CHAPTER 3

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH) AND MALIC ENZYME IN THE REGENERATING TAIL OF THE SCINCID LIZARD, MABUYA CARINATA

Some of the past and recent studies on the regenerating tissues of vertebrates have proved the existance of glycolytic cycle, hexose monophosphate (HMP) shunt pathway and reduced TCA cycle as the main energy yielding reactions during the various phases of regeneration. These investigations tend to highlight the dominant role of HMP shunt pathway in the regeneration blastema. The shunt pathway, as already known, is the major metabolic pathway identifiable in the embryonic cells. The existence of this pathway and its role in the metabolism of animal tissues have been established by Chefurka (1957, 1958); Hoskin (1959) and Rossi et al (1963).

Glucose-6-phosphate dehydrogenase (G6PDH) is the first enzyme of a series, which brings about the direct oxidation of glucose at the level of glucose-6-phosphate to liberate reduced co.enzyme II ($NADPH_2$) and hexose pentose phosphates necessary for the synthesis of fatty acids and nucleotide metabolism

respectively (Abraham et al., 1954, Beaconfield, 1964a & b). The histochemical observation of G6PDH which affords an undoubtable clue to the existence of the pentose phosphate cycle has been carried out in the regenerating limb of adult newts, Triturus viridescens by Wolfe and Cohen (1963) and in Diemictylus viridescens by Schmidt and Weidman (1964). These investigators have reported a high activity for G6PDH in the wound epithelium, blastema and differentiating chondrocytes and a low activity of this enzyme in the limb striated muscle as compared to that of other dehydrogenases such as LDH, MDH and αC-GPDH. The presence of lipids and nucleotides during regeneration (Schmidt, 1964a, b & c; Chakko, 1967) further lend strong evidence to the important role of this enzyme in regeneration.

It was the intention of the present work in this context to farther the insight into the possible metabolic pattern of the regenerating tail of reptiles, by studying the histochemical localization of G6PDH together with malic enzyme, known to play an equally important and identical role in the production of reduced nicotinamide adenine nucleotide diphosphate and, also to ascertain the validity of the concept

arrived at in chapters one and two in the regenerating tail of the lizard, Mabuya carinata.

MATERIALS AND METHODS

The selected adult Mabuyas were maintained on a diet of young cockroaches in the laboratory. The autotomy of normal and regenerating tails were carried out by pinching off the tail 1-2 inches distal to the vent. The wound surfaces were blotted to remove blood and tissue fluids and the autotomized normal and regenerating tails were immediately fixed on a microtome chuck of a cryostat maintained at -20°C. Longitudinal and transverse sections of 12-18 μ thickness were cut and incubated for about 30 minutes at room temperature in the respective incubation media prepared as follows and maintained at a pH of 7 - 7.4.

Glucose-6-phosphate dehydrogenase

Substrate (Glucose-6-phosphate disodium salt, 1 M)		0.1 ml
Triphospho pyridine nucleotide	(0.1 M)	0.1 ml
Nitro-BT	(1 mg/ml)	0.25 ml
Sodium azide		0.1 ml
Sodium fluoride		0.05 ml
Magnesium chloride	(0.05 M)	0.1 ml

Tris buffer, pH 6.8 - 7	(0.2 M)	0.25 ml
Polyvinylpyrrolidone		75 mg

Malic enzyme

Substrate (Sodium malate, 1 M)		0.1 ml
Triphospho pyridine nucleotide	(0.1 M)	0.1 ml
Sodium cyanide	(0.1 M)	0.1 ml
Magnesium chloride	(0.05 M)	0.1 ml
Nitro-BT	(1 mg/ml)	0.25 ml
Phosphate buffer, pH 6.8 -7	(0.06 M)	0.25 ml
Polyvinylpyrrolidone		75 mg

Control: Control sections were incubated in the respective substrate blank media and some were heated in water at 80°C before incubating in the normal media. Sections were then washed thoroughly in distilled water and fixed in 10% buffered neutral formalin at 4°C for about 2 hours. These were again washed in distilled water and mounted in glycerine jelly.

OBSERVATIONS

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

Skin:

The outermost layers of the skin, the beta and alpha cells were enzyme negative due, mainly to the

process of keratinization. Appreciable activity of the enzymes was noticeable in the cells of the stratum germinativum and also in the scutogenic cells. In the dermis and the subcutaneous adipose tissue, the concentration of the enzymes was poor.

Muscle:

The muscle fibres showed a moderate activity of these two enzymes though not much appreciable. The activity was discernible in the form of both fine mitochondrial as well as diffuse cytoplasmic. Of the muscle fibres, the peripheral fibres in each fasciculus were more prominant in enzyme activity as compared to the inner bigger fibres which showed a low level of activity. Although the activity of these two enzymes in the muscle fibres of the normal tail was distinctly less as compared to LDH and MDH, neverthless, the muscle fibres could be distinguished into three types with regard to the mitochondrial number and enzyme concentration as with LDH and MDH distributions, reported in Chapter 2.

