CHAPTER 6

A HISTOCHEMICAL STUDY ON SOME ASPECTS OF RESPIRATORY MECHANISM DURING REGENERATION IN THE TAIL OF THE SCINCID LIZARD, MABUYA CARINATA

Invariably, all living tissues are dependant on metabolism for their existence. This dynamic life sustaining phenomenon encompasses both anabolic as well as catabolic reactions, manifest in the form of growth and yield of energy respectively. A continuous supply of energy is of primary importance not only for the multiple day to day functioning of the various cellular organizations but also for the innumerable synthetic activities at work. The biological energy currency is represented by the adenosine triphosphate (ATP) molecules. During anaerobic glycolysis, the ATP molecules are formed as a result of direct phosphorylation of adenosine diphosphate(ADP) whereas during aerobic TCA cycle oxidations this phosphorylation is coupled to the respiratory chain or the electron transport chain and is denoted by the term oxidative phosphorylation. Cytochrome oxidase is well recognized as the terminal enzyme in the electron transport chain wherein it catalyzes the transfer of

electron(s) from cytochrome.c to molecular oxygen. This enzyme by its intimate association with oxidative phosphorylation is not only involved in the cellular energetics but a study of it could as well denote the presence or absence of oxidative metabolism. Even though this enzyme is purported to be widely distributed in the tissues of various vertebrates, its activity in the regenerating tissues has been poorly understood. In fact, to-date there appears to be only two isolated cases of investigation in this respect; that of Niwelinski (1960) in the regenerating limb of adult Triturus viridescens and that of Magon (1970) in the tail of Hemidactylus flaviviridis. It was thus deemed fit to study the distribution of cytochrome oxidase in the normal and regenerating tail of the scincid lizard, Mabuya carinata.

Besides, another aspect found interesting to investigate was that of diaphorases. For the efficient working of all metabolic reactions, co.factors play no less an important role. Co.enzymes I and II (NAD and NADP) are inevitably associated with most of the dehydrogenase catalyzed metabolic reactions. They fill in the role as efficient hydrogen acceptors set free as a result of dehydrogenase activity. The sustained

rhythmic pace of the metabolic processes can be maintained only by making available to the dehydrogenases a continuous supply of NAD and NADP. The living tissue has circumvented this problem by two specific flavoprotiẹn enzymes NAD and NADP diaphorases (NAD.D and NADP.D) which reoxidize the reduced co.enzymes ($NADH_2$ & $NADPH_2$) and thus replenish them continually. The term diaphorase was coined by Corran, Green and Straub (1939). The operation of NAD.D and NADP.D was discovered by Green, Dewan and Leloir (1937), Von Euler and Helstrom (1938) and Alder et al., (1939) respectively. Lately Massey (1960) has related NAD.D to lipoyl (lipoamide) dehydrogenase. Eventhough de Duve et al., (1962), and Long (1961) have questioned the validity of the existence of NADP.D, histochemists for all practical purposes have readily demonstrated the two diaphorases by their distinct staining patterns (Pearse, 1960; Burstone, 1962). The histochemical demonstration of the two diaphorases was integrated in the present investigation to evaluate the possible functioning of them in the biochemical interactions taking place during lacertilian tail regeneration specifically and in regeneration generally as there is an acute paucity of such information felt of such a study. Moreover, their operation during

the various phases of regeneration could not only establish the presence of two distinct diaphorases in regenerating systems but could also be correlated with reflected on to the activities of the corresponding co.enzyme dependant dehydrogenases as ∉ confirmatory evidence.

MATERIAIS AND METHODS

The adult Mabuyas selected for the experiment were maintained on a diet of young cockroaches. The normal and regenerating tails were autotomized by pinching off 1-2 inches away from the vent. The autotomized tails were blotted to remove blood and tissue fluids and were fixed on a chuck of a cryostat microtome maintained at -20°C. Longitudinal and transverse sections of 12-18 μ thickness were cut and incubated at room temperature for about 45 minutes in the respective incubation media prepared as follows. For demonstrating histochemically the cytochrome oxidase in the normal and regenerating tail of Mabuya carinata, the incubation medium was prepared as per the method adopted from the reports of Moog (1943) and Nachlas, Crawford, Goldstein and Seligman (1958). The Nadi mixture was composed of the following reagents.

