GENERAL CONSIDERATIONS

Epimorphic regeneration as exemplified by vertebrate appendages is a fascinating phenomenon involving evocation of developmental potencies in adult organisms. Regeneration of the limb and tail of larval and adult amphibians, and the tail of lizards represents a restricted and localised potency to reactivate developmental events. Obviously regeneration affords unparallelled opportunities to evaluate and comprehend many developmental events such as wound healing, dedifferentiation, proliferation, tissue interactions, differentiation and growth, all of which occur as a continuum in a precisely synchronised sequential order. Of these various events, proliferation, tissue interactions and differentiation can be considered as true developmental events as they form the major crux of embryonic development. The other events such as wound healing and dedifferentiation represent developmental events of post-embryonic origin. The process of differentiation as it occurs during regeneration, though a primary event of development, represents a second order differentiation not only due to its re-enactment a second time, but also due to its occurrence in close structural and physiological association with an adult body, and not in isolation as it occurs during embryonic development. Mechanics of wound healing as well as the process of dedifferentiation have their own individualistic importance in applied aspects of research. Dedifferentiation is an event also related to tumerogenesis or carcinogenesis and hence forms an important topic of study in Oncology. Since the dedifferentiation associated with carcinogenesis demands release of cells from the firm bondage of tissue organisation essentially provided by the intercellular cementing substance, there are obvious spatial restrictions which makes its progress very slow. However, it proceeds rather quickly during regeneration due to the ample spatial opportunities made available at the cut end. Moreover, the act of wound closure which involves the formation of a single layered epithelium with no underlying dermal substance (a characteristic feature of regeneration associated wound healing) provides a conducive closed enclosure for the process of dedifferentiation and proliferation to proceed. Regeneration as it occurs is however a composite one including a conglomerate of events inexorably linked together in a definite fashion.

