CHAPTER 7

GENERAL CONSIDERATIONS AND CONCLUSIONS WITH A COMPARATIVE IDEA THEREOF BETWEEN THE REGENERATING REPTILIAN AND AMPHIBIAN APPENDAGES

It was owing to the lack of a proper understanding of the metabolic patterns and reactions underlying the process of reptilian regeneration, that the present study was undertaken. Even though the morphological and anatomical patterns of regeneration in this group have been well attended to, the metabolic patterns and adaptations have been less explored and illuminated. Investigations on these lines are far and few and are more on the amphibian appendages. The major brunt of this line of investigations has been done by Schmidt (1960-1966) who on a comprehensive note studied the various enzymes and metabolites in the regenerating limb of the newt, Diemictylus viridescens. Some other reports along these lines are those of Wolfe and Cohen (1963) and Niwelinski (1960) also on regenerating amphibian limbs. Though the above works have thrown some light on the biochemical aspects of regeneration in the amphibian

appendages, there is little known ~~on these aspects~~ about in the regenerating reptilian appendages. It was in this wake that studies on these lines have been initiated in this laboratory. The present thesis is part of such a study wherein some of the enzymes of intermediary metabolism demonstratable histochemically, chiefly dehydrogenases have been investigated in the normal and regenerating tail of the scincid lizard, Mabuya carinata. The present histoenzymological observations have yielded sufficient clues to give an insight into the metabolic patterns representing the subcellular biochemical adaptations during regeneration in the tail of Mabuya carinata.

Due to the lack of work along these lines so far, the study of other systems are!

Patterns of enzyme distribution observed herein project the fact that there is a metabolic flux with regard to the preferential choice of the metabolite as the source of energy during the process of regeneration. Carbohydrate appears to be the selective metabolite in the normal tail tissues of Mabuya carinata. This aspect seems to be well exemplified by the observed strong localization of enzymes concerned with carbohydrate metabolism such as aldolase (Chapter 1), α-glycerophosphate dehydrogenase (Chapter 4) and lactate dehydrogenase (Chapter 2).

In contrast, the poor localization of β-hydroxybutyrate dehydrogenase (Chapter 4) and tricarboxylic acid cycle enzymes such as succinate and isocitrate dehydrogenases (Chapter 5) together with a poor response of glucose-6-phosphate dehydrogenase (Chapter 3) speak for themselves the representation of an inadequate machinery for lipid metabolism. Shah and Radhakrishnan (unpublished) have in their study noted that the chief stored metabolite in the various tissues excepting the adipose tissue in the normal tail of Mabuya carinata to be glycogen and not lipid. High phosphorylase and low lipase activities were another important features observed by them. These observations are indicative of an active anaerobic glycogenolysis. Presumably, the normal tail is capable of sustaining itself solely on the energy yield from glycolytic reactions. A similar picture is available from the studies of Chakko (1967) and Magon (1970) on the tail tissues of the geckonid lizard, Hemidactylus flaviviridis. Glycogen and phosphorylase were reported to be more than lipid and lipase in the various tail components of Hemidactylus flaviviridis (Chakko, 1967). Magon (1970) working on the same tail has observed pronounced activities of enzymes such as LDH, MDH, aldolase and αC-GPDH and

low level activities of BDH and G6PDH. Considering the above evidences, Magon (1970) also has suggested anaerobic glycolysis to be an active process in the normal tail of Hemidactylus flaviviridis. From the above reports it could be safely assumed that in the normal tail tissues of lizards metabolic reactions characteristic of carbohydrates take preceedence over those of others. Notably, there is a suppressed or a low level operation of reactions involving lipids. Could this be taken to represent an attempt at adaptation in both economy and conservation by the lizard tails capable of undergoing regeneration as there could be a distinct advantage in cutting down the number of chemical reactions ?

