CHAPTER - III

CHEMICAL SYMPATHECTOMY WITH 6-HYDROXYDOPAMINE ENHANCES TAIL REGENERATION IN THE LIZARD, *HEMIDACTYLUS FLAVIVIRIDIS*

Catecholamine (CA) are involved in several neuroimmunomodulatory functions and participate in maintenance of body homeostasis. The cellular actions of CA are closely linked to the cyclic nucleotides. The modulatory functions of CA have been found in regulation of various developmental (reviewed by McMohan, 1974) and regenerative events (Sicard, 1983; Taban and Cathieni, 1988). In the newt, *Notophthalmus Viridescens*, accumulation of CA has been recorded within the regenerated limb (Taban *et al., 1978*). Destruction of sympathetic synapses by chemical sympathectomy and inhibition of CA biosynthesis by alpha-methyl-para-tyrosine (AMPT) both have been affected adversely the process of limb regeneration in newt (Taban and Cathieni, 1988). These evidences strongly support the involvement of CA during limb regeneration in urodele amphibians. It has been observed that in the regenerating tail, the sympathetic trunk also regenerates along with the nerve fibres on either side of the caudal artery(Terni, 1922). However, no experimental reports are available regarding the adrenergic functions in regeneration of saurian tail.

It has been found that peripheral administration of catecholamines has no positive influence on the tail regeneration in lizards (chapter-II). However, it is suggestive that a stage-specific CA sensitivity might be occurring during the regenerative phases. Therefore, in the present experiment an attempt has been made to evaluate the adrenergic function during tail regeneration through 6-Hydroxydopamine(6-OHDA)-induced chemical sympathectomy. 6-OHDA is a specific catecholamine neurotoxin which depletes the CA content in adrenergic nerve fibres by selective destruction of nerve terminals leaving the cell bodies intact (Thoenen and Tranzer, 1973). Moreover, chemical sympathectomy with 6-OHDA does not alter the brain CA levels as the drug is impermeable through the blood-brain barrier. Thus chemical sympathectomy enables us to study the adrenergic functions in animals. Two series of experiments were conducted to delineate CA functions, (1) the animals were sympathectomised at pre-autotomy level and then the regenerative performance was assessed till early growth phase (2) sympathectomy was carried out at preblastemic and blastemic stages to ascertain the stage specific effects.

MATERIALS AND METHODS

Animals. Adult wall lizards, *Hemidactylus flaviviridis* of both sexes, with intact tail and an average body weight of 10 ± 1 gm were procured from commercial animal dealer. The animals were caged in the laboratory for a period of seven days prior to the experiments. The animals were fed as and when required (2-3 times a week) and water was given daily.

Experiment-1. One group of lizards was divided into two sets of 12 each and caged separately with a balanced sex ratio. The lizards in first set were given intraperitoneal injection of 6-Hydroxydopamine hydrobromide (6-OHDA Hbr, Sigma chemical company, MO, USA) in 0.6 % saline containing 1 % ascorbic acid as stabilizer. The drug was prepared immediately before use. A total of 700 mg / kg body wt of 6-OHDA was administered to each animal in four equal doses. 6-OHDA at a dosage of 350 mg/kg body wt was given five days prior to autotomy at 24 hrs interval in two equal doses. Second set of lizards which was given equal amount of vehicle served as control animals. Five days after the administration of first dosage of drug, the animals were autotomized by pinching off the tail leaving three segments intact by exerting mild thumb pressure. On 5th day and 12th day after autotomy, another dosage of 6-OHDA (175 mg/kg b wt) was given to the first set of animals. The extent of sympathetic denervation was assessed through the histofluorescence localization of CA in the cornea of lizards according to the method described by Terro (1977) (chapter-1). The growth of the tail regenerate was measured at specific intervals of 10,15,20,25 and 30 days of post-autotomy. The time taken by both groups of animals to attain various arbitrary stages of the regeneration was also recorded. For histological studies, the regenerate with one segment of the stump was removed under anaesthesia and processed as described in chapter-I.

Experiment-2. In this series of experiments, the animals were sympathectomized at specific stages of regeneration, i.e., in preblastemic stage (wound epithelium) and blastemic stage. A total of 80 lizards were autotomized and allowed to regenerate. The WE stage is marked by shedding of the scab and appearance of smooth shining epithelium. Blastema is characterised by a conical elevation (average length 2 mm) from the stump. Only those animals which attained the specific stages on the same day were selected and grouped.

Series-A: Injection of 6-OHDA at WE stage : A total of 30 animals were used and they were divided into two sets of 15 each. First set of animals received an ip. injection of 6-OHDA.Hbr at a dosage of 300 mg/kg b wt. (in 0.6% saline containing 1.0 % ascorbic acid as stabilizer) in a single dose during WE stage. The second set served as controls. The growth of the regenerate was measured at 48 hrs and 96 hrs after injection.

Series-B : Injection of 6-OHDA at BL stage. A total of 30 lizards which attained the BL stage on the same day were selected and divided into two set of 15 each. First set received a single ip.dose of 6-OHDA.Hbr (300 mg/kg body wt; drug preparation as above) while second group that received vehicle served as controls. The growth of the regenerate was measured at 48 hrs and 96 hrs post-injection. From both A and B series the regenerates were cut at 48 hrs and 96 hrs, fixed in Bouin's fluid and processed for histological studies (chapter-I).

