CHAPTER 4

HISTOCHEMICAL RESPONSE OF THE NORMAL AND REGENERATING TAIL OF THE SCINCID LIZARD, MABUYA CARINATA TOWARDS αC -GLYCEROPHOSPHATE DEHYDROGENASE (αC -GPDH) AND β-HYDROXY BUTYRATE DEHYDROGENASE (BDH)

Dehydrogenases are the most important enzymes catalyzing various metabolic reactions. Glycogen and lipids being the chief metabolites for the biological tissues, their synthesis and breakdown in the animal tissues as per need are to be expected undoubtedly. The multitude of biochemical reactions forming the essence of the above processes and even the intricate interconversions and interconnections between the metabolites are all within the realms of dehydrogenase catalysis. Owing to the importance of dehydrogenases, several workers have studied the dehydrogenases in different tissues of different animals (Mammals - Pearse, 1960; Birds - George and Scaria, 1958; Dubowitz et al., 1960; Cooper and Konisberg, 1961; George and Talesara, 1961; Reptiles - Stolk, 1961; Chakko, 1967; Amphibians - George and Scaria, 1958; Dubowitz et al., 1960a & b; Lofgren et al., 1960; Niwelenski, 1960; Stolk, 1961; Schmidt, 1963; Schmidt and Weidman, 1964;

Fishes - Hughes, 1956; Stolk, 1961; George and Bokdawala, 1967; and Insects - Pearse and Scarpelli, 1958; Hess and Pease, 1961). Recently, attention has been focussed on the role of dehydrogenases during regeneration and a number of dehydrogenases have been studied in the regenerating tissues (Geczik and Wolsky, 1959; Wolfe and Cohen, 1963; Schmidt and Weidman, 1964). αC-glycerophosphate dehydrogenase catalyzes the reversible reaction between αC-glycerophosphate and dihydroxyacetone phosphate with Co.enzyme I as electron acceptor wherase β-hydroxy butyrate dehydrogenase catalyzes the reactions of fatty acid degradation. The only study of αC-glycerophosphate dehydrogenase in the regenerating tissues is that of Schmidt and Weidman (1964) and Shah and Magon (1969) in the regenerating limb of adult newt, Diemictylus viridescens and the regenerating tail of the lizard, Hemidactylus flaviviridis respectively. On the basis of their findings, Schmidt has ascribed both ancillary as well as subsidiary role in glycolysis for αC-GPDH. He has characterized it as puzzling the absence of αC-GPDH and presence of large amounts of lipids in the blastema which, he considers within the realm of αC-GPDH oxidation. Apart from this, no other reports on αC-GPDH

during regeneration is available. This has prompted the present study on αC-GPDH activity during regeneration in the tail of Mabuya carinata. Moreover, the appearance and utilization of lipids during regeneration and the possibility of a significant role for αC-GPDH in linking carbohydrate and lipid metabolisms, is a probability, and thus requires investigation the need to be explored. Some lipids are shown to be present in the normal and regenerating appendages of amphibians (Hess, 1957; Schmidt, 1966a & b) and reptiles (Chakko, 1967; Shah and Hiradhar, unpublished; Shah and Radhakrishnan, unpublished), and as lipids serve as an excellent fuel for metabolic energy for the maintenance and functions of cells (Fredrickson and Gordon, 1958; Rossiter and Strickland, 1960), an investigation on some enzymes concerned with lipid catabolism would obviously be informative and interesting. Lipase and esterase activities were found to vary during different phases of tail regeneration in Hemidactylus flaviviridis (Chakko, 1967). Hence, a study of the enzyme β-hydroxy butyrate dehydrogenase (BDH) which could not only provide an index of lipid utilization but also play an useful role in fatty acid catabolism was deemed worthwhile to project a better picture on

lipid metabolism during regeneration in the tail of the scincid lizard, Mabuya carinata.

MATERIALS AND METHODS

The adult Mabuyas selected for experiment were maintained in the laboratory on a diet of young cockroaches. The autotomy was induced by pinching off the normal and regenerating tails at least one to two inches away from the vent. The wound surface of the autotomized tail was blotted to remove blood and tissue fluids and was immediately fixed on a chuck of a cryostat microtome maintained at -20°C. Longitudinal as well as transverse sections of 12-18 μ thickness were cut and the sections incubated for 40 minutes at room temperature for both the enzymes in the respective incubation media prepared as given below which were adjusted to a pH of about 6.8 to 7.2.