After autotomy, from wound healing onwards through preblastema and blastema upto early differentiation, a constant increase of lipid content was the main feature. This increase has been reported in both the lizards, Mabuya carinata and Hemidactylus flaviviridis tails (Shah and Radhakrishnan, unpublished; Chakko, 1967) as well as in the amphibian limb (Schmidt, 1966a & b). A concomitant depletion of glycogen was also observed through wound healing upto preblastema and glycogen and phosphorylase were noted

to be nil in the blastema. Shah and Radhakrishnan (unpublished) in Mabuya carinata, Chakko (1967) in Hemidactylus flaviviridis and Schmidt (1960, 1962) in Diemictylus viridescens have all reported the same pattern. In the course of the present study in Mabuya carinata, all the enzymes viz., LDH, MDH, αC-GPDH, BDH, malic enzyme, G6PDH and diaphorases were found to record higher levels of activity from wound healing onwards excepting for aldolase, which maintained constant, its normal level of activity; cytochrome oxidase which remained unrepresented and SDH and ICDH which made their appearance during the blastemic phase from an almost nonexistent condition in the normal tail. Excepting for a slightly reduced activities ~~for~~ of aldolase and αC-GPDH, Magon (1970) has also observed increasing activities of LDH, MDH, G6PDH and BDH in Hemidactylus flaviviridis. Increasing activity of malic enzyme and low levels of SDH from wound healing to blastema have been noted in Hemidactylus flaviviridis by Shah and Hiradhar (unpublished) and Shah and Chakko (1969) respectively. Schmidt and Weidman (1964) working on Diemictylus viridescens have also reported increasing activities of all the above mentioned enzymes excepting

for aldolase, αC-GPDH and SDH which they found to drop down to a negative level in the blastema. In the case of ICDH too, a similar drop has been observed by Wolfe and Cohen (1963) whereas Niwelinski (1960) in Triturus vulgaris observed a very high activity of cytochrome oxidase. Accumulation of lipids in the regeneration blastema appears to be a distinct feature in all the cases. The increasing activities of G6PDH and malic enzyme observed in the present work are definite indications of activation of the synthetic machinery for lipids. The concomitant depletion of glycogen and the high activity of glycogenolytic enzymes noted through wound healing and preblastema phases further lead us to believe that the intermediary products of carbohydrate metabolism are being diverted towards pathways of lipid synthesis. A high activity of G6PDH together with aldolase ensures the complete operation of hexose monophosphate shunt (HMP) thus yielding a ready supply of reduced co.enzyme II ($NADPH_2$) and pentose sugars, important cofactors for both fatty acid synthesis as well as nucleotide metabolism. The presence of aldolase at this stage of regeneration in Mabuya carinata seems to be of pivotal importance as according to the time and needs it may be of utility in both the

continuity of glycolysis as well as in the successful operation of the HMP shunt. Another important aspect of aldolase catalysis at this juncture could be to channelize a part of its product towards glyceride synthesis mediated via the operation of αC-GPDH. In fact, the high incidence of αC-GPDH at this stage of regeneration when viewed in the wake of increasing concentration of lipids is self explanatory. By its active participation during the early phases of regeneration αC-GPDH not only entails a steady supply of glycerides for esterification with the readily available fatty acids, but, also keeps open the avenue of phospholipid production. Concrete evidences are available by now to identify αC-glycerophosphate derived from glycolysis as the important starting point for phospholipid synthesis. Kornberg and Pricer (1953); Rossiter et al.,(1957); Kennedy (1953, 1954, 1957a & b) are some of the investigators who have helped make impressive progress in elucidating the mechanisms involved in the biosynthesis of phospholipids. Kennedy (1957a & b) after conducting extensive studies has reasonably well established phospholipogenesis from glycerophosphate produced by αC-GPDH in conjunction with glycolysis as the most potent one. Since regeneration