Submuscular adipose tissue:

There was noticeable localization of the enzymes in the peripheral part of the cells of submuscular adipose tissue. However, the development

of bluish colouration in the adipose tissue could be due to tetrazoleum adsorption.

Vertebral column and nerve cord:

Excepting for the cartilage cells at the articulating surfaces of the centrum, the rest of the cells of the vertebrae and the matrix failed to show any response towards these enzymes whereas in the nerve cord, the grey matter evoked a positive response and the white matter a negative one.

REGENERATING TAIL

Wound healing phase: (Figs.3, 5 & 3A, 5A)

The wound epithelium together with the cut end of the stump tissues exhibited a pronounced activity of the enzymes when compared to that in the normal tissues of the original tail.

Blastemic phase: (Figs.4, 6 & 4A, 6A)

The pronounced activity of the enzymes noticed in the wound epithelium was further enhanced and could be seen as a gradual increasing activity which paralleled in correspondence to the progressive stratification of the wound epithelium as it continued getting changed into the blastemic epithelium. The mesenchymal cells filling up the blastemal cap also showed enhanced enzyme activity

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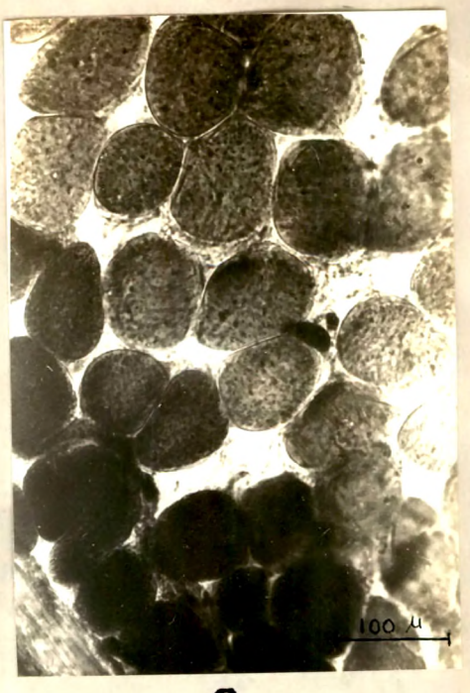

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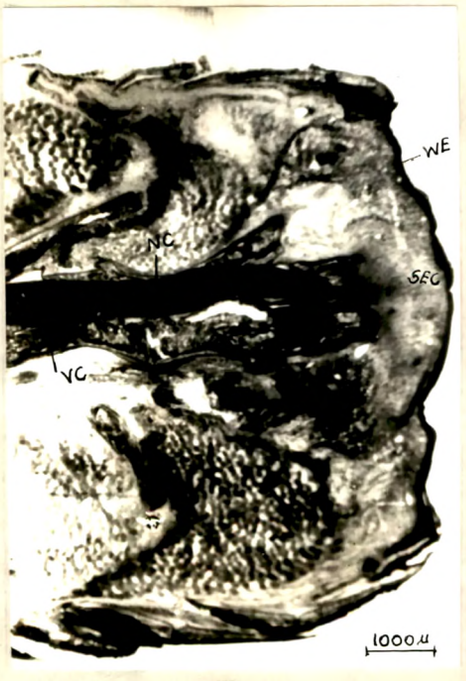

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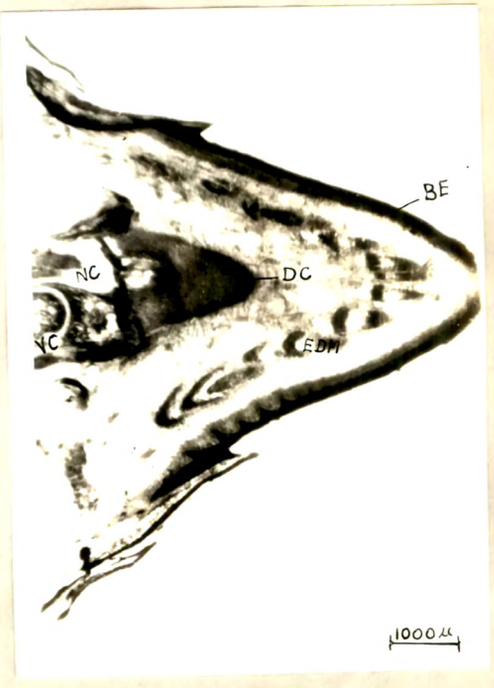

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EXPLANATIONS FOR FIGURES

Fig. 1. Photomicrograph of T.S. of normal tail skin showing G6PDH activity in the stratum germinativum.