Incubation medium for αC-GPDH

Substrate (αC-glycerophosphate sodium salt, 1 M)		0.1 ml
Nictinamide adenine dinucleotide	(1 mg/ml)	0.1 ml
Nitro-BT	(1 mg/ml)	0.1 ml
Sodium cyanide	(0.1 M)	0.1 ml
Magnesium chloride	(0.05 M)	0.1 ml

Tris buffer, pH 7.2	(0.2 M)	0.45 ml
Distilled water		0.05 ml
Polyvinylpyrrolidone		75 mg

Incubation medium for BDH

Substrate (sodium salt of β-hydroxy butyric acid, 1 M)		0.1 ml
Nicotinamide adenine dinucleotide	(1 mg/ml)	0.1 ml
Nitro-BT	(1 mg/ml)	0.1 ml
Sodium cyanide	(0.1 M)	0.1 ml
Magnesium chloride	(0.05 M)	0.1 ml
Phosphate buffer, pH 7.4	(0.06 M)	0.45 ml
Distilled water		0.05 ml
Polyvinylpyrrolidone		75 mg

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

OBSERVATIONS

NORMAL TAIL (Figs. 1-3 and 1A, 2A)

The outer beta and alpha layers of cells in the skin were enzyme negative. Both αC-GPDH and BDH were not detectable in the cells. However, the enzymes

were present in the cells of stratum germinativum. The BDH activity was not found to be very prominant in the cells of stratum germinativum whereas the activity of αC-GPDH in the cells of this layer of skin was moderately high. The scutogenic cells also showed positive enzyme activity comparable to that of stratum germinativum. The enzyme activity was negligible to nil in the dermis and associated components of the skin for both the enzymes.

Of the various tissue components in the normal tail, the muscles depicted the highest enzyme activity. Amongst the muscle fibres distinction between the peripheral smaller fibres and inner larger fibres could be ~~drawn~~ seen by the pattern of enzyme distribution and localization. Both mitochondrial and sarcoplasmic localization were evident. The smaller peripheral fibres in each fasciculus showed a more pronounced activity than the inner bigger fibres. Between the two, αC-GPDH was found to be very active in the muscles with BDH demonstrating a very feeble activity, almost negligible. In addition to the outer smaller fibres with higher activity and the inner bigger fibres with milder activity, some other fibres with intermediary size and

enzyme activity could also be ~~however~~, noticed.

The submuscular as well as subcutaneous adipose tisue failed to show any activity either for αC-GPDH or BDH. However, the tetrazoleum adsorption by the fat in these tissues was noticeable, more so, in the case of αC-GPDH.

The matrix and the osteocytes of the vertebral column were enzyme negative but the cartilage cells at the articulating surfaces of the centrum showed a positive response. αC-GPDH activity was noticeably higher than that of BDH in these cells. In the nerve cord, the grey matter was more enzyme active than the white matter. Again, the αC-GPDH activity was relatively more than BDH which was quite poor.

REGENERATING TAIL

Wound healing phase: (Figs.4 and 3A, 5A)

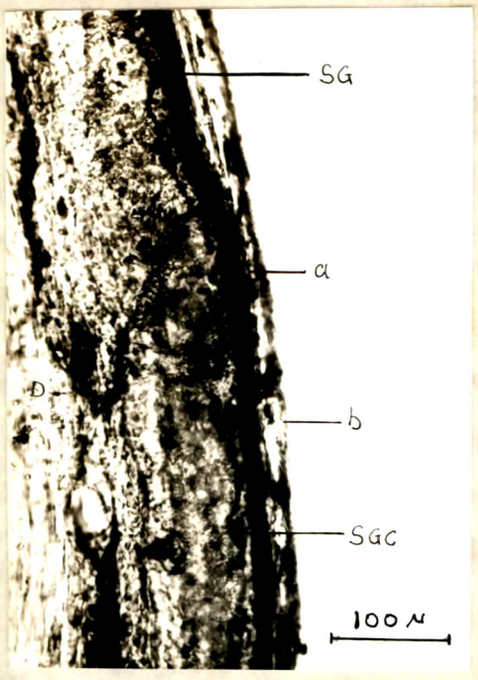
The wound epithelium showed marked enzyme response towards both the enzymes. A comparison, easily revealed the αC-GPDH activity to be more prominant than BDH. Even the cells accumulated below the wound epithelium demonstrated appreciable activities of the enzymes. The cut end of the tail tissues also demonstrated an increased enzyme activity.

Blastemic phase: (Figs.5-7 and 4A-6A)

Alongwith the progressive stratification of the wound epithelium trasforming itself into the blastemic epithelium, there was a simultaneous increase in the activity of these two enzymes. Both the enzymes registered a high activity. The mesenchymal cells of the blastema too, were enzyme active. Both the enzymes seemed to be well represented in the blastemal cells. The activity of BDH during the blastemic phase when compared with its activity in the normal tail tissues, seemed to create the impression, that the enzyme really makes its appearance at this stage. The highly pronounced activity of αC-GPDH also seemed to be more than that observed in the normal tail tissues. The cut end of the tissues still showed the pronounced activity even at this stage.

Differentiation phase: (Figs.8-10 and 7A-9A)

From the blastemic phase onwards, allthroughout the differentiation phase, the activities of the two enzymes seemed to increase continuously and steadily. The differentiating epidermis together with its scales showed pronounced αC-GPDH and BDH activities.