The process, a commendable feat of re-enactment has great biological interest. Hence, much scientific scrutiny had been directed towards this phenomenon in order to understand its morphology, physiology and biochemistry. Initial studies had concentrated on understanding the morphology and anatomy of the extremity endowed with the power of regeneration, as the tail in lizards. With the establishment of the topological features of the tail, gross anatomical and histo-anatomical studies led to the discovery of specialised arrangement of muscle bundles and the plane of break in the centrum of caudal vertebrae etc., which enabled a lizard to cast off its tail by breaking at these prefixed planes of autotomy (Bellairs and Bryant, 1985). However, such prefixed planes of breakage, as they occur in the lizard tail, is not an essential requisite for regeneration to occur and is clearly illustrated by the ability of the limbs of urodelian amphibians to undergo regeneration when amputated at any level. Perhaps it is very appropriate, that some of the early investigators to take fancy for regeneration, attempted to evaluate the influence of environmental factors on the process of regeneration and succeeded in showing the positive influence of nutrition, temperature and humidity on the process (Licht, 1967; Maderson and Licht, 1968; Tassava, 1969; Magon, 1970; Schauble, 1972; Schauble and Nentwig, 1974; Maier and Singer, 1977). Microscopic anatomy of the various stages of regeneration formed a very essential aspect of many of the investigations which enabled in establishing the histo-architectural features of the regenerating system. The importance of histo-architectural features can be gauged by the fact that the parts undergoing regeneration are packages of heterogeneous tissue populations, and the new tissues form effective structural associations not only between themselves but also between the new and the old at the cut end. Though the studies at the morphological and anatomical level did provide some very useful information, the underlying biochemical and molecular mechanisms nevertheless remained unknown. But soon there occurred an awakening of interest on these aspects and a flurry of investigations erupted in the mid 20th century. Many investigators directed their attention on metabolic and experimental aspects of regeneration. Starting with Okuneff (1933) and Niwelinsky (1958), Wolfe and Cohen (1963), Johnson and Singer (1964), Schmidt (1968) down to Procaccini et al. (1973), with their extensive investigations on varied aspects of metabolic physiology, had helped establish the biochemical make-up and metabolic profile associated with amphibian limb and tail regeneration. Concurrently, the experimental studies of a few investigators successfully demonstrated the importance of the apical epidermal cap and neuronal contribution in the early phases of urodelean limb regeneration (Singer, 1952; Singer and Caston, 1972; Jabaily and Singer, 1977; Globus et al., 1980). The endocrine participation in regeneration also received considerable attention right from the beginning, and the many studies on this aspect in amphibian regeneration have not provided a clear answer regarding the specific roles of major endocrine secretions (Schmidt, 1968; Vethamany-Globus and Liversage, 1973a&b; Liversage and Brandes, 1977; Liversage and Korneluk, 1978). Lately, mechanisms of morphogenesis and location of positional information have gained impetus (Carlson, 1974, 1975; Dinsmore, 1981a,b, 1982; Glade, 1978; Lheurenux, 1975, 1976). On a comparative note, except for the indulgence of a few workers like Singer (1961), Maderson and Licht (1968), Maderson and Salthe (1969) and Simpson (1970) regenerative ability in reptiles as represented by lacertilian caudal regeneration has not been subjected to critical and detailed experimental scrutiny. Apart from a pristine interest, study of regeneration in reptiles is of greater significance in evaluating the regenerative potential in homeotherms as the reptiles represent the basic stock from which the aves and mammals have evolved. It was this nagging thought as well as the relatively poor attention paid to the regenerative process in lacertilians that provided justification in initiating studies in this respect in our laboratory just about 2 decades thence. Two of the lizard species that have been used extensively to this end are the Gekkonid lizard, *Hemidactylus flaviviridis* and the Scincid lizard, *Mabuya carinata*. The initial studies were devoted to an understanding of the morphological and anatomical structural features of the lizard tail (normal as well as the regenerating) (Chakko, 1967; Magon, 1970; Hiradhar, 1972; Radhakrishnan, 1972; Ramachandran, 1972). Once having established the basic and fundamental features of the anatomy of the lizard tail, the stage was set for undertaking studies on the metabolic features of the lizard tail and its adaptive changes during the course of regeneration. Extensive application of a combination of histochemical and quantitative techniques has provided a well knit cohesive picture of the <u>in loco</u> sub-cellular metabolic adaptations characteristic of lacertilian tail regeneration (Chakko, 1967; Magon, 1970; Hiradhar, 1972; Radhakrishnana, 1972; Ramachandran, 1972). The studies which involved detailed evaluation of the metabolic distribution and enzymological make-up of the pre- and post-autotomic tail have shown the lizard tail to be primarily adapted for anaerobic metabolism, which becomes more pronounced in the immediate post-autotomy period (3-5 days) corresponding to the wound healing phase. The most crucial aspect of the metabolic strategy adopted by the regenerate is the shift in emphasis from anaerobic to aerobic in the late regressive and early progressive phases of regeneration. The necessary biochemical modulations in this connection are established during the preblastemic phase (5-7 days) and put into operation during the blastemic and differentiation phases of regeneration (7-25 days). This is followed by a period of co-participation of both oxidative and glycolytic reactions (25-40 days) and the final, gradual shift back to the anaerobic pattern through growth to the fully regenerated state of the tail (40-60 days). The occurrence of such metabolic transformations once having established raised the question of the adequacy of the post-autotomic tail stump to generate enough resources for the sustenance of the process of regeneration for such a prolonged period. The possible systemic participation in this connection became a logical and plausible possibility needing experimental scrutiny. The possible systemic involvement becomes all the more obvious when viewed in the context that the regenerative ontogeny occurs in total physical association with the parent body unlike the embryonic ontogeny which occurs in isolation within the confines of the fertilised egg with a full store of energy rich nutrients. This provided impetus for studies on systemic participation during regeneration which led to the discovery of extensive haemodynamic and metabolic adjustments in the body of the lizard (Kothari, 1977; Kinariwala, 1977). The extensive haemodynamic adjustments more pronouncedly expressed by Mabuya included increased RBC count and haemoglobin content (Shah _et_ _al_., 1980c) development of lymphocytopoietic nodules in the liver, spleemic white pulp hypertrophy and establishment of haemophoietic activity in the visceral fat-bodies