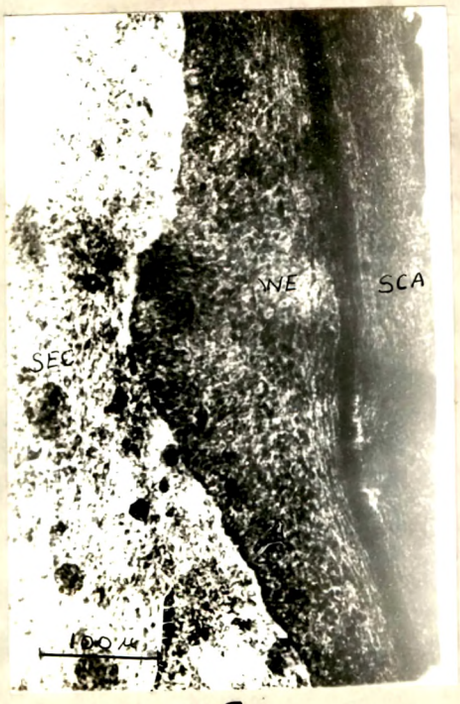
Fig. 2. Photomicrograph of T.S. of normal tail muscles showing enzyme activity.

Fig. 3. Photomicrograph of L.S. of wound healing tail showing increased G6PDH activity. Note the enzyme activity in the wound epithelium.

Fig. 4. Photomicrograph of L.S. of blastema showing high G6PDH activity.

ABBREVIATIONS

a	-	alpha cells
b	-	beta cells
BE	-	Blastemic epithelium
D	-	Dermis
DC	-	Differentiating cartilage
EDM	-	Early differentiating muscle
MF	-	Muscle fibres
NC	-	Nerve cord
SC	-	Scute
SEC	-	Subepithelial cells
SG	-	Stratum germinativum
SGC	-	Scutogenic cells
VC	-	Vertebral column
WE	-	Wound epithelium



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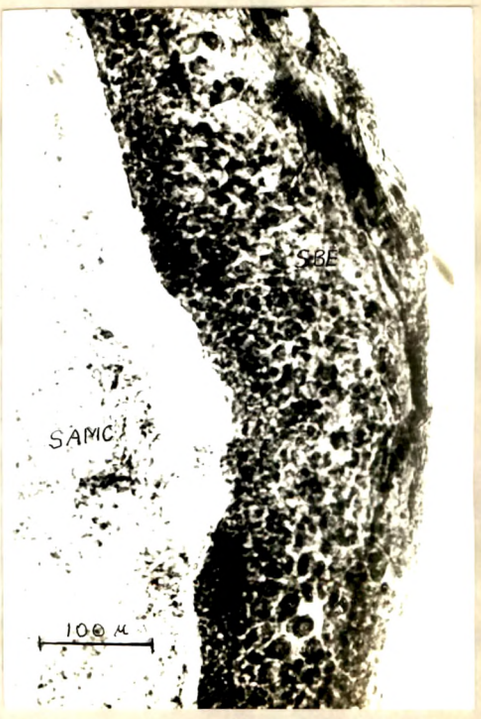

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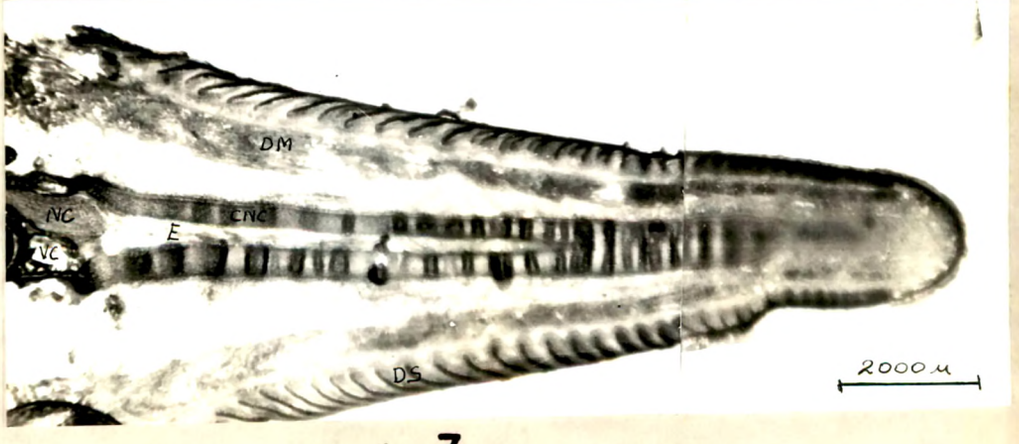

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Fig. 5. Photomicrograph of wound epithelium with subepithelial cells showing enzyme activity.