Histofluorescence localization of nucleic acids. The regenerate from both series at 48 hrs and 96 hrs were cut and immediately transferred into a cryostat microtome at -20°C, embedded in Tissue Tek-II and sectioned at 12 μm. The sections were stained with acridine orange and observed under fluorescence microscope equipped with epi-illumination with filter settings of 440 nm excitation filter and 510 nm barrier filter.

Statistical analysis: Data on the growth of the regenerate are expressed as mean ± SD. A P value of 0.05 or less was considered as significant using Student's 't' test.

RESULTS

Experiment-1. Chemical sympathectomy in the lizard, *H.flaviviridis* was found to enhance the tail regeneration (table-2, fig.1.). Histofluorescence of CA in cornea of the sympathectomized lizards have shown that CA depletion was at near total level (fig.5 & 6). The process of wound healing occurred simultaneously in both control and 6-OHDA treated lizards. Measurement of tail length revealed a boost in growth in the early events of differentiation which persisted throughout regeneration. This early stage was in correspond with blastema cell proliferation and differentiation. In sympathectomised lizards, the duration to reach various stages of regeneration was shorter than that in control (table-1). In all stages, the growth rate in 6-OHDA treated animals was at a significantly higher level (fig.2). Per day growth rate of the tail (fig.3) showed a peak between 10-20 days corresponding to the active phase of differentiation.

Table:1. Number of days taken to reach various stages of regeneration in control and chemically sympathectomised lizards.

Groups	WH	BL	ED	MD	LD	GR
Control	5-6	8-9	11-13	13-16	17-25	25 Onwards
6-OHDA	5-6	7-8	9-11	12-15	16-22	22 Onwards

Table:2. Length of tail regenerated in control and chemically sympathectomised lizards in a period of 30 days. The tail growth (in mm) is presented as mean ± SD.

Days	Control	6-OHDA
		***
10	3.6 ± 0.69	4.9 ± 0.28
		***
15	12.6 ± 1.35	14.9 ± 1.10
		***
20	21.6 ± 1.35	24.8 ± 2.15
		NS
25	26.5 ± 1.33	27.2 ± 2.31
		***
30	27.8 ± 0.69	30.7 ± 1.33

*** $P < 0.001$; NS - Nonsignificant.

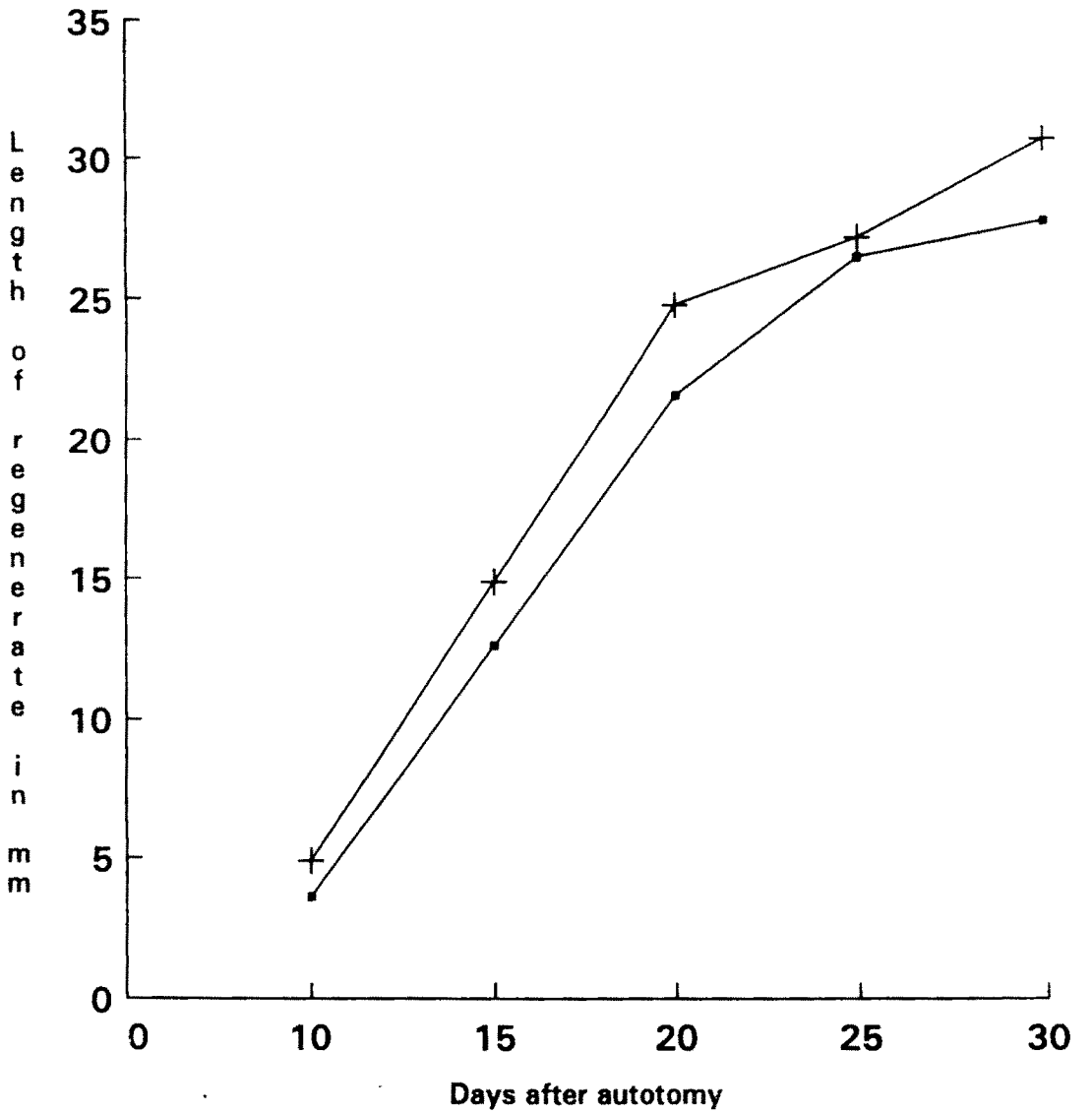


Fig.1. Length of tail regenerated in control and chemically sympathectomised lizards in a period of 30 days. The tail length is presented as mean +/- SD. N-12 animals per group.