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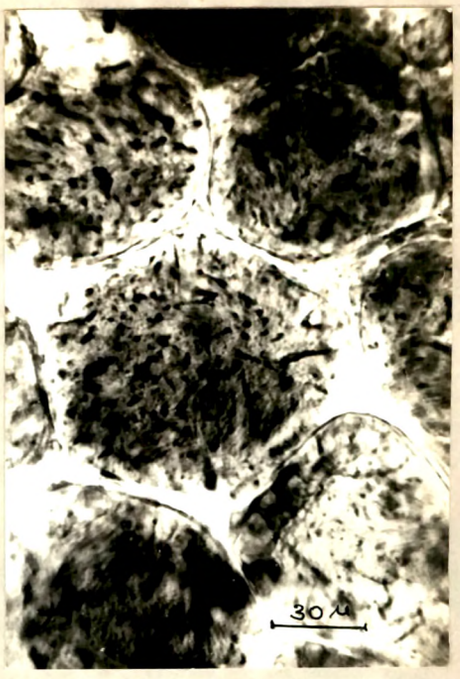

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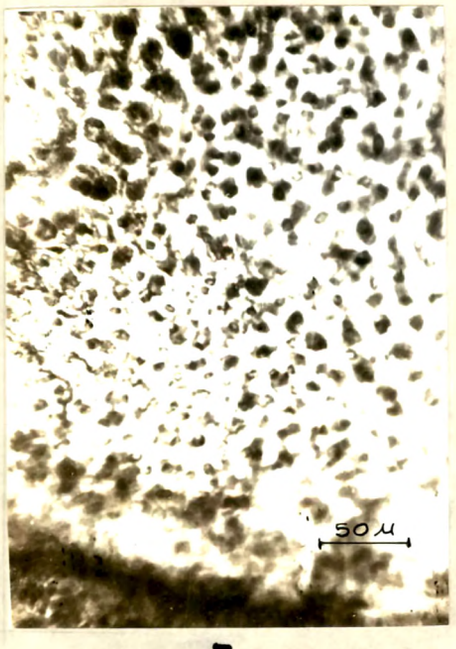

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EXPLANATIONS FOR FIGURES

Fig. 1. Photomicrograph of T.S. of normal tail skin showing strong αC-GPDH activity.

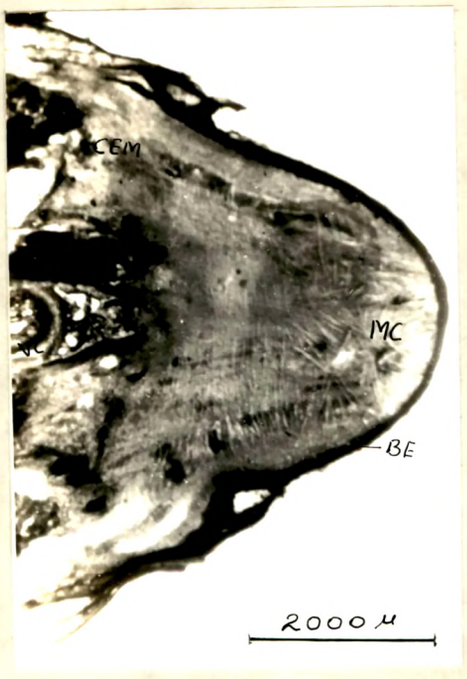
Fig. 2. Photomicrograph of T.S. of normal tail muscles showing both mitochondrial as well as cytoplasmic localization of αC-GPDH.

Fig. 3. Photomicrograph of cartilage cells at the articulating surfaces of centra showing αC-GPDH activity.

Fig. 4. Photomicrograph depicting strong enzyme response in the wound epithelium and subepithelial cells.

ABBREVIATIONS

a	-	alpha cells
b	-	beta cells
D	-	Dermis
SC	-	Scute
SCA	-	Scab
SEC	-	Subepithelial cells
SG	-	Stratum germinativum
SGC	-	Scutogenic cells



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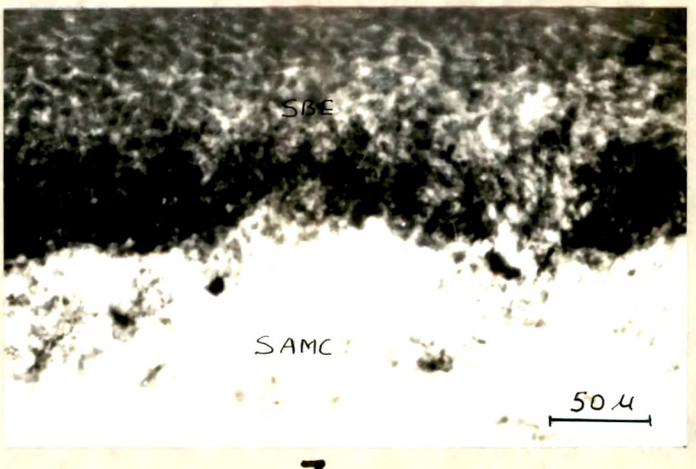

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Fig. 5. Photomicrograph of L.S. of Blastema showing pronounced αC-GPDH activity in the blastemic epithelium and mesenchymal cells.

Fig. 6. Photomicrograph of the region differentiating into cartilaginous neural canal showing enzyme activity.

Fig. 7. Photomicrograph of blastemic epithelium denoting αC-GPDH activity.