(Shah *et al.*, 1982b; Ramachandran *et al.*, 1985) and the increase in circulating lymphocyte population (Shah *et al.*, 1980c), all of which were correlated with the establishment of an aerobic environment and the possible role of lymphocytes in populating the regeneration blastema. On the metabolic front, alterations involving the metabolite content (glycogen, lipids, and proteins) of liver, muscle and fat-bodies were documented suggesting resource mobilisation from systemic sources to the local site of regeneration as well as increased energy demands systemically as well, due to an overall physiological activation pervading the body during the course of regeneration.

The revelation that extensive systemic participation in the form of physiological activation and metabolic modulations accompany the *in loco* changes during regeneration, gave sufficient motivation to evaluate endocrine participation as a logical follow-up. The recent studies of Kothari (1977), Ramachandran *et al.*(1984), Swamy (1981)and Valsamma (1982) have delved into this problem. A cursory review of their salient findings indicate the involvement of pancreatic hormones in modulating carbohydrate metabolism and in providing blood glucose as the provision of choice metabolite to the regenerate (Kinariwala, 1977; Shah *et al.*, 1982a, 1987) and the retardation in regenerative tail elongation under hypothyroidic condition (Kothari *et al.*, 1979; Ramachandran *et al.*, 1984). This retardation was however shown to be reversed by either replacement with T_4 (Ramachandran et al., 1984) or testosterone (Kothari et al., 1979). Subsequent work of Valsamma (1982) on the influence of bilateral gonadectomy (during the reproductively active months) on tail regeneration in Hemidactylus could not reveal any inhibitory influence or retardation in tail elongation. Obviously, the normal regenerative potential exhibited by hypothyroidic lizards replaced with testosterone is understandable in the light of the reports indicating the ability of testosterone to mimic the actions of thyroxine in reptiles (Thapliyal et al., 1974a,b, 1975; Oommen, 1978, 1980). Moreover, unilateral adrenalectomy was found to have no influence on the course of tail regeneration except for an initial delay which got nullified in the later period, and was attributed to the compensatory hypersecretion from the intact adrenal (Valsamma, 1982).

The present study has attempted to further our knowledge about Saurian regeneration. Since regeneration involves a reactivation and re-enactment of early developmental events, some in loco factor(s) can be considered to play a crucial role in the initiation of regeneration and factors such as the neurotrophic factor in amphibians and a product of the ependymal cells lining the spinal cord in reptiles have been suspected to play an important role in initiating the process of regeneration (Singer, 1952; Simpson, 1968. 1970, 1977). Though the exact identity of this neuronal moiety remains yet elusive, acetylcholine or acetylcholinesterase (Singer et al., 1960), non-specific cholinesterase (Ramachandran et al., 1981b) and even NGF (nerve growth factor) have all been implicated. However, some regeneration specific protein or proteins can definitely be considered to play some crucial role in the process of developmental reactivation characteristic of embryonic organogenesis. A number of studies involving electrophoretic separation of proteins of the limbs of urodeles prior to and after amputation have been conducted in this context (Schmidt, 1966a; Dearlove and Stocum, 1974; Garling and Tassava, 1984), and though almost all of these studies have demonstrated the appearance of regeneration specific proteins in the amputated limb, the number of such proteins induced has not shown any uniformity. No comparable studies have ever been conducted on reptilian regeneration and hence electrophoretic separation of soluble proteins of unautotomised and autotomised tail of the lizards, H. flaviviridis has been attempted (Chapter 6). The study has shown appearance of four specific proteins in the autotomised tail not represented in the unautotomised condition. Whereas one of them was very short living and transitory, one was permanent, and two were long living. Though some functional involvement of these proteins in one or more of the events associated with lacertilian tail regeneration can be presumed, it is nevertheless not possible to ascribe any specific function to them with certainty. It is also difficult to associate any one of them with NGF or any other neuronal principles. An added dimension is the appearance of four new proteins in the liver as well, with one appearing on the third day and as many as three on the fifth day. The relation between the new proteins of the liver and those of the tail are however difficult to assess and the previous report of Ramachandran et al. (1980) suggesting the possibility of liver elaborating some regeneration specific proteins can neither be discounted nor validated. A characterisation of these proteins and identification of their source of origin could be ideal and very useful in assessing the functional involvement of these proteins in regeneration.