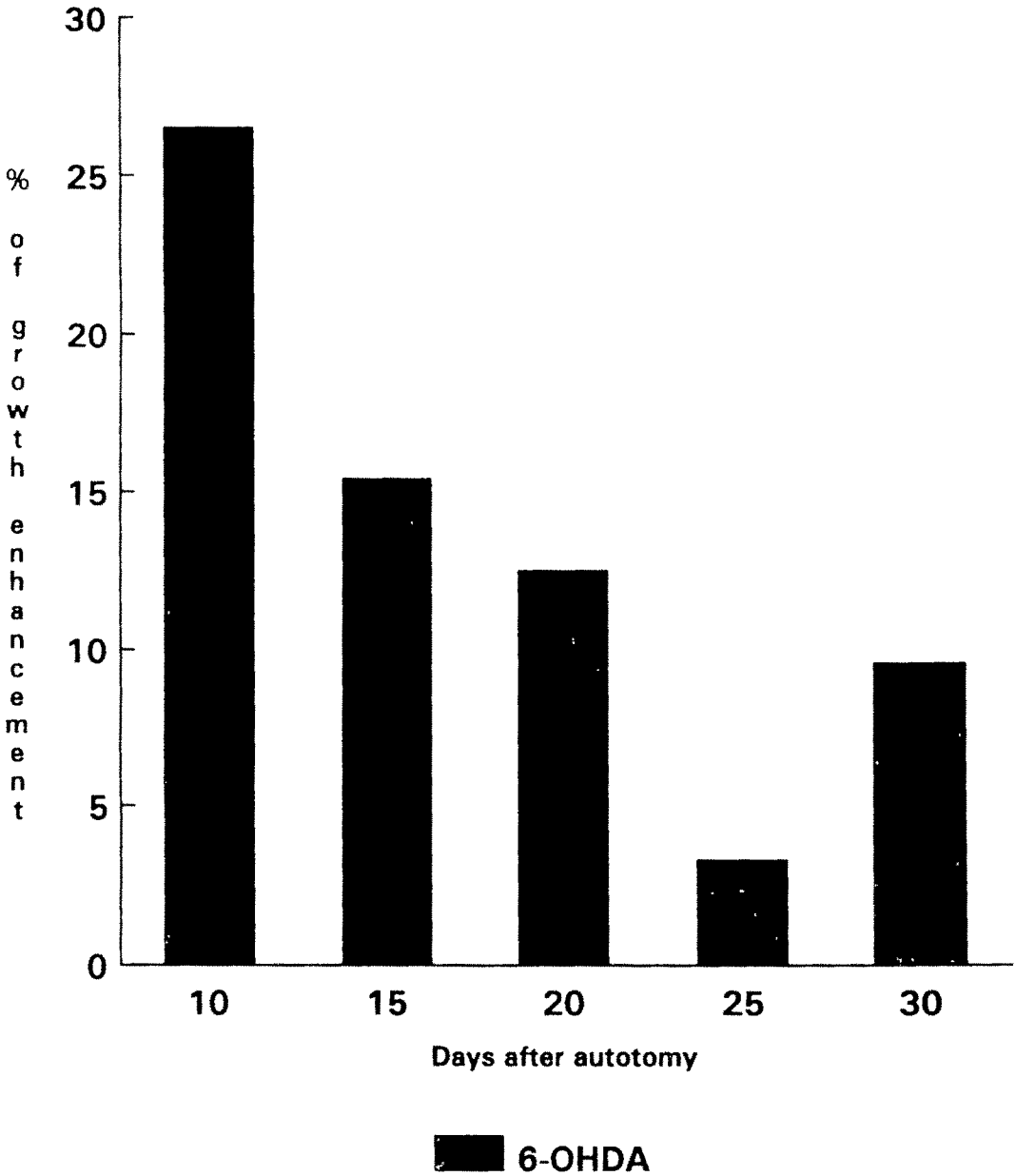


Fig.2. % of growth enhancement recorded in sympathectomised animals at intervals of 5 days for a period of 30 days.