calls for much cellular turnover by division and differentiation during blastema and early differentiation phases an obvious increase in phospholipid production is rather pertinent as it is necessary for laying down the structural framework of the actively proliferating cellular elements. Apart from its significance in contributing for the structural entities of the cells its other attribute as a metabolite for energy yielding reactions should not be overlooked. At all events, the increase in phospholipid contents is justifyable and has in fact been recorded in all the regenerating systems mentioned above (Shah and Radhakrishnan, unpublished; Chakko, 1967 and Schmidt, 1966b). In the absence of an active TCA cycle, the mitochondrial oxaloacetate formed from pyruvate may be visualized to undergo multifarious changes. The changes that could be undergone by the mitochondrial oxaloacetate during the blastemic phase are (1) conversion to citrate, its extramitochondrial transport and the subsequent breakdown by citrate cleavage enzyme yielding acetyl co.A the building block of fatty acids, (2) its participation in the pyruvate centered short cycle (Chapter 2). During this cycle, oxaloacetate is converted to malate by MDH and then back to pyruvate

by malic enzyme. The role of malic enzyme and its catalysis in this cycle appears to be ~~bi~~three fold; (a) maintenance of a steady pyruvate pool and (b) to act as a subsidiary mechanism for the production of $NADPH_2$ for fatty acid synthesis, and (3) some part of oxaloacetate could as well be used up in the production of aspartate, an aminoacid. The production of pentose sugars and aminoacids are again of acute importance during blastemic and early differentiation phases as nucleotide and protein metabolisms too are vital for the efficient processes of division and differentiation forming the essence of regeneration. The pentose sugar requirement for nucleotide metabolism is being effectively met by the HMP shunt. At the same time the important sources of aminoacids are pyruvate, oxaloacetate and α-ketoglutarate. Since pyruvate and oxaloacetate are freely available the production of aminoacids from them does not seem to pose a problem. α-ketoglutarate is a very important source for a number of aminoacids and is a product of ICDH catalysis. The stronger ICDH activity in comparison to SDH (Chapter 4) may be indicative of at least a low level operation of ICDH based catalysis, depicting the selective adaptation at the subcellular

level. In totality, the striking situation unveiling in blastema, a short living stage during regeneration where the old and the new meet, is especially, one, full of multifarious synthetic activities. Anabolic reactions characteristic of this stage of regeneration build up indigenous lipids, nucleic acids and proteins to attain self sufficiency for the big events to follow. Blastema is in fact, a dynamic phase striking a parallel with a miniature embryonic world, appearing as a bud during regeneration endowed with potentialities for multitude channels of restorative development. With the ill established TCA cycle and a carbohydrate supply which is at a premium, right from the time of autotomy uptil the formation of blastema, a systematic and well planned programme to extract all of the energy from the sparse glucose molecules with the maximum economy evident in the form of lipid biosynthesis is a clear cut indication of a preparatory phase wherein a definite attempt is being made to pile up a reserve source of energy for the progressive phases of regeneration lying ahead.

The biochemical knowledge obtained so far, enlightens one point, and that is the association of lipid synthesis with wound healing of regeneration.

It is rather pertinent in this wake to ponder as to whether this lipogenesis is a factor of distinction significance between regenerative and nonregenerative wound healing. If it be considered as a factor of significance, then, it is to be assumed that there exists in all animals endowed with the gift of regeneration a master plan to initiate this process of lipogenesis controlled either locally or distally at or before the time of wound healing. An interesting query that could be raised in this connection is whether this process of lipogenesis as such and or the lipid molecules so synthesized can be purported to supply the necessary stimulus or impetus for the process of regeneration ? More comparative works on these lines in both regenerating and nonregenerating animals of the same group might be helpful in deciding a possible significance of lipid molecules in the molecular ecology of regenerating systems.

