General Considerations

Regeneration, no doubt is an adaptive phenomenon evinced primarily to restore functional capacities by recreating the lost structures due to any reason. This definitely, calls for the enactment of some of the early ontogenetic mechanisms impinging at the cellular and molecular levels and, gradually extending to spatial and temporal interactions, ultimately resulting in the development of a fully differentiated adult body extremity. Whereas the cell lineage during the normal ontogenic development could be traced to the fertilized eggs, in the case of regenerative development, the cell lineage represents the fully differentiated adult tissues. These tissues have to nevertheless undergo a reversal of their differentiated state, specifically designated as dedifferentiation, which releases them from the constraints and restraints imposed by the differentiated state thereby, permitting them to proliferate in a certain gay abandon. Released from the cudgels of their differentiated state these cells now totally free of the memory of their past, are ready to undertake any of the cell specific phenotypic pathways of differentiation (metaplasia), depending on the input of instructive information dictated by their special positioning in the system as a whole. These events require, so to say, "the opening of shut off vaults" resulting in the reactivation of some of the genetic programmes which had been repressed during the previous ontogenic differentiation. Unlike the ontogenic developmental system, where the entire genetic repertoire is at the disposal of the developing system, during

regeneration of an adult structure, the availability of the necessary genetic programmes has to be generated by specific reactivation of the repressed systems. It is in this respect, that the factors of endogenous origin released from the animal body in response to the stress due to the loss of a body extremity, can be conjured to indulge in the above.

Hormones emanating from the various endocrine sources could be potential candidates amongst the factors of systemic origin, which could be implicated in the reactivation of the developmental process. Apart from the reactivation and expression of the molecular mechanisms, even metabolic support for the increased energy demands, is also a matter of great consequence. In this respect too, hormones could play effective roles. Since hormones could exert pleotropic extracellular, intracellular and subcellular effects in tissue specific and developmental stage specific manner, they are the ideal candidates to trigger off and maintain the regenerative process. Only a few hormones can be expected to undertake the role of regulation of regenerative process, which again depends on the evolutionary status of the organism. A screening of the various vertebrate hormones had identified a few of them as having the potential to influence regeneration. The amphibians, which have been recognized as a group of vertebrates with the maximum regenerative endowment do, also exhibit another developmental phenomenon in the form of metamorphosis. The metamorphic process among anurans has been widely studied and has been shown to be influenced

by two hormones mainly, prolactin and thyroxin. Of the two, prolactin has been implicated to be the growth promoting hormone which tends to prevent metamorphic transformation (Etkin and Lehrer, 1960; Berman *et al.*, 1964; Grant and Cooper, 1965; Nicoll *et al.*, 1965; Remy and Bounhiol, 1965; Bern *et al.*, 1967; Etkin and Gona, 1967; Derby and Etkin, 1968; Brown and Frye, 1969; Gona and Etkin, 1970; Medda and Freeden, 1970, 1972; Cohen et al., 1972; Platt, 1976; Clemons and Nicoll, 1977) while thyroxine has been identified as the metamorphic hormone (Frienden and Just, 1970; Atkinson, 1971; Cohen *et al.*, 1978). Interestingly, the potential to generate the limbs and tail in anurans remains restricted to premetamorphic stages while, this ability is totally lost after metamorphosis suggesting a negative influence of thyroxine on regeneration in anurans. Since the regenerative potential in these animals is manifested in the premetamorphic stages, a positive correlation between regeneration and prolactin can easily be assumed as, the PRL level in this phase of their life cycle is at its maximum. In contrast, the urodelean amphibians exhibit a wide spectrum of regenerative adeptness. The most thoroughly studied, of the various structures capable of undergoing regeneration is, the regeneration of the limbs and tail of newts. Unlike in the case of anurans, in the urodeleans, the thyroid hormone seems to be an essential ingredient of the supporting hormonal '*milieu*' favouring regenerative growth. Both thyroxine and prolactin have been implicated in controlling regenerative outgrowth in newts (See Chapter 6).