ABBREVIATIONS

BE	-	Blastemic epithelium
CEM	-	Cut end of muscles
EDC	-	Early differentiating cartilage cells
MC	-	Mesenchymal cells
NC	-	Nerve cord
SAMC	-	Subapical mesenchymal cells
SBE	-	Stratified blastemic epithelium
VC	-	Vertebral column

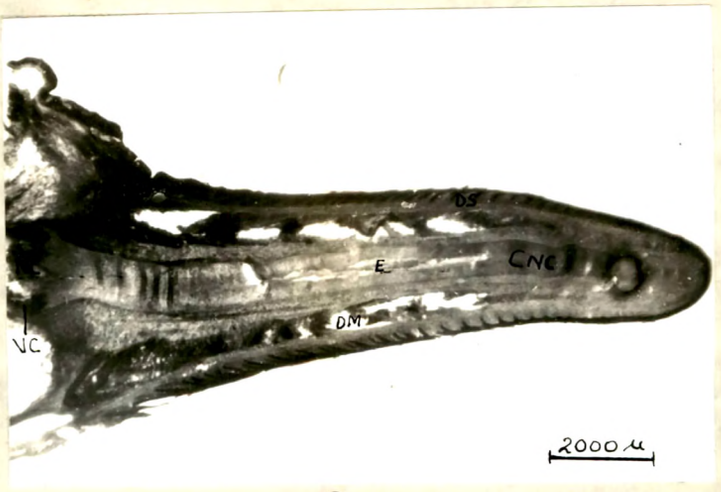

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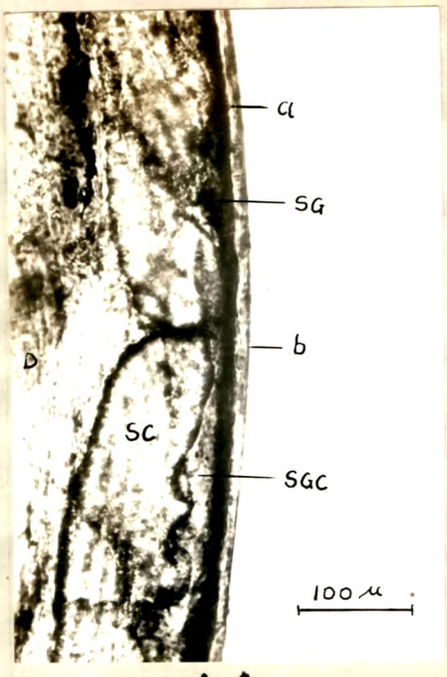
Fig. 8. Photomicrograph of L.S. of differentiating tail showing high αC-GPDH activity.

Fig. 9. Differentiating scales and muscles showing pronounced enzyme activity.

Fig.10. Cartilaginous neural canal with ependyma demonstrating enzyme activity.

ABBREVIATIONS

CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma
NC	-	Nerve cord
VC	-	Vertebral column



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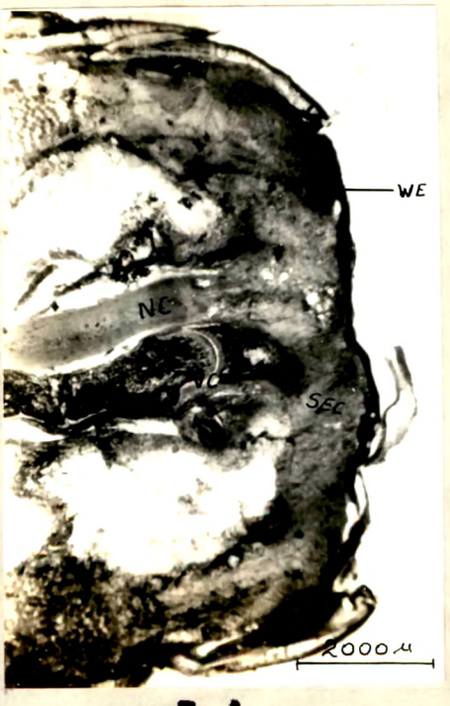

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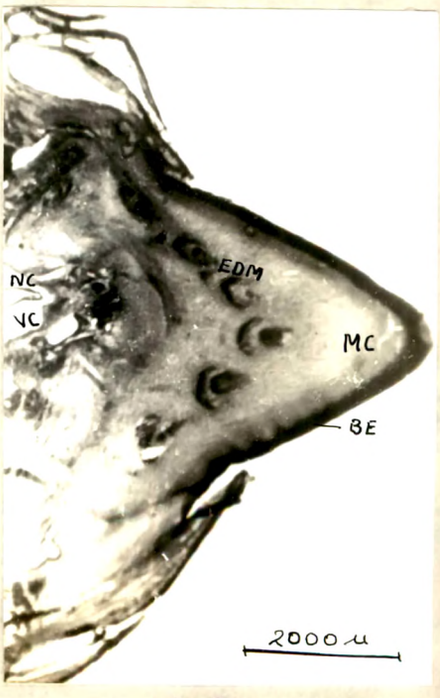


4 A

Fig. 1A. Photomicrograph of T.S. of normal tail skin showing BDH activity.