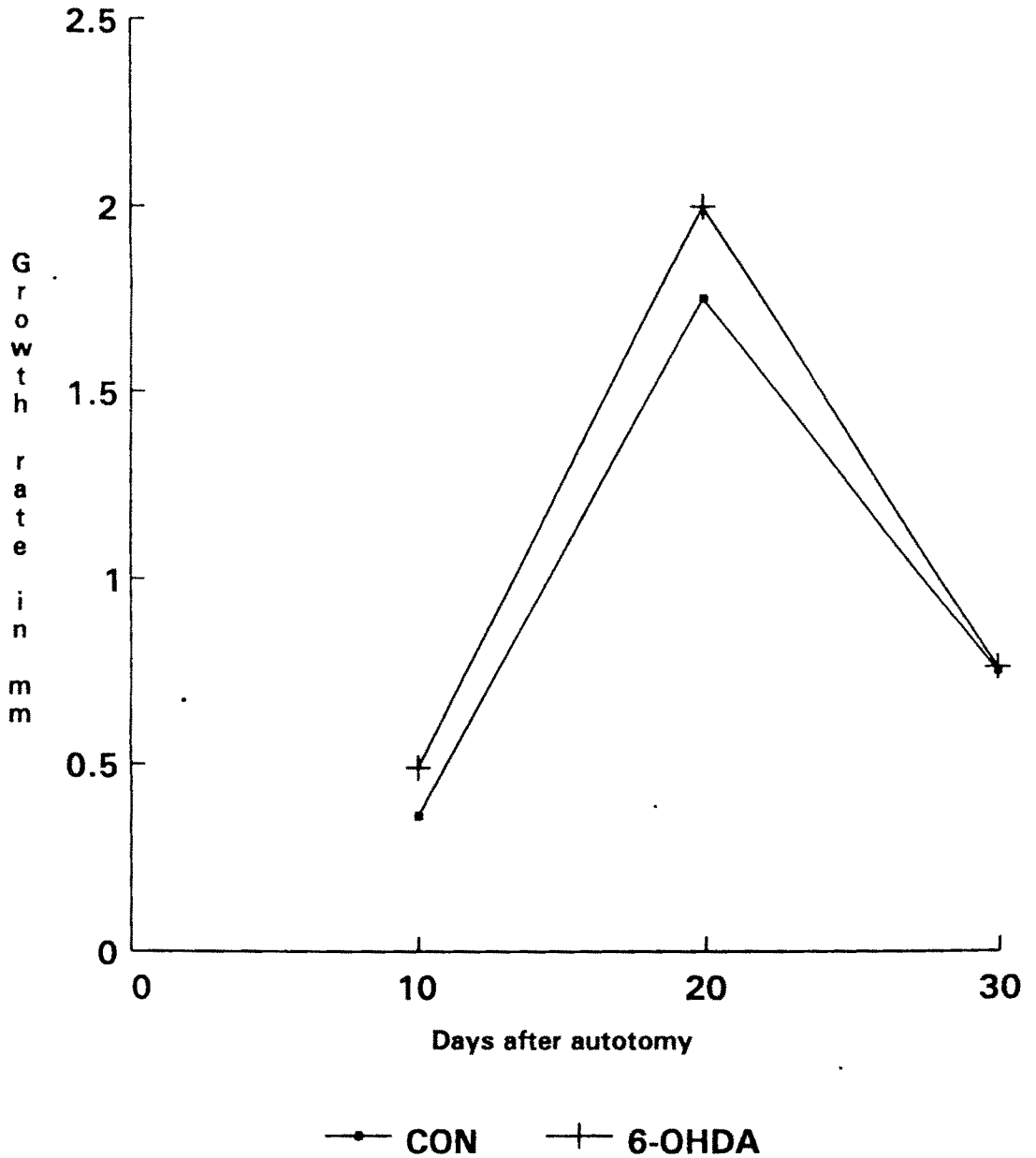


Fig.3. Average per day growth rate of the tail in control and chemically sympathectomised lizards.

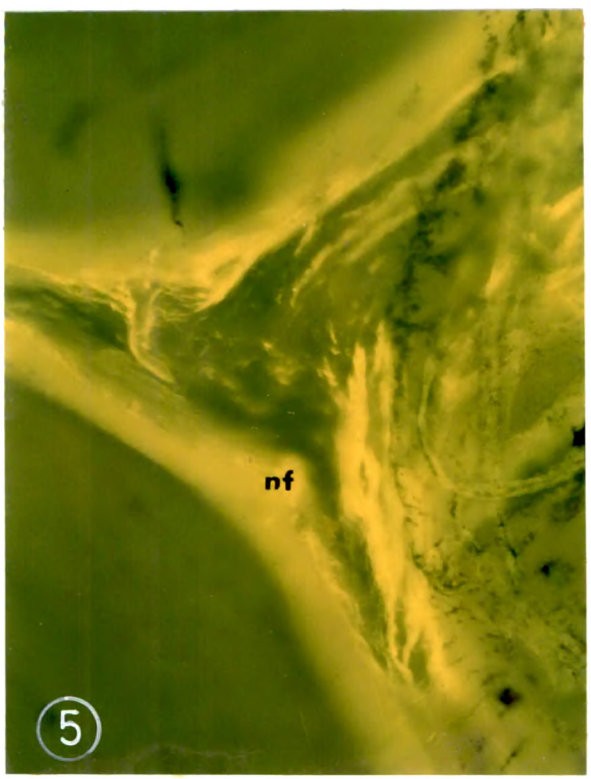
Explanation for Figures

Fig.5. Fluorescence of catecholamine in cornea of lizards. Note the intense fluorescence of CA in nerve fibres. X 184.

Fig.6. The CA levels are appreciably reduced after 6-OHDA-induced chemical sympathectomy. X 184.

Fig.7. An Advanced blastemic stage of the tail regenerate. The proximal area shows early signs of differentiation. Distal area shows proliferating blastemal cells. X 11.5.

Fig.8. Tail regenerate at blastemic stage in chemically sympathectomised lizards with 6-OHDA. The distal area shows accumulation of myogenic cells(arrows). The apical epidermis is thickened. X 11.5.