Based on haematological and enzymological studies coupled with quantitative evaluation of metabolite contents (Ramachandran et al., 1979, 1980, 1983, 1985; Shah et al., 1980a,b,c, 1982a,b,d; Swamy et al., 1982a,b), establishment of an aerobic environment in loco as well as systemically was presumed to occur during the blastemic and differentiation phases of regeneration in M. carinata. This was further confirmed by the appearance of oxidative LDH_1 and LDH_2 isozymic forms between the 7th and 25th days post-autotomy. Similar enzymological studies in Hemidactylus have also provided tentative evidence for a shift in metabolism from anaerobic to aerobic pattern (Chapter 7). The presently observed changes in LDH isozymes provide strong evidence for increased oxidative potential in the regenerate as well as in the liver and skeletal muscle of Hemidactylus. Moreover, the oxidative forms were very prominently expressed even in the unautotomised tail, as well as in liver and muscle in comparison to the pattern in Mabuya where the oxidative bands were very weak in the tissues prior to autotomy. Apparently, greater oxidative ability of the tissues of Hemidactylus can be inferred, though in general, the lizards are considered to be better adapted for anaerobic mode of metabolism (Pough, 1969; Shah and Ramachandran, 1970; Bennet, 1972; Bennet and Dawson, 1972, 1973; Bennet and Licht, 1972; Shah and Hiradhar, 1974; Shah and Ramachandran, 1976b; Shah et al., 1982d; Swamy et al., 1982b). This presumed greater oxidative potential of the tissues of Hemidactylus is further emphasised by the observed presence of LDH 'C' isozyme in the tissues of Hemidactylus. These changes indicate a definite difference in the metabolic strategy adopted by the two lizards which may be in keeping with their differences in habit and habitat.

Owing to the fact that the regenerate grows in intimate association with the body, factors emanating from the systemic source are likely to influence the course of in loco events. Majority of the humoral signals are known to act through their specific surface or cytosolic receptors. Those which act via the surface receptors exert their actions by making use of intracellular second messengers such as free calcium or cAMP. Hence an evaluation of such second messengers could provide useful clues regarding the possible participation of humoral factors of systemic origin. Quantitative evaluation of cAMP and cGMP contents during newt limb regeneration has been done by Jabaily et al. (1975) and Liversage et al. (1977) respectively. Both these studies indicated elevated cyclic nucleotide contents in the early phases of regeneration. Owing to the practical limitations in a direct assay of cAMP content, the present study has attempted to evaluate alterations in cAMP content during tail regeneration in Hemidactylus indirectly by way of the quantitative assay of PDE activity (Chapter 3). Based on the activity levels of PDE, elevated cAMP content has been inferred in the immediate post-autotomy periods corresponding to wound healing and pre-blastema. Active participation of cAMP even during the blastemic and differentiation phases is envisaged from the observations of low PDE activity. This indicates sustained high cAMP content till the 25th day of tail regeneration in Hemidactylus which is in slight contrast to an earlier observation in Mabuya wherein a biphasic increment in cAMP content was inferred : one during the first seven days and the other between the 15th and 40th days of regeneration. Both these studies on lizard tail regeneration have given indications of the possible involvement of PDE mediated modulations of cAMP content.