Phosphate buffer (0.1 M; pH 7.4)	3 ml
∝C-Naphthol (1 mg/ml in 1% NaCl)	5 ml
Dimethyl.p.phenylene diamine hydrochloride (1 mg/ml in 1% Nacl)	5 ml
Cytochrome C (3 mg/ml)	2 ml

All reagents were freshly prepared, mixed together and filtered just before use. Control sections were treated with sodium azide and sodium cyanide before incubation as suggested by Moog (1943). After incubation sections were thoroughly washed in 0.6% NaCl solution and mounted in saturated potassium acetate. The incubation media for NAD and NADP diaphorases were prepared by the method of Ogata and Mori (1964) as given below.

Ingredients	NAD.D	NADP.D
Reduced co.enzyme ($NADH_2$)	5 mg	--
Reduced co.enzyme ($NADPH_2$)	-	5 mg
Phosphate buffer (0.2 M; pH 7.6)	1 ml	1 ml
Nitro-BT (5 mg/ml)	1 ml	1 ml

Control: A few sections treated with water at 80°C before incubation and a few other sections incubated in substrate blank medium served as the controls.

OBSERVATIONS

NORMAL TAIL (Figs.1, 1A & 2A)

Of the various tail components, epidermis and muscle depicted the highest activities of all the three enzymes under investigation. In the epidermis the stratum germinativum showed the maximum followed by the alpha and beta layers of cells. Of the two diaphorases, NAD.D was the most active whereas NADP.D revealed a relatively weak activity. Cytochrome oxidase was the least active of the three and its activity was found to be almost negligible.

The maximal activity for cytochrome oxidase was registered by the muscle fibres and only in the peripheral ones in each of the fasciculus. Even this maximal cytochrome oxidase activity in the muscles is subminimal when compared to diaphorases. NAD.D was highly active in muscles and was found to be more than NADP.D. The localization of both, NAD.D and NADP.D was mitochondrial as well as cytoplasmic. NADP.D though discernible in the muscles was nevertheless found to be poor. In the vertebral column and nerve cord, cytochrome oxidase was more or less nonexistent. NADP.D was poorly localized whereas NAD.D was well

localized in the cartilage cells of the centrum and, in the nerve cord, where in the grey matter, it was more concentrated than in the white matter.

REGENERATING TAIL

Wound healing phase: (Figs.2, 3A & 1B)

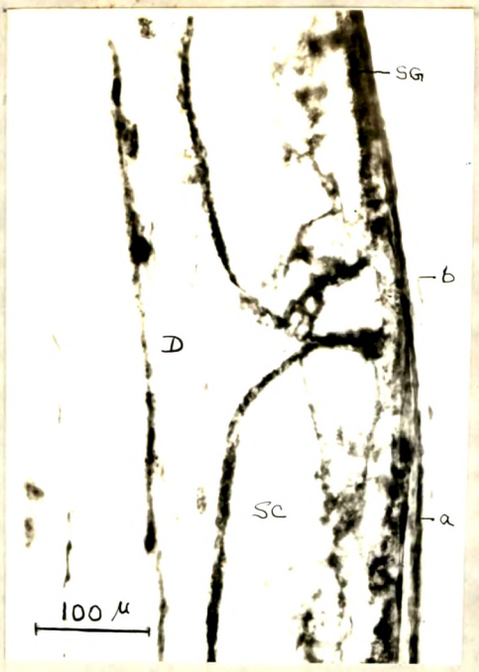
Cytochrome oxidase activity did not reveal any difference from that in the normal tail. Neither the wound epithelium nor the underlying cellular assemblage responded for this enzyme. NAD.D activity was prominant in both the wound epithelium as well as subapical cells below the epithelium. In contrast, the NADP.D again was demonstrated though noticeable but at low levels of activity.