Differentiation, the next stage in the linear progression of regeneration is marked by a tremendous rate of cellular proliferation and differentiation. As though in concert, along with this spurt of cellular activity, there was also a concomitant spurt in the activities of all the enzymes viz., LDH, MDH,

malic enzyme, G6PDH, BDH, αC-GPDH, SDH, ICDH, aldolase and diaphorases as noted in the previous chapters in the tail of Mabuya carinata. A similar increase has been observed with respect to LDH, MDH, αC-GPDH, aldolase, G6PDH and BDH activities during differentiation in the tail of Hemidactylus flaviviridis (Magon, 1970). Stepped up activities of SDH and malic enzyme have been reported in the same tail by Shah and Chakko (1969) and Shah and Hiradhar (unpublished) respectively. A well functioning TCA cycle denoted by the stronger activities of SDH and ICDH together with BDH were suggestive of lipid oxidations. Shah and Radhakrishnan (unpublished) and Chakko (1967) in their studies on distribution of lipids and lipase in Mabuya carinata and Hemidactylus flaviviridis respectively have in fact observed a gradual depletion of lipids and strong lipase activity from early to late differentiation phases. Persistence of stepped up activities of LDH, MDH, αC-GPDH and aldolase even in the absence of glycogen during blastema and early differentiation phases were construed to denote a process of gluconeogenesis during the differentiation phase. This interpretation arrived at in the course of the present work gains validity by the reported appearance of glycogen in the various

differentiating components of the tail regenerates (Shah and Radhakrishnan, unpublished; Chakko, 1967). Unfortunately, the distribution pattern of enzymes in the differentiating amphibian limbs are not available as it has not been given much prominance.

The distribution pattern of enzymes unveiled by the present works on the regenerating tail of Mabuya carinata is that there is a peak activity of all enzymes studied so far during the early phases of differentiation to face up to the metabolic challenges followed by diminishing activities of all the enzymes during the later phases till their attainment in the fully regenerate tail the original level of activity characteristic of the corresponding normal tail.

The above observations lend credulence to the fact that towards the completion of the process of regeneration with the attainment of morphological and anatomical restoration, a simultaneous effort at biochemical normalisation as well is in progress evident by the decreasing activities of the various enzymes and the metabolic shift with regard to the type of metabolite as the principal source of energy. The shift is well exemplified by the increasing titre of glycogen in the differentiating tissues (Shah and

Radhakrishnan, unpublished; Chakko, 1967) and the concomitant depletion of lipids. The above observation when viewed in light of the presently observed higher activities of all the glycolytic enzymes during differentiation highlights the fact that apart from their utilization for high energy requirements lipids are also being consumed for gluconeogenesis by a reversal of the glycolytic process. It could thus be well within the realms of speculation to conclude that the process of gluconeogenesis is another important subcellular biochemical adaptation in the unfolding metabolic spectrum of regenerating reptilian tails. In this respect it could be assumed that a part of the oxaloacetate is being continually used up for the functioning of the TCA cycle whereas another part is being utilized for the formation of phosphoenolpyruvate for gluconeogenesis. With the TCA cycle in operation, citrate, α-ketoglutarate and malate can serve as effective sources of extramitochondrial oxaloacetate. The role of malic enzyme at this stage could well be identified with the continuous replenishment of a pyruvate pool for oxaloacetate production. Studies on factors regulating the rate of gluconeogenesis in animal tissues have well established the importance of lactate,

EXPLANATIONS FOR FIGURES

Fig. 1. Synoptic chart summarising the metabolic events in the normal tail of Mabuya carinata based on the observations from the present study.

Fig. 2. Diagramatic representation of the metabolic reactions in the blastema of the regenerating tail of Mabuya carinata based on the data obtained from the present work.

Fig. 3. Possible metabolic scheme during differentiation in the tail of Mabuya carinata based on the present investigations.

N.B.: Black arrows (⟶) indicate the enzymes studied and their thickness represent the intensity of activity.

acetoacetate and acetyl co.A in the acceleration and proper operation of the gluconeogenetic process (Krebs et al., 1963). The peak activities of BDH and LDH noted herein may in this regard be considered as rather impressive and suggestive. Channelization of glycerol moities set free during lipid utilization into the gluconeogenetic process could well be postulated as a distinct possibility within the realms of high OC-GPDH activity (Chapter 4). Further more judicious work along this line should prove useful in not only confirming the operation of this biochemical process during regeneration but also in the identification and elucidation of the underlying controlling factors.