The reptilian group as a whole is less remarkable in terms of the expression of regenerative potential and, at best, this potential is restricted mainly to the tail of a few lizards. (See Bellairs and Bryants 1985). However, reptiles have not proved to be that popular amongst Developmental biologists foraging into the study of regenerative phenomenon. Physical aversions and difficulty in maintenance and experimental manipulations could be, the detracting factors in this respect. Nevertheless, they hold great interest due to their ancestral position linking the modern aves and mammals. This evolutionary linkage could be very useful in deciphering the reasons for the loss of regenerative potential during the transition, from poikilothermy to homeothermy. Moreover, being closest to the mammals, an understanding of the mechanisms of the process, as well as, the regulatory factors involved thereat, could provide us with some clues regarding the loss of the potential or, even the ways and means by which the potentials could be reactivated in the mammals. Ironically, though histomorphological features of the regeneration process have been evaluated in different lizards, the physiological and regulatory mechanisms did not somehow catch the imagination of the Developmental biologists of the past. The very few studies conducted on these lines have been only on a single lizard, *A. carolinensis*.

This gave the necessary fillip to initiate studies on these lines in this laboratory. The two tropical lizards which were used in the past in this context were the Gekkonid lizard, *H. flaviviridis* and the Scincid lizard, *Mabuya carinata*. Earlier studies were

focused mainly on the enumeration of the histomorphological aspects (Shah and Chakko, 1968; Radhakrishnan and Shah, 1986).

This spade work which laid the basic foundation, then served as a launching pad for further searching evaluations. A sizeable quantum of studies provided an insight into the metabolic and biochemical intricacies occurring at the local site as well as systematically, during the process of tail regeneration (Kinariwala, 1978; Swamy, 1982; Valsamma, 1983; Chacko, 1987; Ramachandran, 1996). As a consequence of such studies, there emerged a concept of a local competence and an adaptive systemic support for successful initiation, maintenance and completion of the regenerative process.

Having established the adaptive local and systemic responses, stage was set for exploring the regulatory factors involved thereat. Owing to the importance of thyroxine as a principal metabolic hormone and in the light of its known role in the initiation of tail regeneration in lizards, the regulatory influence of this hormone was evaluated in relation to the observed metabolic intricacies both *in loco* and systemic. To this end, hypothyroidism was induced in lizards to evaluate its consequence on the process of regeneration, as well as, the underlying metabolic and biochemical schema. A series of studies in this line, implicated thyroid hormone as an important mediator of the various metabolic and biochemical alterations in the overall scheme of

regeneration specific responses (Kinariwala, 1978; Swamy, 1982). Apart from the effects on biochemical and metabolic profiles, hypothyroidism also demonstrated retardative effect on tail regeneration in both *Mabuya carinata* and *H. flaviviridis* (Kothari et al., 1979, Ramachandran et al., 1984; Ramachandran and Abraham, 1990).

Since the poikilothermic vertebrates respond to exogenous cues of environmental origin and even use these to adjust their endogenous rhythms and functions, attention was then shifted to an understanding of the influence of environmental agents on tail regeneration. Since photoperiodism plays an important role as an external *zeitgeber* in most of the vertebrates, influence of different photic schedules on regenerative performance in lizards was studied. Such studies showed that light has a favourable influence on regeneration and that increasing photic schedules speed up and decreasing photic schedules slow down tail regeneration (Ramachandran and Ndukuba, 1989a; Ndubuka and Ramachandran, 1991 a,b). This photic influence on tail regeneration was shown to be mediated through pineal rather than the eyes, as both pinealectomy and light deprivation to the pineal, prevented the favourable response of light while, blinding was ineffective (Ndubuka and Ramachandran, 1988). Further studies provided evidence for polactin as a hormone mediating the effects of photoperiodism on regeneration, and further, that both dopaminergic and serotonergic activity, both amenable to photoperiodic changes, regulate the PRL release (See Chapters 2, 4, 5).

Some preliminary studies also revealed, the influence of pineal hormone, melatonin, to affect the course of regeneration either in a positive or negative way, by exogenous administration of the hormone. The time dependent influence of exogenous melatonin was also purported to be acting by way of altering prolactin secretion (See Chapters 4, 5). The above studies though provided new insights and understanding, nevertheless, left many lacunae and also generated new queries thus creating new avenues for experimentation.

