells. This conclusion shows considerable similarity with previous studies (Mookerjee and Bhattacharjee, 1966) in which interstitial cells were found as the chief sites of RNA synthesis. It could be assumed that it is due to the tremendous capacity of interstitial cells to differentiate into other types of cells in the organism that they are enriched with the capacity for synthesizing RNA molecules during the regeneration processes. The exclusive relationship between RNA synthesis and activities of differentiating cell lines has been discussed elsewhere in this thesis.