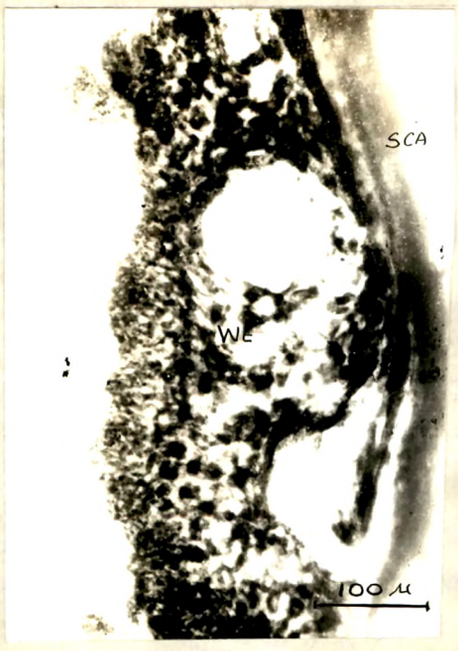
Fig. 2A. Photomicrograph of T.S. of normal tail muscle showing BDH activity.

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

Fig. 4A. Photomicrograph of L.S. of blastema showing enhanced BDH activity.

ABBREVIATIONS

a	-	alpha cells
b	-	beta cells
BE	-	Blastemic epithelium
D	-	Dermis
EDM	-	Early differentiating muscles
MC	-	Mesenchymal cells
NC	-	Nerve cord
SC	-	Scute
SEC	-	Subepithelial cells
SG	-	Stratum germinativum
SGC	-	Scutogenic cells
VC	-	Vertebral column
WE	-	Wound epithelium



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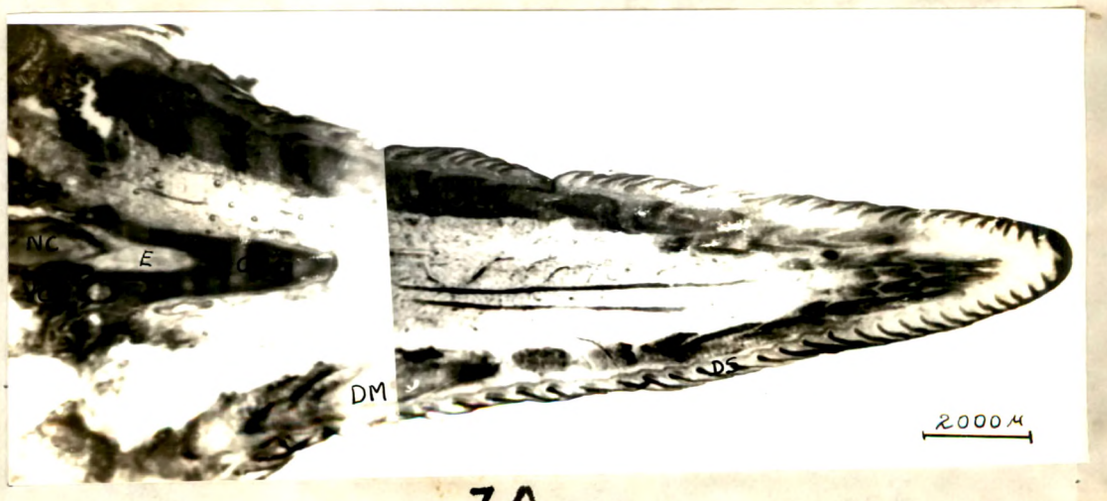


7 A

Fig. 5A. Wound epithelium depicting strong BDH activity.

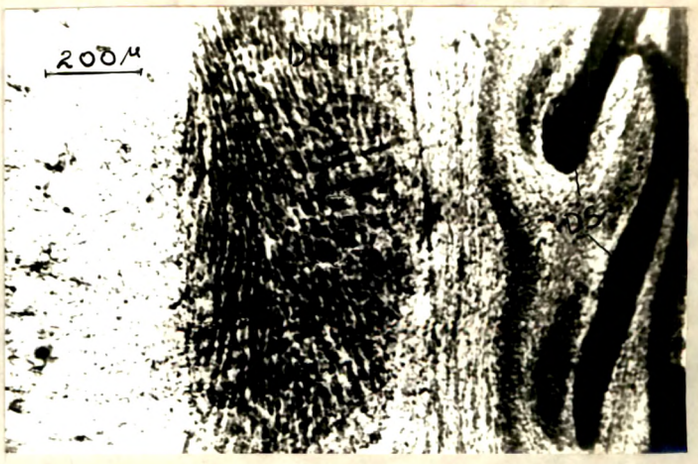
Fig. 6A. Blastemic epithelium denoting a strong response towards BDH.

Fig. 7A. Photomicrograph of L.S. of differentiating tail demonstrating high enzyme activity in all the differentiating structures.