The present study essentially undertaken as a sequale to the above, has tried to seek answers to some queries like,

1. What is the basis for the seasonal difference in regenerative performance ? what is the role of light and temperature in the same and which is relatively more important?
2. What will be the time dependent influence of various methoxy indoles on the process of tail regeneration?
3. Is prolactin involved in the photothermal and methoxy indoles induced alterations in tail regeneration?
4. What is the relative importance of serotonergic and dopaminergic mechanisms of prolactin release?
5. Is thyroxine and prolactin involved in the season specific variation in tail regeneration?

6. What is the relative importance of thyroxine and prolactin in tail regeneration in lizards?

Experiments conducted to seek answers to the above questions have provided some tangible results and also generated new queries and avenues of future investigations.

The experiments have clearly brought out the fact that there is a definite seasonal influence on regeneration with maximum regenerative performance being characteristic of summer months and the minimum in the winter months. The monsoon months produced an intermediate performance. Both light and temperature are important mediators and, increasing temperature and increasing duration and intensity of light, can all enliven the process of regeneration. The temperature effect was seen to be essentially gradient in nature, with increasing temperatures producing consistent increments in regenerative growth. An important observation was that lizards without any exposure to light also showed the gradient effect of temperature like the lizards exposed to a normal light-dark cycle. In fact, the lizards in continuous darkness despite having a very poor regenerative growth at low temperatures, manifested the gradient effect more effectively with the result that at the highest temperature, the regenerative performance in DD lizards overshot the NLD lizards, albeit, marginally. This suggests that lizards deprived of one of the factors for

regeneration, depicts a compensatory hyperresponsiveness to the other. This was further proved by the experiments involving exposure of lizards at lower temperature to increasing light schedules. Here again, light produced a compensatory hyperresponse. Apparently, both light and temperature are of consequence in regenerative performance, however, temperature seems to be the dominant factor. Different species of vertebrates show variations in intensities of light to which they become responsive. Based on the present observations on *H. flaviviridis*, the minimum intensity of light at which they show a noticeable change in regenerative response is, 300 lux units. Upto 300 lux units, there was no regenerative response, while higher intensities of light produced significant regenerative growth. It is necessary to understand the actual mechanisms of transduction of photothermal input into endogenous humoral or other responses favouring regenerative growth. Since it was speculated earlier, that prolactin is a growth promotor favouring regenerative tail elongation in lizards, it is likely that the photothermal changes by affecting release mechanisms of prolactin could alter its level and sensitivity.

Some of the experiments performed in the course of the present study, provide justification to the above as both 5-HT blockade as well as antagonising the action of DA affected the regenerative growth significantly. Starting from fishes to mammals, there are by now, documented evidences to show 5-HT mediated PRL release and also for light induced increase in PRL release essentially by increasing the hypothalamic

5-HT content (See Chapter 2). In this respect, the increased regenerative response elicited by increasing photoperiod can be easily related to the light induced PRL release triggered by the serotonergic system. Justifiably, in the present study, treatment of lizards with the potent 5-HT_2 antagonist, cyproheptidine retarded regenerative growth. This effect was manifested by administration of cyproheptidine either in the morning or in the evening to lizards obeying a normal light-dark cycle. Interestingly, the retardatory influence of cyproheptidine was effective only in the morning in lizards experiencing photoperiodic extremes. There appears to be a circardian thythm of 5-HT and DA turnover in the hypothalamus with, diurnal 5-HT peak and nocturnal DA peak. The observed retardation in regeneration caused by 5-HT antagonism in NLD lizards is related to, on the one hand, the suppression of 5-HT mediated diurnal PRL secretion and, on the other hand, to the increased DA tone mediated, dampening of PRL release, as a consequence of 5-HT suppression. In animals in continuous light, presumably, a diuranl amplitude of 5-HT rhythm is greatly elevated while the amplitude of nocturnal DA rhythm is greatly suppressed. Hence the morning specific suppression of regeneration by 5-HT blockade is understandable and, the ineffectiveness of the same in the evening is apparently due to the already maximally reduced DA tone in continuous light. Conversely, animals in continuous darkness can be expected to have a higher DA content and tone. Hence the relative ineffectiveness of 5-HT blockade in the evening in animals in continuous

darkness is essentially due to the minimum increase in the DA tone that can be caused. The fact that 5-HT blockade in the morning could dent the regenerative response in these animals is, a pointer to the persisting 5-HT rhythm even in continuous darkness. An actual evaluation of circardian variation in hypothalamic 5-HT and DA contents in animals exposed to various photoperiodic regimens would be a fruitful exercise.