Blastemic Phase: (Figs.3-4, 4A & 2B)

A distinct feature of this phase was the high activity of NAD.D and a poor localization of NADP.D. Once again cytochrome oxidase remained unrepresented in both the mesenchymal cells as well as the blastemic epithelium.

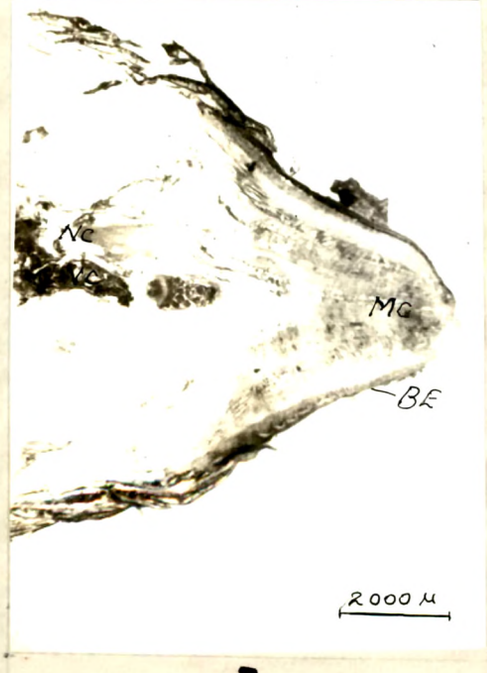
Differentiation phase: (Figs.5-7, 5A-7A & 3B-5B)

Once the cells started differentiating into various components of the regenerate, there was a marked



1

2



3

4

EXPLANATIONS FOR FIGURES

Fig. 1. Photomicrograph of T.S. of normal tail skin showing cytochrome oxidase activity.

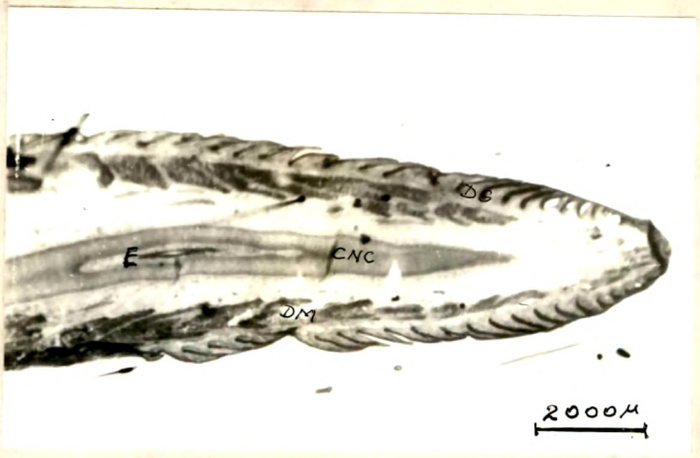
Fig. 2. Photomicrograph of wound epithelium showing cytochrome oxidase activity.

Fig. 3. Photomicrograph of L.S. of blastema showing poor cytochrome oxidase activity.

Fig. 4. Poor localization of cytochrom oxidase in the blastemic epithelium.

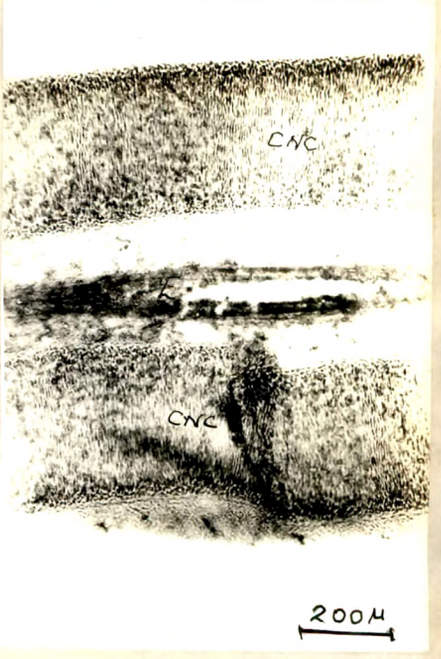
ABBREVIATIONS

a	-	alpha cells
b	-	beta cells
BE	-	Blastemic epithelium
D	-	Dermis
MC	-	Mesenchymal cells
NC	-	Nerve cord
SC	-	Scute
SCA	-	Scab
SEC	-	Subepithelial cells
SG	-	Stratum germinativum
VC	-	Vertebral column
WE	-	Wound epithelium