ABBREVIATIONS

BE	-	Blastemic epithelium
CNC	-	Cartilaginous neural canal
DM	-	Differentiating muscles
DS	-	Differentiating scales
E	-	Ependyma
NC	-	Nerve cord
SCA	-	Scab
VC	-	Vertebral column
WE	-	Wound epithelium

8 A



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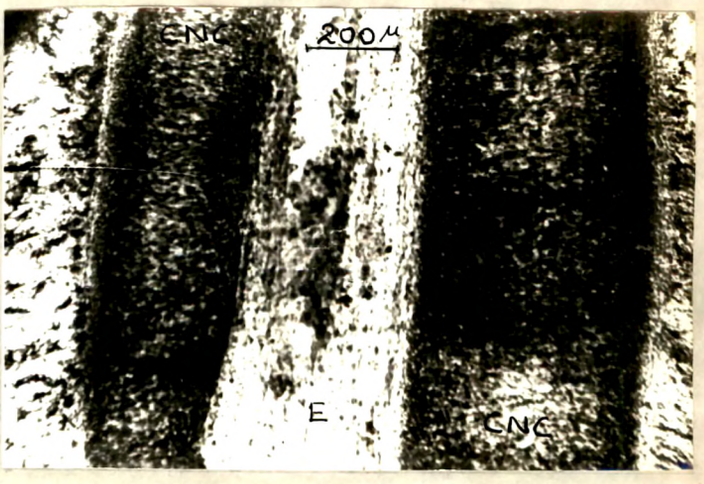


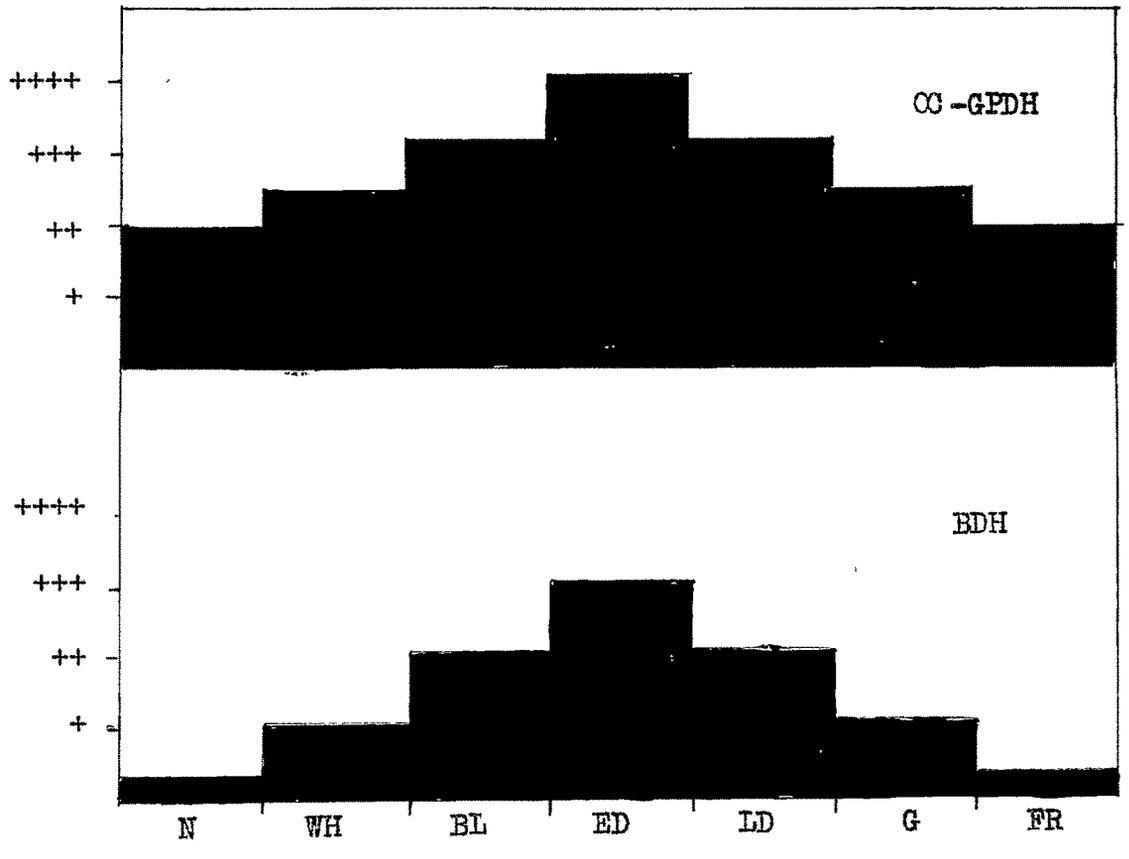
Fig. 8A. Strong BDH activity elicited by differentiating scales and muscles.

Fig. 9A. Strong BDH activity in 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
∝-GPDH & BDH 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.

The differentiating muscles ~~too~~ also registered increasing activities of αC-GPDH and BDH. Both of the enzymes were also present in chondrocytes during chondrogenesis whereas the extending ependyma gave a mild response.

Growth phase:

As in the case of other dehydrogenases, at this stage both αC-GPDH and BDH started their slow and steady descent in activity. Throughout the growth phase, the enzymes registered decreasing activities in all the tissues. The growth phase was marked by the gradual attainment of the morphological and physiological maturity of the already differentiated cellular components of the regenerate. Closely alignated to this attainment of maturity was the tendency to get set to approach the original levels of enzyme activity.