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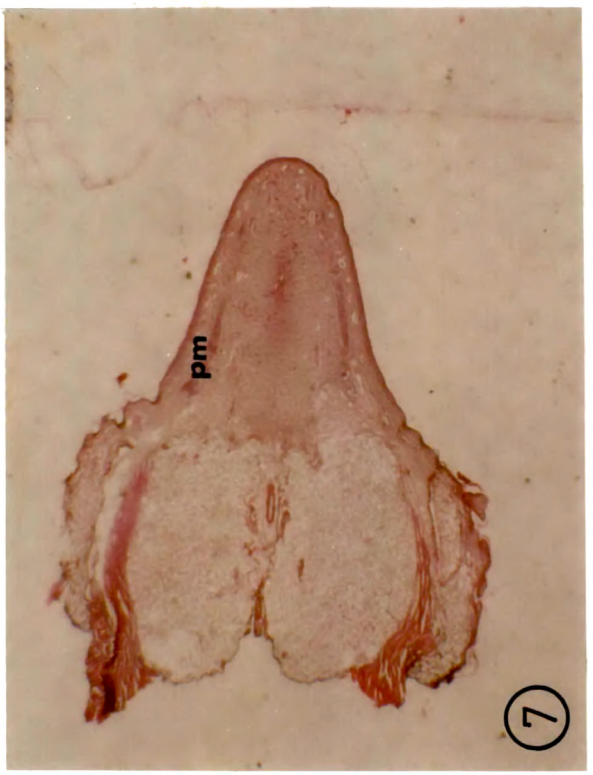

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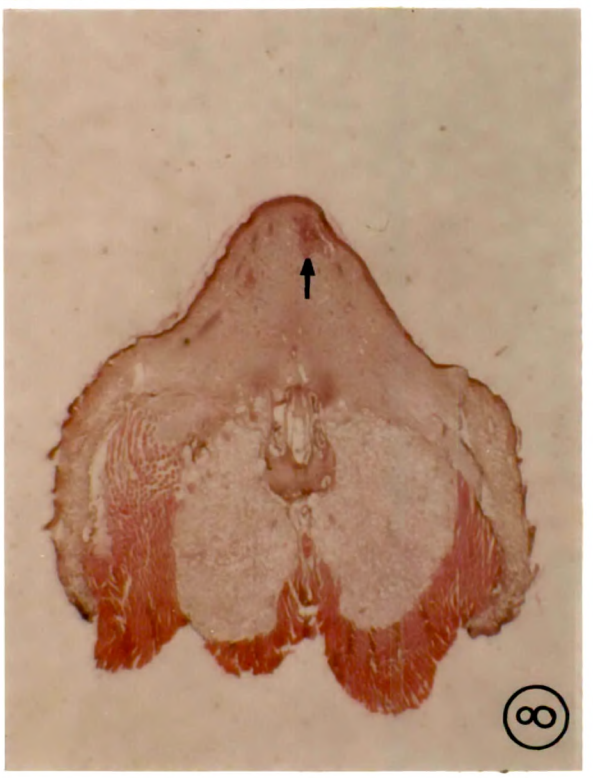

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Explanation for Figures

Fig.9. Tail regenerate at 12 days of growth. The process of differentiation extends proximo-distally. In proximal area the myomeres, cartilage tube and ependymal canal are visible. The distal tip shows proliferating cells. X 9.

Fig.10. Tail regenerate at 12 days of growth in chemically sympathectomised lizards with 6-OHDA. The process of differentiation is distinct, myomeres extend proximo-distally. Cartilage tube and ependyma elongates, nerves from spinal ganglia extends into the mesenchyme(arrows). X 9.0.

Fig.11. Magnified area of the tail tip (fig.9) showing the proliferating cells. Promuscle aggregates are visible. X 108.

Fig.12. Magnified area of the tip of the regenerate (fig.10) showing enhanced rate of differentiation. The myomeres are visible in bundles. The epidermal cap is thickened. X 108.

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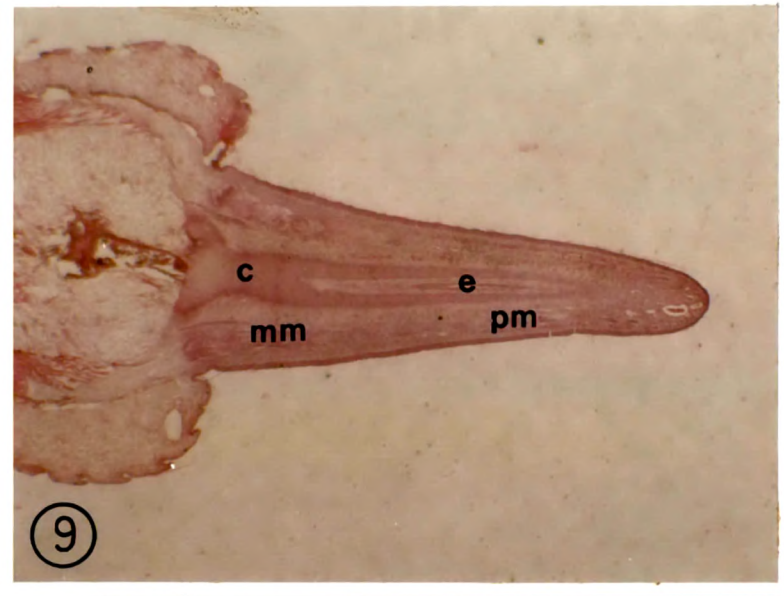