Fig. 6. Photomicrograph of blastemic epithelium showing G6PDH activity. Note the enzyme activity in the subapical mesenchymal cells.

Fig. 7. Photomicrograph of the L.S. of differentiating tail showing G6PDH activity in the various differentiating elements.

ABBREVIATIONS

CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma
NC	-	Nerve cord
SAMC	-	Subapical mesenchymal cells
SBE	-	Stratified blastemic epithelium
SCA	-	Scab
SEC	-	Subepithelial cells
VC	-	Vertebral column
WE	-	Wound epithelium

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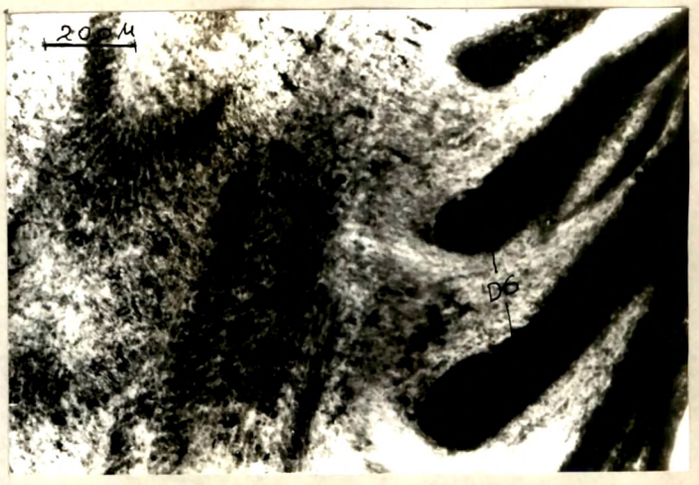

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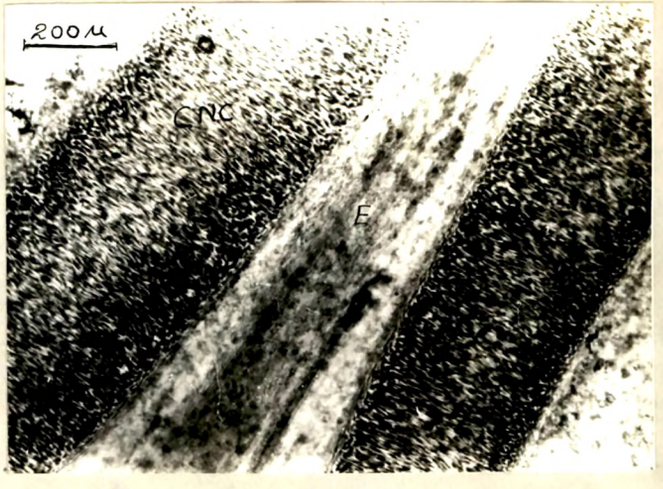

Fig. 8. Photomicrograph of differentiating scales and muscles demonstrating high G6PDH activity.

Fig. 9. Photomicrograph of cartilaginous neural canal and ependyma with high enzyme activity.

ABBREVIATIONS

CNC	-	Cartilaginous neural canal
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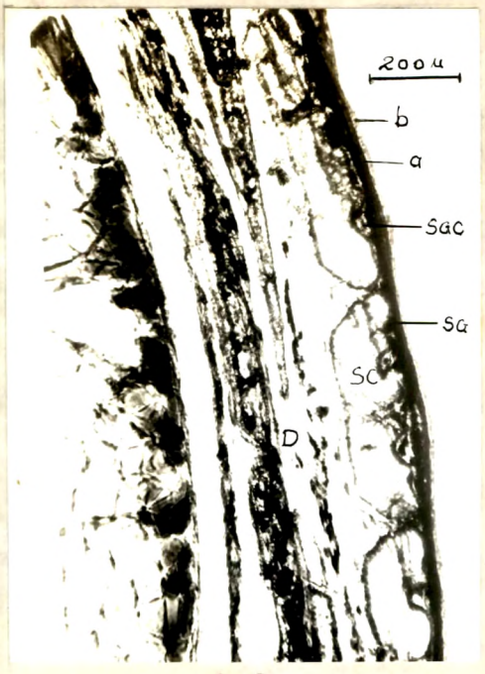
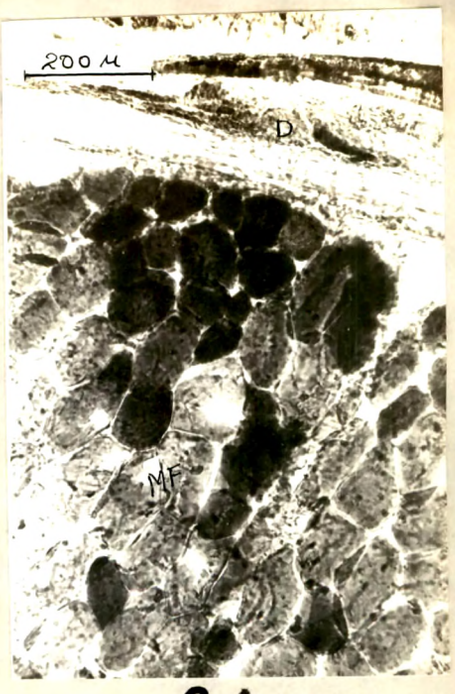