5

6



7

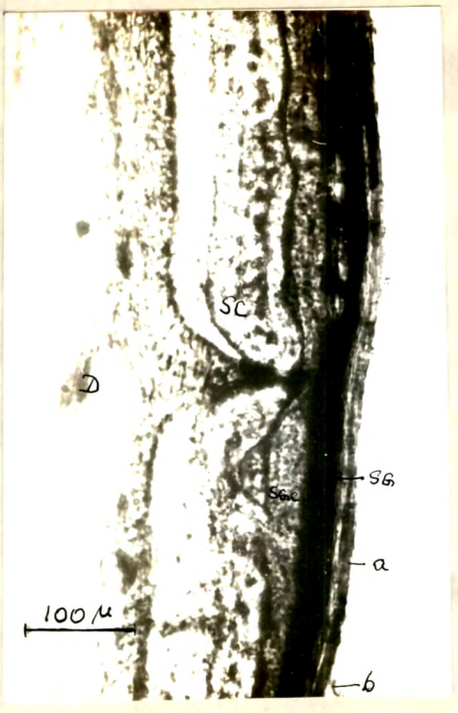
Fig. 5. Photomicrograph of L.S. of differentiating tail showing the maxium cytochrome oxidase activity.

Fig. 6. Differentiating scales and muscles showing cytochrome oxidase activity.

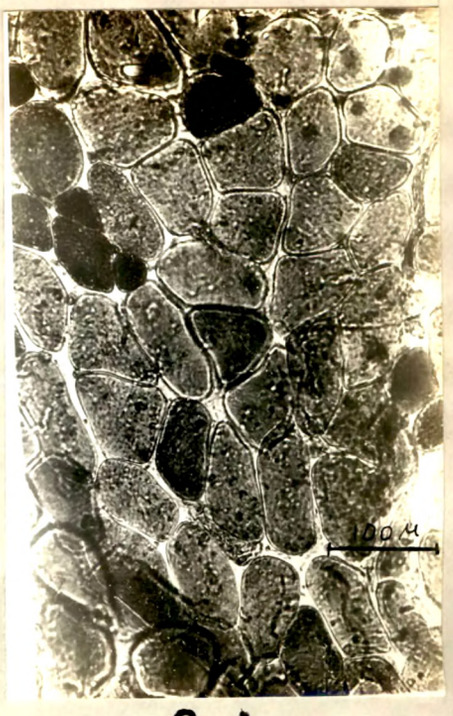
Fig. 7. Enzyme activity in the cartilaginous neural canal and ependyma.

ABBREVIATIONS

CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma

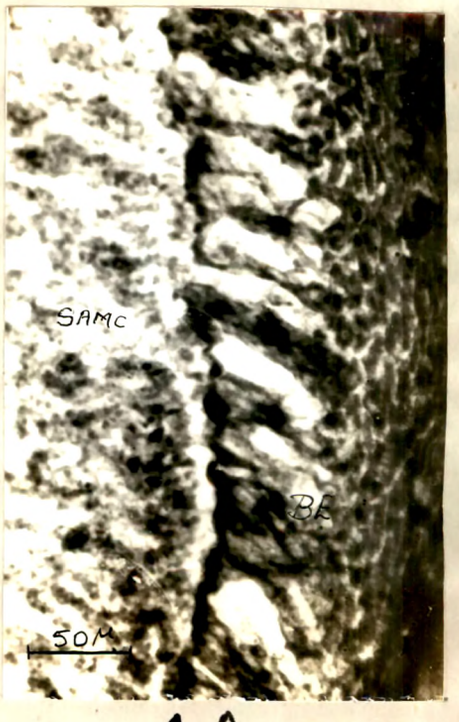


1 A



2 A

3 A



4 A

Fig. 1A. Photomicrograph of T.S. of normal tail skin showing DPN.D (NAD.D) activity.