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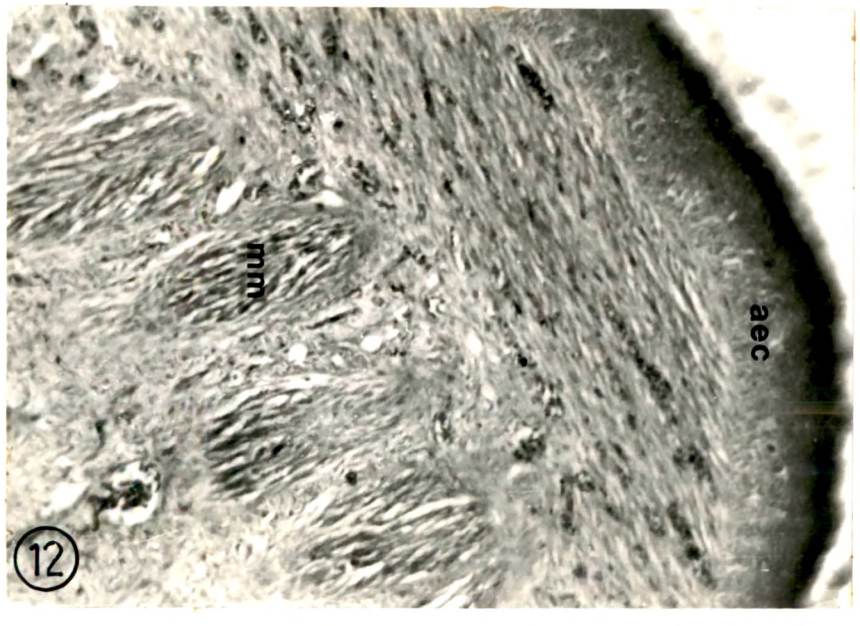

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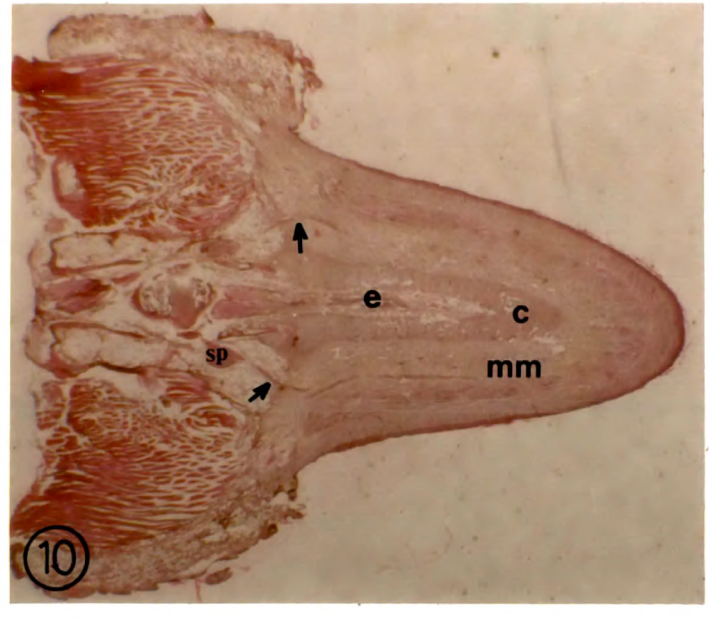

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Histological observations. Histological features of 6-OHDA treated regenerate include a thickened apical epidermal cap (AEC) and increased myogenesis (fig.7 & 8). The number of promuscle cells increased in the 6-OHDA treated regenerate and the myoblast fusion was found to be enhanced after sympathectomy. Differentiating stages have shown an increase in myomeres even at the tip of the regenerate which was not observed in the normal regenerate (figs. 9-12). In general, it has been found that the process of differentiation hastened in sympathectomized animals.

Experiment-2 A. (*Injection of 6-OHDA at WE stage*). Sympathectomy at WE stage significantly retarded the growth rate of the tail (table-3, fig.4a). Sympathectomised animals showed 40% and 45% inhibition in growth of regenerate as compared to control at 48 hrs and 96 hrs respectively.

Histological observations. *Control*: A well-formed blastema could be seen by 48 hrs while the proximal area showed the signs of early differentiation (fig.13). By 96 hrs, the process of differentiation proceeded proximo-distally (fig.14). The proximal areas showed fusion of myotubules and signs of chondrogenesis. The distal areas were occupied by proliferating mesenchymal cells.

Experimental: 6-OHDA treatment : Though the length of the regenerate was found to be decreased in the 6-OHDA treated animals at this stage, histological features revealed that the process of differentiation was unaffected. By 48 hrs post-injection, the outgrowth of the regenerate was considerably reduced and the process of dedifferentiation was found to be at a low pace (fig.15). However, the proliferation and the differentiation of the regenerate was found to be enhanced which was evident at 96 hrs (figs. 16-24). The regenerate at 96 hrs showed an acceleration in the process of differentiation, particularly evident in the differentiation of myogenic cells. The proportion of myogenic cells increased within the regenerate and these myoblast cells differentiated immediately and fused to form the myomeres. The pro-muscles were present at the distal end of the regenerate. In some cases, the proportion of myomeres increased considerably which resulted in abnormal regeneration. The apical epidermal cap (AEC) was also found to be thickened.

Experiment-2 B. (*Injection of 6-OHDA at blastema stage*). Catacholamine-depletion at BL stage enhanced the growth rate of the tail (fig. 4b). The tail length increased notably by 48 hrs after 6-OHDA administration that persisted upto 96 hrs. Histological features

Table:3 Length of tail regenerated in control and 6-OHDA treated lizards at two specific stages (preblastemic and blastemic) of regeneration. The growth of the tails were measured at 48 hrs and 96 hrs after treatment and represented (in mm) as mean ± SD.