An intimately associated aspect with photoperiodic changes is, the synthesis and secretion of pineal melatonin. It is by now a universally accepted dictum that every vertebrate animal shows a rhythmic secretion of pineal melatonin, marked by a nocturnal elevated melatonin level as a function of the duration of the dark phase. Though photoperiod seems to be the principal cue that drives the melatonin rhythm, of late, evidences are forthcoming to implicate temperature as a factor in controlling the amplitude of the nocturnal melatonin peak at least in poikilothermic vertebrates. Obviously, both light and temperature are of crucial significance in regulating the overall melatonin rhythmicity in poikilotherms. A matter of consequence that impinges in the present study, is the ability of melatonin to influence PRL release. Conceivable, photothermal changes, apart from a direct action on the 5HT and DA content and tone, could also influence regeneration indirectly, by their effect on the melatonin level. How melatonin affects PRL release, is a matter of conjecture. The available experimental observations provide circumstantial evidences or incriminating clues for the ability of melatonin to affect the hypothalamic 5HT and DA content and

tone. This aspect of melatonin function seems to be, apart, from its many other subtle neuroendocrine influences. Of late, there is a surfeit of observations and flurry of investigation, which all tend to relate pineal and melatonin with the multitude of neuroendocrine, endocrine, metabolic and biochemical activities in different vertebrate species. Viewed in the restricted sense in relation to the present study, the role of melatonin is in influencing pituitary PRL secretion and, ultimately the regenerative performance. One of the means of testing the perception that photoperiodic changes exert control on regeneration, also through melatonin, is to evaluate the impact of exogenous administration of melatonin on regenerative performance. As a sequale to this thinking, exogenous administration of melatonin was tried out at different times of the day. These experiments revealed an antiregenerative effect of melatonin when administered only in the morning or noon or noon and evening or morning and noon or morning, noon and evening. As against these, a single injection of melatonin in the evening produced a pro-regenerative effect. There are reports in literature suggesting differential sensitivity to melatonin during the 24 hrs cycle, and even reports of down-regulation of melatonin receptors during the early half of the day (See Chapters 4, 5). However, the observations made in the present study, indicate a continuous sensitivity to melatonin on a 24 hr. basis, which may be manifested in the form of a pro-regenerative or anti-regenerative effect. Even studies on birds conducted in this laboratory also corroborate this. It is likely

that in reptiles and birds unlike in mammals, melatonin sensitivity is not restricted to any part of the day but, can manifest any at time during the day. Though melatonin sensitivity in lizards in terms of regenerative response is demonstrated on a continuous basis, a causative explanation is needed. Viewed in a simplistic way, it is natural that, an evening injection of melatonin by coinciding with the endogenous nocturnal elevation, would result in a greater amplitude of the melatonin signal. The question is, how this high amplitude melatonin can be related to a favourable regenerative response? Since no direct action of melatonin on pituirary or even on regenerating system has ever been demonstrated, it is conjured that melatonin must have some influence on PRL profile by its effect on the hypothalamus. In this context, melatonin binding to different hypothalamic sites has been documented in many species of vertebrates. This hypothalamic sites of action of melatonin as related to the present study could have bearing on the purported 5-HT and DA rhythms. Interestingly, there are reports suggesting both a PRL increasing action as well as a PRL decreasing action of melatonin. There are also reports showing increased hypothalamic 5-HT turnover in response to melatonin administration, especially in the evening. Keeping this in mind, the currently recorded proregeneration effect of evening injections could be a consequence of elevated 5-HT production, leading to grater diurnal prolactin release. Conversely, the antiregenerative effect of single or multiple injections of melatonin at other times of the day, could make the lizards to read it as an extended