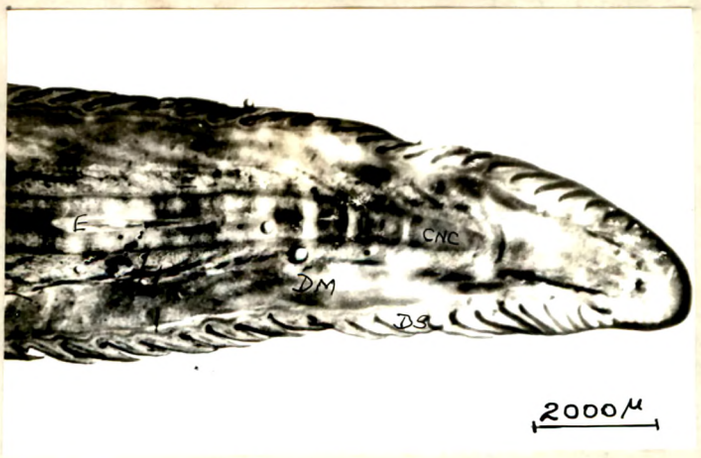
Fig. 2A. T.S. of tail muscles depicting NAD.D activity.

Fig. 3A. Wound epithelium showing prominent enzyme activity.

Fig. 4A. Increased NAD.D activity in the blastemic epithelium.

ABBREVIATIONS

a	-	alpha cells
b	-	beta cells
BE	-	Blastemic epithelium
D	-	Dermis
SAMC	-	Subapical mesenchymal cells
SC	-	Scute
SEC	-	Subepithelial cells
SG	-	Stratum germinativum
SGC	-	Scutogenic cells
SWE	-	Stratified wound epithelium



5 A

6 A

7 A

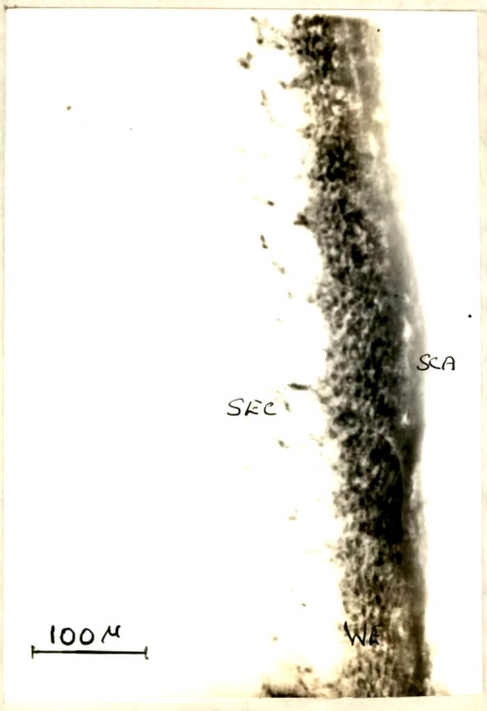
Fig. 5A. Photomicrograph of L.S. of differentiating tail showing high NAD.D activity.

Fig. 6A. Differentiating scales and muscles with strong localization of NAD.D.

Fig. 7A. Cartilaginous neural canal and ependyma showing NAD.D activity.

ABBREVIATIONS

CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma



1 B

2 B

3 B

Fig. 1B. Wound epithelium showing NADP.D activity.

Fig. 2B. Blastemic epithelium showing localization of NADP.D. Note the absence of NADP.D in the mesenchymal cells.

Fig. 3B. Photomicrograph of L.S. of differentiating tail showing pronounced NADP.D activity.