Treatment	Preblastemic stage		Blastemic stage	
	48 hrs	96 hrs	48 hrs	96 hrs
Control	2.50 ± 0.43	6.58 ± 0.90	4.16 ± 0.53	7.16 ± 0.93
	***	***	**	NS
6-OHDA	1.45 ± 0.39	3.58 ± 0.66	4.75 ± 0.75	8.12 ± 1.43

** P < 0.02; *** P < 0.001; NS - Nonsignificant

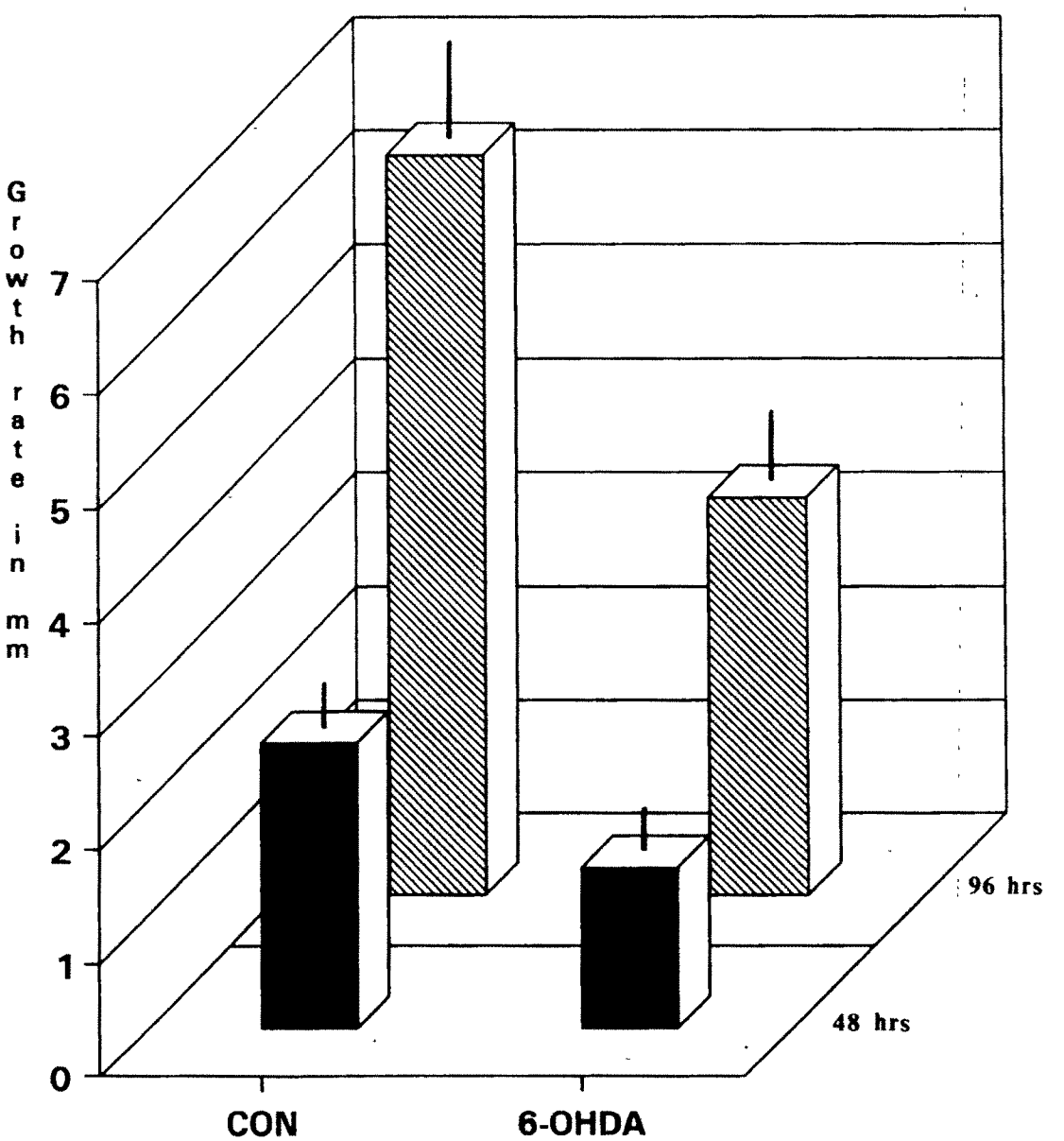


Fig.4a. Tail growth measured in control and 6-OHDA treated lizards at WE stage. Tail length was recorded at 48 and 96 hrs. N-15 animals in each group.