dark phase, or even as a short photoperiod, all of which could have a dampening effect on PRL release by virtue of an increased dopaminergic activity. Validity to this perception is provided by the reports of morning injections of melatonin making a prolonged dark phase signal in seasonally breeding mammals as also, from our own observations of darkness induced retardation in regeneration to be due to decreased PRL secretion mediated by increased DA activity. A perception that emerges from the present findings is that, increased melatonin levels above the photophase level, which tend to reduce the difference between the scotophase and photophase levels, could result in increased hypothalamic dopaminergic activity and attenuated PRL release. Both these influences of 5-HT induced PRL release due to evening injections of melatonin and, DA induced decreased PRL release by injections at other time of the day, stand verified by the nullifying influences of 5-HT antagonism or by DA agonism on the proregenerative effect of evening melatonin and, that of DA antagonism on the antiregenerative effect of day time melatonin injections. Neuropharmacological evidences from the present study thereby confirm the role of melatonin also, as a potential candidate in modulating PRL release. As discussed earlier, temperature is considered to be an important factor in controlling the amplitude of the nocturnal melatonin signal. Some of the experiments on the reptilian species have shown that higher day time temperatures could increase the amplitude of the nocturnal melatonin and also, the potency of temperature in entraining the melatonin rhythm. In this

context, the increased regenerative performance seen in the summer months and, the fact that the progerenerative influence of evening melatonin injections is less pronounced in summer months than the monsoon months, are in concordance with this.

A minor offshoot of the present investigation is, the inhibitory influence of cyproheptidine, a 5-HT antagonist, on tail regeneration, when administered locally at the regenerating site. Since the early regenerate is free of nervous innervations and neurotransmiter functions, much like the early embryos, this observed effect is a bit unorthodox. There are reports in literature denoting the localization of neurotrasmitter substances even in prenervous embryos where nervons functions are not yet established and, these observations have proclaimed functional garbs other than as neurotranssmiters, like, in controlling cell permiability and ionic transport, intercellular communication and macromolecular synthesis, all of which are essential events characteristic of developmental processes. Hence, the observed retardation of tail regeneration by cyproheptidine could be due to an interference with serotonin mediated functions of this type. However, the observation that, this effect was restricted to only evening injections and not to morning injections, is enigmatic, and the only reasonable explanation could be, some sort of circardian rhythmicity in the local turn over of serotonin and its purported functions. This is an aspect which could be very interesting and, which needs to be explored further to understand the degree

and extent of involvement of such neurotransmitter substance and their functional implications in early developmental events.

Since both thyroxine and prolactin have been identified as major enactors in the control of regeneration process in reptiles, it was necessary to understand their functional involvement not only in relative terms but, also in terms of already inferred photothermal and pineal mediated effects. A very interesting fact that emerged from a paraphrenalia of experimental combinations is that, the already inferred seasonal influence on regeneration has a definite relation with thyroid and lactotroph activity and, sensitivity towards these hormones. What emerges as a consensus is that, both the circulating titres of PRL and thyroxine as well as, the sensitivity towards them, reflected in terms of regenerative performance, are the greatest in the summer months (higher temperatures) and, abysmally low during the winter months (lower temperatures). Reports are aplenty, relating increased thyroid activity with higher temperatures and decreased thyroid activity with lower temperature in poikilotherms including reptiles. However, additional features that have been gleaned form the spectra of experimental observations made herein are, a low temperature induced insensitivity towards thyroid hormone as well as, low temperature induced decreased lactotroph activity, PRL secretion and sensitivity. It becomes obvious that, atleast in *Hemidactylus flaviviridis*, the release of thyroxine and PRL and the consequent increase in their serum titres and tissue sensitivity are, to a great extent dependent on

temperature. Both temperature and light, in combination with melatonin, seem to be the pivotal links in the neuroendocrine mechanisms related to thyroid and lactotroph activity and, ultimately regenerative performance. Though it is difficult to explain the insensitivity to exogenously given hormones in the cooler winter months, as an array of unknown reasons could be responsible, one of the reasons perhaps could be the increased content of melatonin. In this respect, it would be worthwhile to investigate the competence of melatonin to alter the sensitivity of the tissues to thyroxine and PRL and also, to see whether it is temperature dependent. The present observations have clearly indicated a predominant influence of temperature as the controlling factor in *H. flaviviridis*. This has been made evident many a time, by the observation that, a sudden change in the daily temperature, either during a month or in between months, used to cause a sudden difference in the regenerative performance. This has been emphatically registered time and again during the course of experimentation during the last three years. It is also made evident that, temperature, influences both phases of regeneration, the regressive phase resulting in the formation of blastmea, as well as, the progressive phase leading to liner growth.