Fully regenerated tail:

This was the ultimate stage of regeneration and at this stage, the length of the now fully regenerated tail was found to be almost equal to that of the normal one. At this stage, the various components such as skin, muscles, submuscular adipose tissue, cartilaginous neural canal and ependyma all displayed an almost similar αC-GPDH and BDH activities as compared to the corresponding components of the normal tail.

DISCUSSION

The observations noted herein seem to reveal a higher activity of αC-GPDH and a relatively poor activity of BDH by the normal tail tissues. BDH being an enzyme connected with the catabolism of fatty acids, its poor activity in the normal tail tissues of Mabuya carinata can be well visualized by the poor content of lipids of the various cellular elements of the normal tail. It is also in conformity with the, by now established, fact that the metabolic activities of the normal tail of Mabuya carinata is exclusively glycolytic. The high incidence of αG-GPDH in the normal tail tissues seems to be, somehow, related to the above fact. Muscles and the cells of the stratum germinativum in the skin were the components which showed a pronounced response towards αC-GPDH. Identical observations were made by Shah and Magon (1969) and Schmidt and Weidman (1964) in the normal tail of the wall lizard, Hemidactylus flaviviridis and in the normal limb of Diemictylus viridescens respectively. The abundance of glycogen in these very cells of Mabuya carinata (Shah and Radhakrishnan, unpublished), Hemidactylus flaviviridis (Shah and Chakko, 1967) and

Diemictylus viridescens (Schmidt, 1962) suggests a probable secondary role for αG-GPDH oxidation in the glycolytic metabolism of the normal tail. The reduction of dihydroxy-acetone phosphate with $NADH_2$ by αG-GPDH yielding αG-glycerophosphate and NAD seems to be of obvious importance as a side machinery in the instantaneous ready generation of NAD, a product of much importance in continous glycolytic metabolism. The ancillary role to glycolysis advocated by de Duve et al., (1962) where, αG-GPDH serves in the reoxidation of glycolytic $NADH_2$ with αG-glycerophosphate as an electron acceptor augments well with the present contention arrived at, based on the observations in the normal tail of Mabuya carinata. It is worthwhile to note here the observations in the skeletal muscles of mammals by Dubowitz et al., (1960) and Pearse (1961) as well as in the case of amphibians by George and Scaria (1958) of a high αG-GPDH activity.

The prominant αG-GPDH activity in the wound epithelium corresponds well with a similar activity of aldolase (Chapter 1) and is suggestive of the continued operation of the glycolytic pathway and the ancillary role of αG-GPDH in it, during the wound healing phase.

At the same time, the noticeable BDH activity in the wound epithelium more than that in the normal skin, reflects the appearance of this enzyme as though a prelude to the important metabolic role to be played by it during the actual process of regeneration to which ensue; and in which the wound healing phase represents the starting point.

A gradual increase of both αC-GPDH and BDH was registered alongwith the progressive stratification of the wound epithelium, which could now be identified as the blastemic epithelium. The cellular aggregation of the blastema also showed a pronounced enzyme response towards both αC-GPDH and BDH. The highly active αC-GPDH in the blastemal cells is suggestive of a significant glycerophosphate based metabolism in the regeneration blastema of Mabuya carinata. It is interesting in this connection to note that Schmidt and Weidman (1964) have reported that there is little or no αC-GPDH reactivity in the cells of the blastema. At the same time (Schmidt, 1964a, b & c) has reported large quantities of lipids including phospholipids at this phase. The presently observed high αC-GPDH in the blastema of the regenerating tail of Mabuya carinata

appears to represent a reflection of the clear cut manifestation at the subcellular level of the preparative metabolism for the progressive phases of regeneration. The operation of glycolysis in the mesenchymal cells at this stage as was evidenced by the presence of aldolase (Chapter 1) readily yields a continuous supply of dihydroxyacetone phosphate. Dihydroxyacetone phosphate, more than an intermediary product, is also the principal substrate for αC-GPDH catalysis in forming αC-glycerophosphate, an important product very much useful in the synthesis of both triglycerides and phospholipids (Kornberger and Pricer, 1953; Rossiter et al., 1957; Kennedy, 1953, 1954 and 1957a & b). Kennedy (1957a & b) has not only identified αC-GPDH as one of the important enzymes in phospholipogenesis but also emphasized the glycolytic pathway as the most potent source of αC-glycerophosphate. In the light of these known facts and observations, it becomes undoubtedly clear that the increased αC-GPDH activity noted during the blastemic phase in the present work, is noteworthy and highlights the participation of αC-GPDH catalyzed route of metabolism for elaborating new lipid molecules by diverting dihydroxyacetone phosphate, an intermediary product of