Previous studies have shown differential rates of tail elongation on a seasonal basis (Magon, 1970; Ndukuba and Ramachandran, unpublished). The above studies have tended to show a parallel relationship between temperature and tail elongation with maximum growth occurring during summer and minimum during winter. Some of the studies on amphibian regeneration have also shown variation in the regenerative potential in relation to environmental temperature with both lower as well as higher temperatures retarding the rate of regeneration (Barfurth, 1891; Ellis, 1909; Pukhal'skaya, 1959; Tassava and Goss, 1966; Maderson and Licht, 1968). Though the influence of season on regeneration has been evaluated mostly at the morphological level, no possible biochemical correlates to the observed changes have ever been attempted. It is this lack of biochemical correlation which had prompted the present studies on the seasonal alterations in carbohydrate and protein metabolisms during tail regeneration (Chapters 1 and 2). The general pattern of changes with respect to carbohydrate and protein metabolisms remained more or less identical during the two seasons though the amount of metabolite utilisation in terms of every millimetre of tail regenerated did show certain interesting variations. Whereas the amount of carbohydrate utilisation was found to be more, the degree of protein utilisation was found to be less in the winter for unit length of tail regenerated. Apparently, an over utilisation of carbohydrate reserves and a relative lethargy in the release of labile pools of systemic protein stores are inferred as the probable biochemical correlates for the observed retardation in tail regeneration in the winter months.

The numerous studies on thyroid in relation to amphibian regeneration have at best given conflicting and contradictory results (Liversage and Brandes, 1977; Liversage and Korneluk, 1978; Korneluk and Liversage, 1978). But the comparatively few studies on reptilian tail regeneration have given a more clear picture and has in general indicated a positive influence of thyroxine on tail regeneration. Turner, (1971), Turner and Tipton (1972), Kothari et al., (1979) and Ramachandran et al. (1984) have shown that the lack of thyroxine induces delay in regeneration by interfering with the growth of the ependyma. Both Kothari et al. (1979) and Ramachandran et al. (1984) had shown the definite retardative influence of hypothyroidism on regenerative elongation in Hemidactylus and Mabuya respectively, though the retardation in the latter case was more marked than in the former. The technique applied for inducing hypothyroidism being different (surgical thyroidectomy in Hemidactylus and chemical thyroidectomy in Mabuya), it was decided to test as to whether that might have had influenced the results obtained, and hence currently the technique of chemical thyroidectomy is applied in the case of Hemidactylus (Chapter 4). The results obtained indicate a 30-35% retardation at the end of 60 days and is quite similar to the observation of Kothari *et al.* (1979). This has been taken to indicate the different degrees of dependence on thyroxine for tail regeneration by the two lizards. It is presumable that Mabuya might require greater degree of thyroxine output and hence a stronger stimulation to mediate the various *in loco* and systemic biochemical and metabolic modulations associated with regeneration while lesser thyroxine output and a lighter stimulus may suffice in the case of Hemidactylus. Some support can be drawn towards this conclusion from the presumed comparatively greater oxidative potential in Hemidactylus (Chapters 7, 11, 12) and the persistence of the oxidative LDH isozymes quite prominently, albeit slightly weaker, during regeneration in the hypothyroidic condition (Chapter 7).

An earlier study in the adult newt, *Notophthalmus viridescens* had shown no change in the circulating levels of either