ABBREVIATIONS

BE	-	Blastemic epithelium
CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma
SAMC	-	Subapical mesenchymal cells
SCA	-	Scab
SEC	-	Subepithelial cells
WE	-	Wound epithelium

4 B

5 B

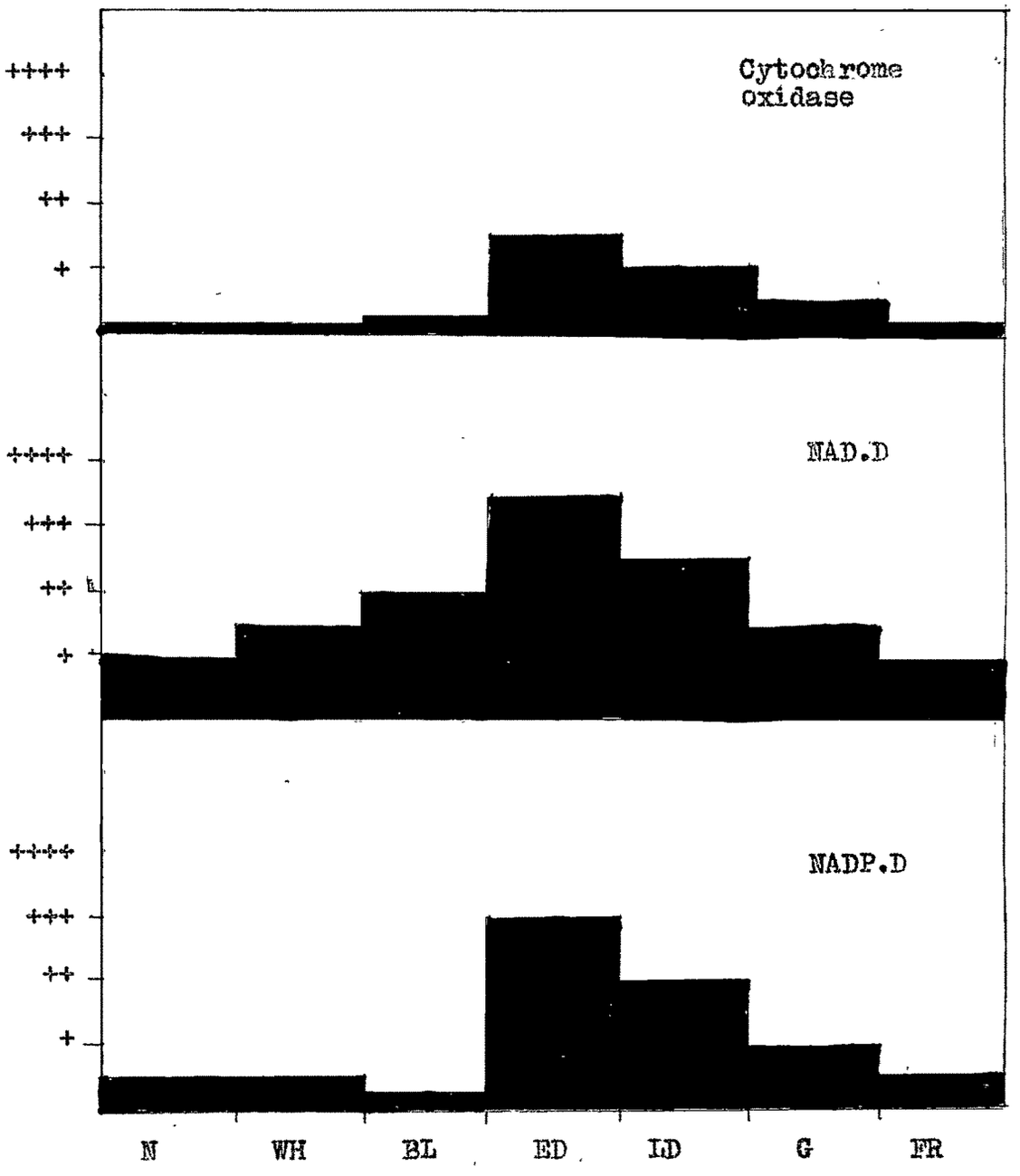
Fig. 4B. Differentiating scales and muscles showing strong localization of NADP.D.