200 µ
b
a
SGC
SG
SC
D

1 A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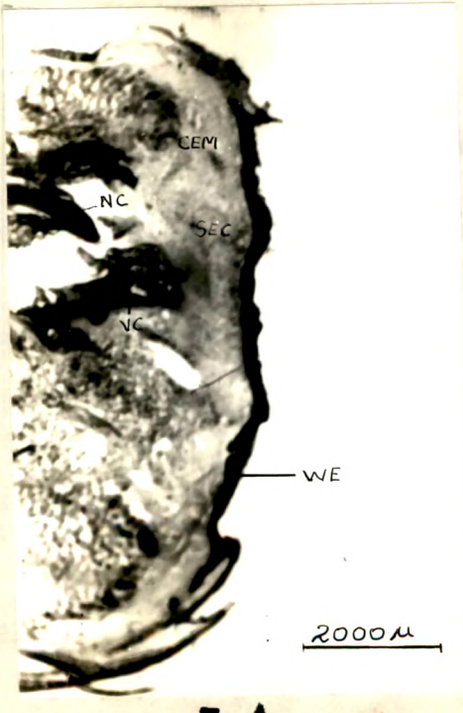
2 A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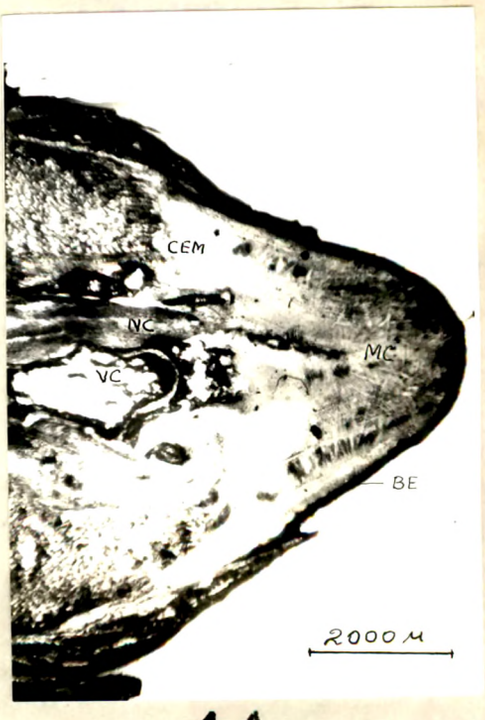
CEM
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4 A

Fig. 1A. Photomicrograph of T.S. of normal tail skin showing malic enzyme activity in the stratum germinativum.

Fig. 2A. Photomicrograph of T.S. of normal tail muscles showing enzyme activity.

Fig. 3A. Photomicrograph of L.S. of wound healing tail showing pronounced enzyme activity in the wound epithelium.

Fig. 4A. Photomicrograph of L.S. of blastema demonstrating increased malic enzyme activity.

ABBREVIATIONS

a	-	alpha cells
b	-	beta cells
BE	-	Blastemic epithelium
CEM	-	Cut end of muscles
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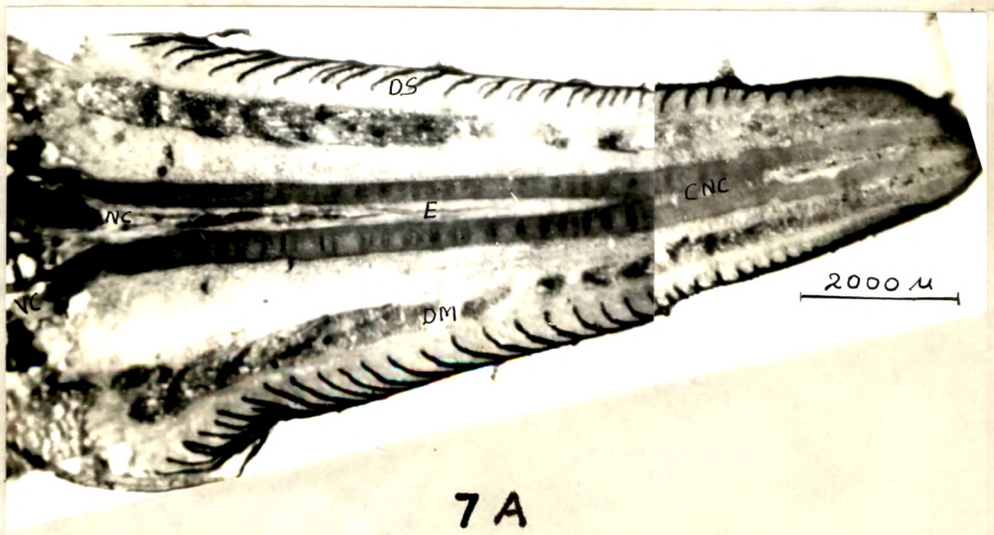
VC	-	Vertebral column
WE	-	Wound epithelium

5 A

6 A



7 A

Fig. 5A. Photomicrograph of wound epithelium with subepithelial cells showing malic enzyme activity.