Present observations on diaphorases and cytochrome oxidase (Chapter 6) have also brought to focus some interesting aspects. The distribution pattern obtained for diaphorases not only indicate the presence of two distinct diaphorases (NAD and NADP) in the regenerating tail of Mabuya carinata but also aid in confirming the observations on dehydrogenases. Whereas poor activity of NADP diaphorase during blastema was obviously in lieu of the direct linking of reduced NADP with fatty acid synthesis, its poor activity in the normal tail was in good conformity with the poor

activities of the corresponding NADP dependant dehydrogenases. With the ceasing of lipid synthesis, NADP diaphorase made itself became prominent during differentiation. In contrast, NAD diaphorase was found to have a parallel distribution pattern with its corresponding dehydrogenases all throughout regeneration. Though all animal tissues in general are known to possess a cytochrome oxidase mediated oxidative metabolism, the normal and regenerating tail of Mabuya carinata presented a total lack of this enzyme. Poor representation of this enzyme in the normal and blastemic phases is understandable in the light of low or negligible levels of TCA cycle operation, its inadequate level during differentiation remains to be explained. The presence of diaphorases and the absence of cytochrome oxidase noted herein tempt one to surmise to surmise the possibility of some other respiratory mechanism in the regenerating reptilian tails as a number of alternate schemes have been widely postulated in both plant and animal tissues. Schmidt (1966) based on the studies in the regenerating amphibian limb has also ~~concluded~~ suggested on a similar possibility. It is of interest to note that ascorbic acid oxidase has been considered

to be an alternative to cytochrome oxidase as a terminal oxidase in plant tissues (James, 1957). Beevers (1954) and Kern and Racker (1954) have suggested the involvement of ascorbic acid itself in the respiratory chain in pea seedlings. Many more discussions on ascorbic acid and ascorbic acid oxidase participation are available (Goodwin, 1960; Mapson, 1953; Meiklejohn, 1953 and Chinoy, 1969a). As ascorbic acid has been shown to be well localized in the normal and regenerating tails of lizards (Shah and Radhakrishnan, unpublished and Shah et al., 1971) and as ascorbic acid is being more and more linked with respiratory metabolism of animal tissues, the possible participation of ascorbic acid in the electron transport mechanisms of regenerating systems cannot be overlooked. Yet another plausible scheme is that of Kikuchi et al., (1959) who after their extensive studies on the electron transport mechanism in Ascaris lumbricoides have identified a cytochrom 'b' factor which after receiving electron(s) from diaphorase can transfer it directly to oxygen without the intervening mediation of cytochrome oxidase. As the existance of a credibility gap on respiratory mechanism is brought out by the present investigations in the regenerating lizard tail, application of suitable

investigations in the light of above postulations may yield both interesting and fruitful revelations.

Finally, on a casual view, the highlights revealed by the present investigations on the normal and regenerating tail of Mabuya carinata could be summarised as follows.

The normal tail which is chiefly glycolytic is followed by a preparatory phase starting with wound healing and culminating at blastema during which there is a primary and quick acquisition of necessary synthetic machinary and synthesis of lipids, nucleic acids and proteins and a secondary slow development of necessary enzymological equipment for lipid utilization. The next phase, differentiation, is marked by two important aspects (1) the active oxidation of lipids for the extra energy needs of the actively proliferating and differentiating tissues and (2) the simultaneous utilization of lipids for gluconeogenesis. Finally, at the conclusion of regeneration, alongwith the attainment of fully regenerate condition which is morphologically and anatomically similar to the normal tail, there is also a biochemical restoration by its reversal back to a glycolytic condition. This change

of metabolite from carbohydrate to lipid and back to carbohydrate thus represents the metabolic shift during reptilian tail regeneration.