glycolytic cycle towards this process. This becomes all the more evident by the appearance of lipids at this phase in the regenerating tail of Mabuya carinata (Shah and Radhakrishnan, unpublished) and also by the identical observations in the tail of Hemidactylus flaviviridis (Chakko, 1967) and in the limb of Diemictylus viridescens (Schmidt, 1964a, b & c). It is interesting to note that Magon (1970) has also observed αC-GPDH activity in the blastemal cells and has suggested a probable function for αC-GPDH in lipid utilization. But, with the more available data and literature, it is the contention of the present author that the role of αC-GPDH in regeneration blastema is more directed towards lipid synthesis than towards its utilization. Unlike these two observations in the lizard tails, that of Schmidt and Weidman (1964) in the urodele limb, is at divergence in that they have failed to localize αC-GPDH activity, but, at the same time noticed increasing lipid contents (Schmidt, 1964a, b & c), which they had felt a bit puzzling and suggested that metabolic routes other than those involving the oxidation of glycerophosphate as of greater importance

to the cells of the regeneration blastema. This discrepancy between the lizard tail and the urodele limb regenerates might represent the tissue specific reactions of the two classes to which they belong. Alongwith the appearance and accumulation of lipids, the cells of the blastema also seem to equip themselves with the necessary mechanism to utilize this metabolite in an efficient manner by the increasing concentration of BDH activity. Coupled with BDH, the increasing titres of lipase and esterse in the blastema of Mabuya carinata (Shah and Radhakrishnan, unpublished) and of Hemidactylus flaviviridis (Chakko, 1967) are definite indications of adaptations at the subcellular level taking place at this stage to meet the tremendous problem of energetics much needed during the ensuing hectic proliferative phase.

With the advent of differentiation, the activities of the two enzymes also registered enhanced activities. In fact, the increase was so tremendous that at late differentiation, the two enzymes displayed their highest peak activity ever observed during the tail regeneration in Mabuya carinata. Throughout the differentiation phase, all the differentiating

elements such as epidermis, scales, muscles and cartilage cells showed a pronounced BDH activity. Concomitant decrease in lipid content with the progression of differentiation has been reported in the various tissues except the adipose ones in the regenerating tail of Hemidactylus flaviviridis (Chakko, 1967) and similarly in the tail of Mabuya carinata (Shah and Radhakrishnan, unpublished). The increased BDH activity and the progressive depletion of lipids during differentiation are indicative of active lipid catabolism and its oxidation via the TCA cycle (Chapter 5). The reports of increased activities of lipase and esterase during differentiation in Hemidactylus flaviviridis (Chakko, 1967) and in Mabuya carinata (Shah and Radhakrishnan, unpublished) lend further support to the same. It could be noted in this connection that Magon (1970) has also reported an identical BDH activity in the differentiating tail of Hemidactylus flaviviridis. Even though no studies have been carried out in the regenerating newt limb, regarding BDH, Schmidt has surmised that lipids are important in the molecular ecology of the regenerating

forelimb. It is also pertinent to note here, similar high BDH activity in the pigeon breast muscle and fish skeletal muscle (Cherian, 1967) and (Bokdawala and George, 1967) respectively, where lipid contents are known to be high. These evidences observations clearly suggest lipids as almost the principal source of energy yielder during the differentiation phase when there is a high rate of cellular proliferation. Cuncurrent to the increase in α-GPDH activity, there was also an increase of phospholipids and neutral lipids (Shah and Radhakrishnan, unpublished) during differentiation in the regenerating tail of Mabuya carinata. Similar data on α-GPDH and lipids are presented by Shah and Magon (1970) and Chakko (1967) respectively in the regenerating tail of Hemidactylus flaviviridis. These observations, when viewed in the light of hitherto reports of α-GPDH catalysis in lipid synthesis (Kennedy, 1957) strongly underscore the important role of this enzyme in the anabolism of lipids during differentiation as during the blastema phase. The synthesis of phospholipids seems to be a continuous process right from blastema ~~upti1~~ until late differentiation; as phospholipids are necessary for laying down the

structural frame work of the organelles of the proliferating and differentiating cells. Neutral lipids on the other hand, appears to be synthesized more during the blastema and early differentiation phases. Neverthless, αC-GPDH may again be attributed of an important role in the laying down of adipose tissue fat during the late differentiation phase. However, the very high activity of αC-GPDH noticed in the present study due to its strategic position in the metabolic map may also aid in glycerol utilization through the glycolytic pathway or even in gluconeogenesis especially during late differentiation when its utility in lipid synthesis may be of a slightly lower value. But its role however meagre, in lipid oxidation through glycolytic pathway by its reversed action during the blastema and early differentiation phases cannot be overruled. Thus αC-GPDH may be considered to play a strategic dual role during differentiation by the participation of its reversible reaction according to the prevailing biochemical necessities at the subcellular metabolic level in the regenerating tail of the lizard.

The growth phase was marked by decreasing activities of both αC-GPDH and BDH in the various

tissues of the regenerate. This could be correlated with the absence of lipids in the tail tissues at this stage (Shah and Radhakrishnan, unpublished; Chakko, 1967). The decrease of both lipid contents, and αC-GPDH and BDH activities during growth phase are indicative of the reduced energy necessities and the reversal of the activities of the two enzymes to the normal level. Thus with the attainment of morphological and functional maturity, the enzyme distribution and localization in the various tissues of the now fully regenerate tail resembled those of the corresponding tissues of the normal tail.