Fig. 6A. Photomicrograph of blastemic epithelium showing enzyme activity. Note the activity in the mesenchymal cells too.

Fig. 7A. Photomicrograph of L.S. of differentiating tail showing high malic enzyme activity in the various differentiating components.

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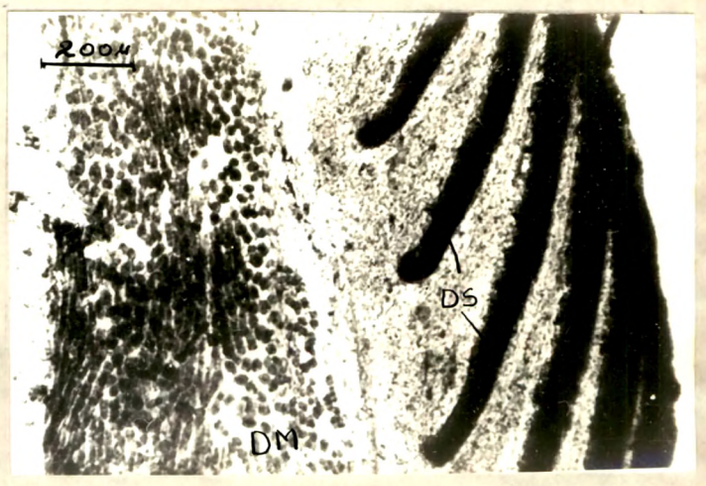
8 A



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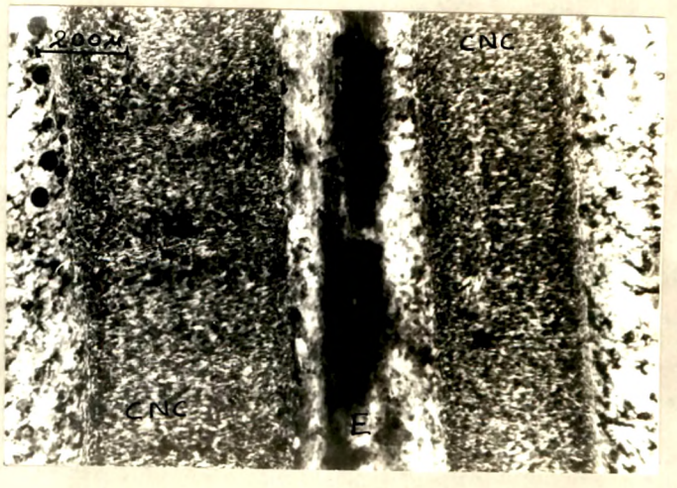

Fig. 8A. Photomicrograph of differentiating scales and muscles showing high malic enzyme activity.

Fig. 9A. Photomicrograph of cartilaginous neural canal with ependyma showing malic enzyme activity.