Fig. 5B. NADP.D activity in the cartilaginous neural canal and ependyma.

ABBREVIATIONS

CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma

Graphic representation of the changes in the cytochrome oxidase, NAD.D & NADP.D distribution pattern during the various stages of tail regeneration



N: Normal tail; WH: Wound healing phase;
BL: Blastemic phase; ED: Early differentiation phase;
LD: Late differentiation phase; G: Growth phase;
FR: Fully regenerated tail.

increase of all the three enzymes. NAD.D which was constantly high, stepped up its activity to a peak level at this phase. At the same time the NADP.D showed a spurt of activity which in its localization, distribution and intensity was at par with that of NAD.D. During regeneration, for the first time cytochrome oxidase made its representation felt at this phase. The enzyme activity was moderate and could be noticed in all the differentiating elements. All the three enzymes showed high activity in the differentiating epidermis, muscles and chondrocytes. It was interesting to note that cytochrome oxidase activity discernible during differentiation was comparatively lower than that of SDH and ICDH noticed during this very phase (Chapter 5).

Growth phase:

During this phase, there was a gradual but definite drop in the activity of all the three enzymes in all the components of the regenerate which, finally attained the level of activity and localization characteristic of the normal tail.

DISCUSSION

The normal tail of Mabuya carinata as seen from

the previous chapters is principally adopted for anaerobic glycolysis. Being chiefly adjusted for glycolysis, a continuous supply of co.enzymes is of utmost importance for its ceaseless operation. In the present study on diaphorases, NAD.D was found to be more active than NADP.D in the normal tail. This seems to reflect well on the distribution of various co-enzyme dependant dehydrogenases. Present investigations have clearly demonstrated that NAD dependant dehydrogenases are very dominant in comparison to the poor display of NADP dependant ones. The parallelism thus noticeable between NAD dependant dehydrogenases and NAD.D on the one hand and NADP dependant dehydrogenases and NADP.D on the other is self explanatory. Wolfe and Cohen (1963) have noticed a similar distribution of NAD.D and NADP.D in the limb striated muscle of Diemictylus viridescens. At the same time, the almost nonexistent activity of cytochrome oxidase might be correlated with the poor SDH and ICDH activities in the normal tail and once again establishing an impregnable evidence that TCA cycle oxidations and cytochrome oxidase system per se have little to contribute towards the normal tail energetics.

During the regressive phase of regeneration i.e., from wound healing onwards upto blastema, both cytochrome oxidase and NADP.D maintained a steady status quo level as noticed in the normal tail. In fact, there was no change at all in the activities of these two enzymes whereas the NAD.D did show a gradual increase. This increase of NAD.D seems to go hand in glove with the increasing activities of NAD dependant dehydrogenases during wound healing and blastemic phases. Similar observation regarding NAD.D has been made in the wound epithelium and blastema of regenerating limbs of adult newts, Diemictylus viridescens and Triturus vulgaris (Wolfe and Cohen, 1963; Niwelinski, 1960). In contrast to this parallelism between NAD.D and corresponding dehydrogenases is the revelation of diametrically opposite distribution of NADP.D and the corresponding dehydrogenases. It is of interest to note that when there was a gradual acquisition and increase of NADP dependant dehydrogenases throughout wound healing and blastemic phases there was no corresponding increase in the activity of NADP.D. In fact, the NADP.D response in both the above phases remained very poor. Niwelinski's (1960) observation on NADP.D wherein he has noticed