T_4 or T_3 during forelimb regeneration (Liversage and Korneluk, 1978), which had forced them to conclude that regeneration does not require hormonal imbalance in the form of excessive over/secretion and that it can proceed under a normal hormonal balance. However, in the present study, definite alterations in the circulating levels of T_4 and T_3 have been registered during the course of tail regeneration with an initial increase in T_4 during the first seven days and a later increase in T_3 during the differentiation phase. These changes have been correlated with the <u>in</u> <u>loco</u> and systemic molecular and metabolic activities occurring during regeneration. In fact such a biphasic thyroxine involvement in regeneration has been hinted at earlier by Ramachandran <u>et</u> <u>al</u>. (1981a) based on their preliminary histological observations in <u>Mabuya</u> <u>carinata</u>. On the evolutionary scale, the loss or restriction in regenerative potential seems to be associated with reduced metabolic and other biochemical activities required to support a normal process of regeneration, apart from the loss of local competence to initiate the process, and is reflected in the comparative difference in thyroid activity during regeneration in amphibians and reptiles. Obviously, the amphibians with a more labile mechanism(?) is able to respond to the regenerative signal even in the background of a near-normal hormonal balance while reptiles with more restrictions imposed on them require a stronger stimulus to bring about the necessary modulations, which though present still, are nevertheless less amenable to the regenerative signal alone. Strong evidence to this concept is provided by the studies of Valsamma (1982) on comparative *in loco* and systemic responses in relation to caudal autotomy and limb amputation in Hemidactylus. The above study showed that either limb amputation (non-regenerating) or tail autotomy (regenerating) could induce identical systemic responses though the *in loco* responses (limb site and tail site) were quite dissimilar thereby suggesting the necessity for both local competence as well as systemic support for regeneration to occur. An earlier observation of Kinariwala (1977) involving a comparative study of *in loco* and systemic responses in response to caudal autotomy in *Mabuya carinata* (regenerating) and caudal amputation in *Calotes versicolor* (non-regenerating) had demonstrated differential *in loco* as well as systemic responses in the two lizards. This suggests the loss of both local competence as well as systemic responses in a lizard with no regenerative potential and provides support to the concept highlighted above.

The endocrine involvement in regeneration particularly with respect to the adrenal hormones has also received considerable attention in amphibians. However, this aspect had not received much attention in relation to reptilian regeneration. The experimental evaluation in this aspect which forms a part of the present study (Chapters 4, 8, 9, 10, 11, 12, 13) have provided some tenable results. The very first observation of interest is the fact that adrenocortical suppression could not inhibit tail regeneration in Hemidactylus but could only retard it to the tune of about 30%. On the metabolic front, an overall reduced carbohydrate and protein utilization could be inferred as the adrenal suppressed lizards had responded to the stress of autotomy and ensuing regeneration by decreased glycogen and lipid depletions (Chapters 8 and 12) and net positive nitrogen balance (Chaper 9). Moreover, a net negative AA balance could also be inferred (Chapter 10). Apparently, adrenal suppression affects the adaptive modulations in metabolite turnover, mobilisation and/or utilisation, and may thus interfere indirectly with the course of regeneration. Similarly the activities of various enzymes like LDH, SDH and BDH have also shown decreased ability to elevate their levels of activity during regeneration, which could also reduce the capacity to utilize metabolic reserves effectively and efficiently and thus affect the various metabolic reactions adversely. On the whole, adrenal corticosterone *per se* does not seem to have a definite influence on reptilian tail regeneration though its permissive influence in bringing about in loco and systemic metabolic transformations can affect the quality of the regenerate produced, which as observed was found to be shrunken and stunted.

In general it can be concluded that the endocrine participation in regeneration is essentially of a permissive nature which is important in bringing about the adaptive metabolic modulations required to support regeneration and that on the evolutionary scale, the dependence on these supportive aspects become more significant requiring stronger stimulation in the form of hormonal factors along with progressive restrictions in regenerative potential and ultimate loss of the same. This may have strong bearing on the fond hope of regenerating the lost extremity of a mammal as though the current attempts in inducing regenerative potential by inducing voltage difference, hormonal priming etc. at the site of amputation may succeed in evoking a favourable response in the form of a spiky or stumpy outgrowth, the fruitful completion and the formation of an aesthetically acceptable regenerate may not be possible without the necessary metabolic modulations and energy output. This may further complicate the matters, as more than 60% of the energy potential of a mammalian body is associated with maintenance of homoeothermy; hence the consequence of diversion of energy potential from this for meeting the requirements of regeneration can easily be guessed - [illegible] inevitable death by freezing. In this context, homoeothermy and major regenerative potential would appear as thesis and antithesis and Nature's readiness to sacrifice one for the other during the course of evolution is easily understandable.

A final query that can be asked is "will the human ingenuity ever find ways and means of overcoming this impasse and ultimately succeed in regenerating the lost extremities in man?".