ABBREVIATIONS

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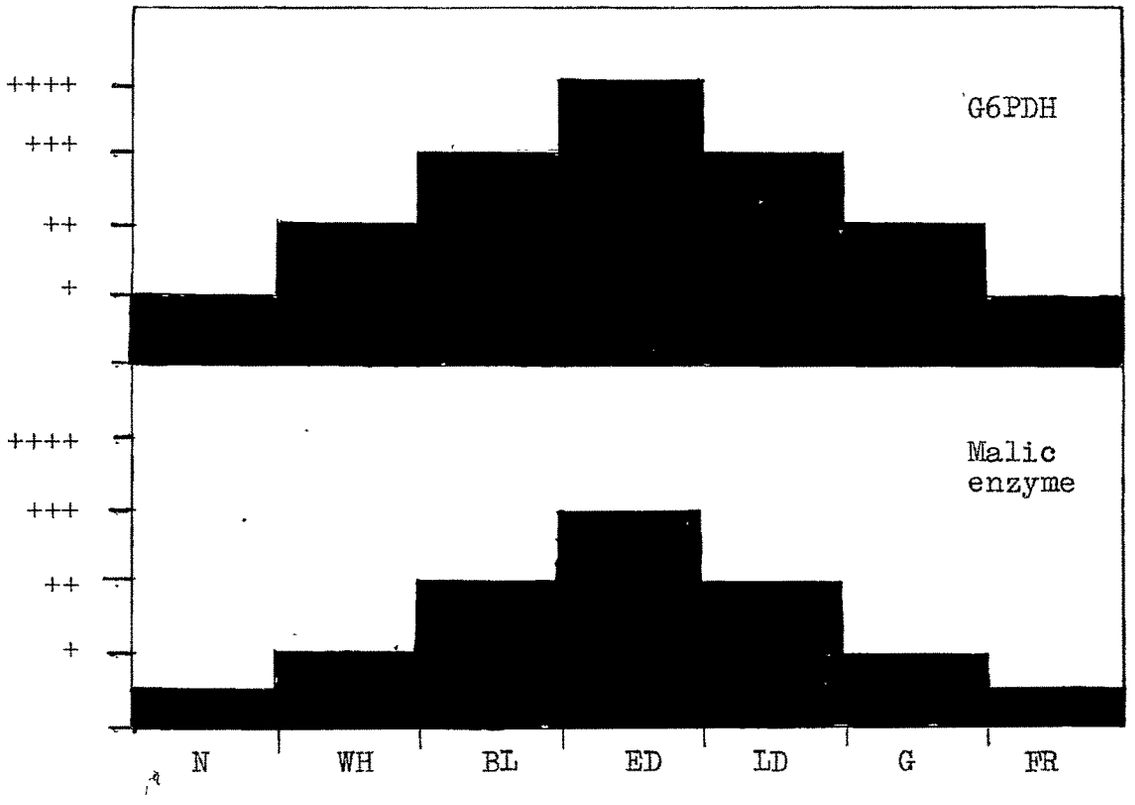
Graphic representation of the changes in the G6PDH & malic enzyme distribution pattern during the various stages of tail regeneration



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.

though slightly lesser than the blastemic epithelium and the cells lying subjacent to it. Moreover, the cut end of the tail tissues showed very high enzyme activities.

Differentiation phase: (Figs.7-9 & 7A-9A)

The late blastemic and differentiation phases were marked by a contrastingly sharp increase of the activity of G6PDH and malic enzyme. All the components of the differentiating tail i.e.,the integument, the scales, the muscles and the cartilage cells showed a persistant pronounced enzyme activity. However, in the ependyma, these enzymes were poorly active.

Growth phase:

The growth phase brought about a slow and steady waning activity of the enzymes in the various tissues, an early indication of which was evident during the late differentiation phase itself. The final growth phase was marked by a decrease of enzyme activity in the various by now well differentiated cellular elements of the now, almost fully regenerated tail.

Fully regenerate tail:

The gradual fall in the enzyme activity

noticeable during the growth phase touched its ultimate low ground value and the tissues of the regenerated tail, along with the attainment of the morphological and physiological maturity also attained a level of enzyme complement, the distribution and localization of which were quite similar and characteristic with those of the normal tail tissues.

DISCUSSION

It is to be noted at the very outset that though both G6PDH and malic enzyme were found to have a parallel distribution and localization during the various stages of tail regeneration, the G6PDH activity was found to be noticeably higher than that of the malic enzyme. These two enzymes together could play a major role in the synthesis of lipids during regeneration. The low level of activity of these two enzymes in the normal tail tissues is in liason with those of Wolfe and Cohen (1963) and Schmidt and Weidman (1964) in the amphibian limb, and Magon (1970) in the tail of the lizard, Hemidactylus flaviviridis. The striking contrast in the activity of these two enzymes noticeable in the normal tail on the one hand, and the blastemic and differentiation phases on the other hand, is highly

noteworthy and highlights the dominant role of these two enzymes during these phases of regeneration.

The tissues in the normal tail which depicted the G6PDH activity were the stratum germinativum of the integument and the muscles. The stratum germinativum, being a layer engaged in constant generation of new cells; a good rate of protein synthesis can well be associated with it. The nucleic acids much needed for protein synthesis seem to be produced in these cells from the reports of high concentration of DNA and RNA in the tail epidermis of the lizard, Hemidactylus flaviviridis (Chakko, 1967) and in the epidermis of Mabuya carinata (Shah and Radhakrishnan, unpublished). The presence of G6PDH and glycogen (Shah and Chakko, 1967 and Shah and Radhakrishnan, unpublished) in the stratum germinativum lead to the surmise that there is a turnover of pentose sugars which are necessary for nucleic acid synthesis by the oxidation of glucose through the HMP shunt pathway.