The present finding of temperature dependence of regenerative performance, in that, even artificial increase in temperature by few degrees in the winter months can immediately and suddenly increase regenerative performance, is in discordance with earlier observations in reference to regeneration in newts. The studies on newts had

shown that, even maintaining newts at higher temperature during winter months, do not accelerate the regenerative process but, when they are injected with PRL, the regenerative performance became equivalent to that of summer months. Obviously two points find identity between newts and lizards

1. PRL is required for regenerative growth and
2. There is a seasonal variation in the PRL level.

Whether there is decreased PRL sensitivity as well, at lower temperatures does not become clear from the above experimental studies on newts, as they were given PRL at higher temperature. The observation on newts had been taken to indicate a circannual rhythm of PRL secretion, which cannot be altered simply by increasing the temperature as, the rhythm was purportedly entrained. But, in the case of lizards, there also appears to be a cirannual variation in PRL secretion nevertheless, they are amenable to temperature changes, indicating no firm entrainment. Apparently, PRL secretion in lizards, atleast in *H. flaviviridis*, is driven by a temperature cycle but, not entrained. This could either be an evolutionary difference in terms of neuroendocrine mechanisms in amphibians and reptiles, or else, it could be related with the habitats and the adaptive strategy associated with *H. flaviviridis*. This aspect would be challenging avenues for future investigators. In terms of control of regeneration, it is clear that, both thyroxine and PRL are of crucial importance. What percolates from

the revelations accruing from the various experimental paradigms, is that, there are overlapping functions of thyroxine and PRL. Though they could be purpoted to exert a sequential action, with thyroxine first, followed by PRL, it is nevertheless clear, that full regenerative expression is the offshoot of interdependent and synergistic effects of the two hormones. No doubt, initial presence of thyroxine in minimal amounts is a must for the formation of a blastema but, simultaneously, thyroxine might also exert a favourable influence on PRL release and potentiate its action while, PRL in turn could exert a similar influence on thyroxine. There is a need for phase dependent optimum relative concentration of these two hormones, to elicit the full regenerative performance.

Though a number of functions can be attributed to thyroxine and PRL during the course of regeneration, one of the potential action of these two hormones during the very early phase of regeneration could be to elicit and mediate the actions of growth promoting substances as, such functions of these hormones are documented during growth processes. In this respect, growth factors loom as prime candidates and as, ironically, studies on growth factors in relation to regeneration are scarce, preliminary investigations with EGF, TGF-β and NGF were undertaken. The final outcome of these studies is the finding, that both EGF and TGF-β inhibit regeneration, albeit by differential mechanisms. EGF, though promoted early wound healing and formed thickened epithilium, nevertheless, retarded the formation of a

blastema and regeneration, by what appears to be its actions by forming collagen material below the epithilium and is influences on precocious myogenic and epithelial differentiation, both these together prevent dedifferntiation and proliferative activities, which are much needed for the formation of blastema. In contrast, TGF-β tend to inhibit dedifferntiation and even if some dedifferentiation and growth occur, regeneration was again stalled, because of its preponderant inductive influence in chondrogenic differentiation and inhibitory effect on myogenic differentiation. TGF-β also promoted erythropoitic activity in the stump adipose tissue, though an unrelated event. By looking at the effects of these two growth factors, it is clear that, they would be involved in tail regeneration process but, under precise temporal and spatial specificity. Interestingly, NGF stimulated regeneration by hastening blastema formation and providing an initial growth spurt. Further, it could even compensate for the absence of thyroid hormone, suggesting a possible link with thyroid hormone and NGF production and its activity locally. It is presumable that, NGF is probably the down stream agent of thyroxine which, provokes the formation of a blastema. It is in this light, that a detailed evaluation regarding influence of thyroxine and prolactin on the formation and action of growth factors as well as their interaction could not only prove academically satisfying but also may throw up many challenging possibilities.