only a gradual increase of NADP.D for the blastemal cells of Triturus vulgaris seems to be more closer to the present observation than the sharply increased NADP.D activity noticed in the blastema of Diemictylus viridescens (Wolfe and Cohen, 1963). Keeping in mind this high NADP.D activity, Schmidt (1966) has propounded the possibility that the NADP produced by the blastemal NADP.D may serve as a co.enzyme to metabolic pathways than has yet been discovered during regeneration. But in the blastema of the tail of Mabuya carinata, the poor activity of NADP.D is a clear cut indication of the subcellular level metabolic adaptation at this phase. Blastema being a synthetic phase, the larger quantities of $NADPH_2$ being produced by the various co.enzyme II dependant dehydrogenases seem to be of high utility value in the various cellular anabolic reactions especially lipogenesis (Chapters 2, 3, 4 & 5) rather than a linkage with the oxido-reduction reactions catalyzed by NADP.D. Even though the TCA cycle per se plays a negligible role in the blastemic metabolism, the presence of SDH and ICDH (Chapter 5) in the cells of blastema and the presently observed unnoticeable cytochrome oxidase activity, however little ~~least~~, should be a matter of some concern and indeed is thought

provoking. However, the intense cytochrome oxidase noticed by Niwelinski (1960) appears to be an opposite extreme and Schmidt (1966) rightly considers it contradictory in view of the incompleteness of the TCA cycle. An interesting study from this laboratory has revealed adequate amounts of ascorbic acid in both the normal tail as well as in the cells of blastema (Shah et al., 1971; Shah and Radhakrishnan, unpublished). With its purported easy redox potential it could be suggested that ascorbic acid may be serving either the same or an identical role as cytochrome oxidase in electron transport in the regeneration blastema of the tail of Mabuya carinata. The possible involvement of ascorbic acid in such a function in living tissues is well discussed in ~~many~~ the scientific literature (Goodwin, 1960; Mapson, 1953; Meiklejohn, 1953 and Chinoy, 1969a).

Alongwith the progression of differentiation the cytochrome oxidase made its appearance and could be discernible in the various differentiating tissues. Moderate activity of cytochrome oxidase during the differentiation phase is suggestive of the fact that alongwith the full functioning of the TCA cycle the normal appointed function of the respiratory mechanism is being assumed by it. With the vigorous activity of all

the enzymes including SDH and ICDH noticed at this stage, the not so high activity of cytochrome oxidase is yet beyond feasibility. It is again interesting to note the five times increase of ascorbic acid recorded in the differentiating tails of Hemidactylus flaviviridis and Mabuya carinata (Shah et al., 1971; Shah and Radhakrishnan, unpublished) respectively. It may be presumed though no convincibility exists that apart from its stepped up role in various synthetic processes ascorbic acid is an additional aider and abetter or even an alternative of cytochrome oxidase in the respiratory mechanics of the regenerating tail. It is worthwhile to note in this connection that Schmidt (1966) has opined the possibility of the operation of other respiratory mechanisms other than that of cytochrome oxidase and which he considers might be the case for the amphibian regeneration blastema. Drawing the attention towards diaphorases at this phase (differentiation) of regeneration, the activities of both the diaphorases were high. With the releasing of reduced co.enzyme necessary for synthetic reactions during differentiation the exigency to replace oxidised co.enzyme II for the highly active co.enzyme II dependant dehydrogenases has become more acute and in

tune, the NADP.D registered a great spurt to meet this challenge. It is safe to assume that both the NAD and NADP diaphorases have, during the differentiation phase, attained their top value, keeping pace with the corresponding dehydrogenases to perform their normal function of replenishing and maintaining continuous supply of oxidized co.enzymes for the continuity of the various oxido-reduction reactions of metabolism with which they are associated.

Finally with the advent of the growth phase all these three enzymes recorded a gradual drop keeping in step with the corresponding physiologically associated dehydrogenases and ultimately attaining in the fully regenerated tail, a concentration and distribution identical to that of the normal tail as with their corresponding dehydrogenases.

The present chapter apart from providing a confirmatory evidence in favour of the various dehydrogenases, has also lent credulence to the existence of two distinct diaphorases at least in the regenerating system.