The activity noticeable in the skeletal muscles of the normal tail can be considered to be moderate. Nene and George (1967) in certain vertebrate muscles; Cherian (1967) in the pigeon breast

muscle and Bokdawala and George (1967) in the fish skeletal muscles have reported high activity of G6PDH. Diculesco et al., (1964) using MTT as an electron acceptor and cobalt chloride as chelating agent could not demonstrate G6PDH activity in the skeletal muscles. Ogata and Mori (1964a & b) in their histochemical study of G6PDH activity in the skeletal muscles of invertebrates and vertebrates did not use any respiratory inhibitor to divert the flow of electrons towards the tetrazoleum salt by inhibiting the oxidation of co.enzyme and hence failed to get the enzyme reaction. Chefurka (1957) working on insect thoracic muscle reported that the presence of cobalt ions greatly inhibited the activity of G6PDH. In the present experiment as suggested by Nene and George (1965) employing Nitro-BT as an electron acceptor and sodium azide as the respiratory inhibitor instead of cobalt chloride and MTT into the incubation medium, it was possible to demonstrate G6PDH activity in the skeletal muscles. The moderate activity seen in the muscles of the normal tail of Mabuya carinata in the wake of the picture unveiling data on elucidations in from Chapters 1, 2 and 5 (i.e.,the dominant glycolytic metabolism in the normal tail) cannot be at this stage envisaged to hold any special

significance.

The increased activity of G6PDH produces $NADPH_2$ and pentose sugars which are utilized for the synthesis of fatty acids and nucleic acids respectively. The malic enzyme together with LDH and MDH (Chapter 2) also yield a ready supply of $NADPH_2$. The lipids synthesised during the blastemic phase can aid in a supply of additional extra energy needed during the progressive phases of regeneration, when there is a high rate of cellular turnover. The nucleotides too play a major role as the process of active cell division and proliferation also calls for a high rate of protein turnover. The increased activity of G6PDH and malic enzyme in the blastema noted by the present worker is in perfect accordance with the presence of lipids during this stage reported by Chakko (1967) in the tail of Hemidactylus flavivirids, Shah and Radhakrishnan (unpublished) in the tail of Mabuya carinata and by Schmidt (1964a, b & c) in the limb of Diemictylus viridescens. The presence of lipids and the large amounts of DNA and RNA during blastema reported by Schmidt (1964), Chakko (1967) and Shah and Radhakrishnan (unpublished) and the increased concentrations of nucleic acids in the differentiation

and blastemic phases (Chakko, 1967; Shah and Radhakrishnan, unpublished) are in complete agreement with the present author's contentions regarding the role of these enzymes during regeneration in the tail of Mabuya carinata. It is worthwhile to note in this connection the works of Abraham and Chaikoff (1959); Glock and Mclean (1954) and Levy (1961) who showed high G6PDH activity and that of Wise and Ball (1963) who have stressed the role of malic enzyme in fatty acid synthesis. Further significant observations are those of Beaconfield (1964a & b) who has shown that the level of G6PDH runs parallel with increase in the nucleic acid synthesis and that of Muskatello and Anderson-Cedergren (1964) who have reported very high protein synthesis in conjunction with high G6PDH activity in the sarcotubular fraction of the frog skeletal muscle.

The later phases of regeneration not only records a reduced activity of the two enzymes but also a fast dwindling of lipid content (Schmidt, 1964a, b & c; Chakko, 1967; Shah and Radhakrishnan, unpublished). The appearance of lipids in the mesenchymal cells of blastema is also accompanied by a concomitant depletion of glycogen content

(Schmidt and Weidman, 1964; Shah and Chakko, 1967; Shah and Radhakrishnan, unpublished). As the glycogen is being utilized through the additional metabolic pathway of the HMP shunt by diverting glucose at the glucose-6-phosphate level so as to ensure the prompt supply of both $NADPH_2$ and pentose phosphates so that the metabolic necessities of the initial phase of active proliferation of cells could be successfully met with. The role of malic enzyme could be well understood by a careful observation of the short pyruvate centered metabolic cycle suggested in Chapter 2 to be operative in the regenerating tail of Mabuya carinata. The synthesis and utilization of lipids during the blastemic and differentiation phases of regeneration seem to be mainly due to a delicate, combined, co-ordinated and active interplay of G6PDH together with the enzymes of the pyruvate cycle viz., LDH, MDH and malic enzyme according to the necessities of the cells during different stages of regeneration. From the present observations it becomes amply clear that malic enzyme during blastema and early differentiation phases aids in the lipid synthesis by supplying $NADPH_2$ while during the late differentiation phase together with LDH and MDH it also aids in

glyconeogenesis by participating in the pyruvate cycle.

The increasing levels of G6PDH during chondrogenesis noticed herein and the concomitant increase in nucleic acid contents in these cells (Chakko, 1967) in Hemidactylus flaviviridis and (Shah and Radhakrishnan, unpublished) in Mabuya carinata seem to favour the probability of the HMP shunt directing its intermediary products towards nucleic acid synthesis.