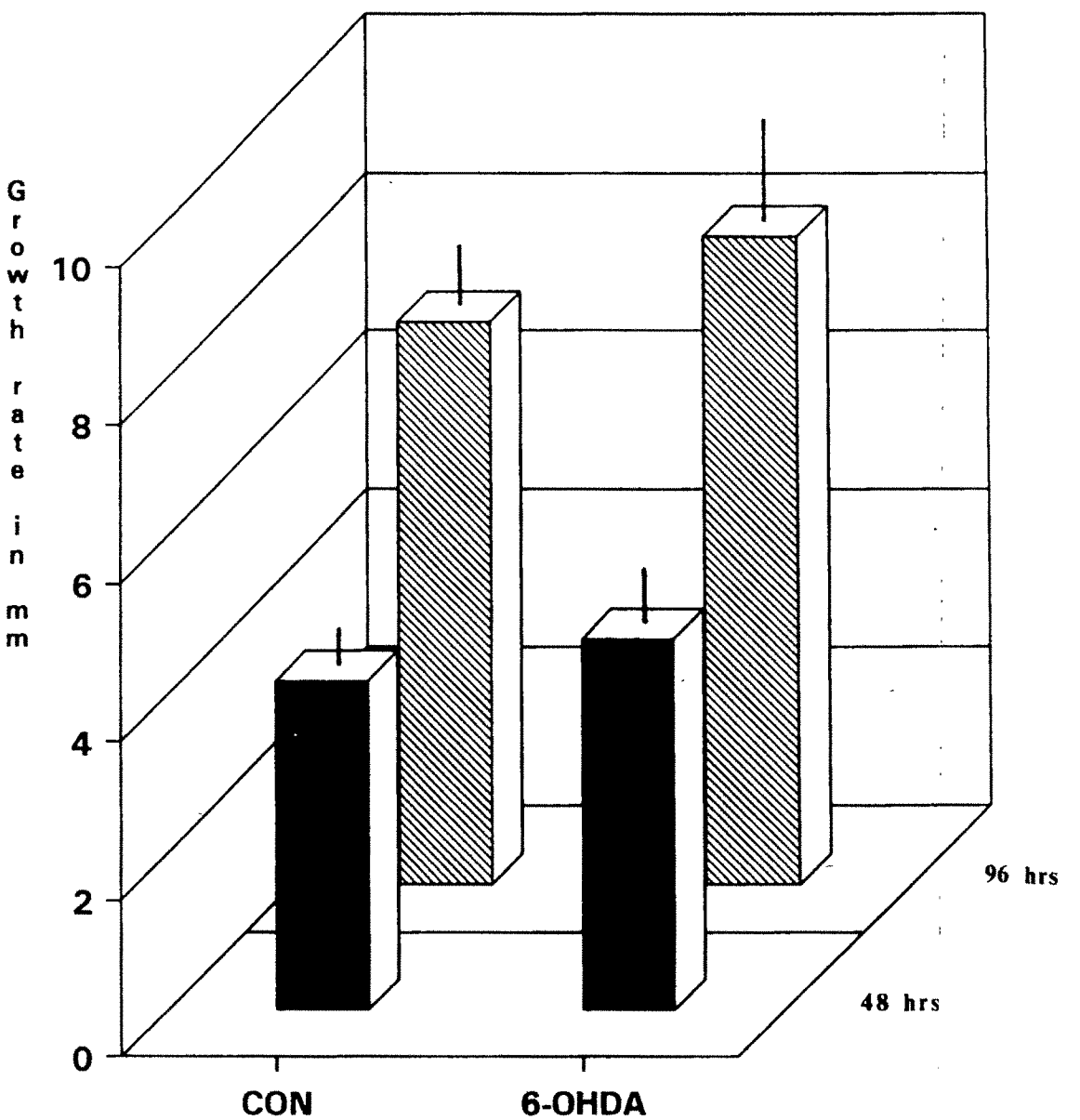


Fig.4b. Tail length measured in control and 6-OHDA treated lizards at BL stage. Growth rate was measured at 48 and 96 hrs. N-15 animals in each group.

Explanation of Figures

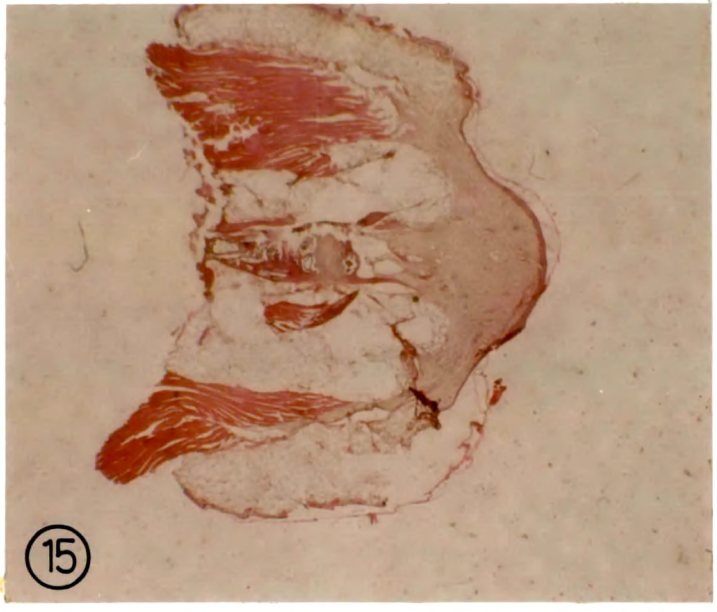
Figs.13-24. Histological features of the tail regenerate in control and chemically sympathectomised (6-OHDA) lizards at preblastemic stage.

Fig.13. A typical regeneration blastema. The mesenchyme consists of proliferating cells, proximal areas show aggregation of promuscle cells(arrows). X 11.5.

Fig.14. Control 96 hrs. The process of differentiation proceeds proximo-distally. X 11.5.

Fig.15. 6-OHDA-induced chemical sympathectomy(48 hrs). A blastema is formed, but the cell numbers are considerably reduced. X 11.5.

Fig.16. 6-OHDA treatment 96 hrs. The process of differentiation is enhanced than the control. Promuscle aggregates are visible even at the tip of the regenerate(arrows), cartilage tube extends to the tip of the regenerate. Nerve fibres from the spinal ganglia extend into the mesenchyme(arrow head). X 11.5.



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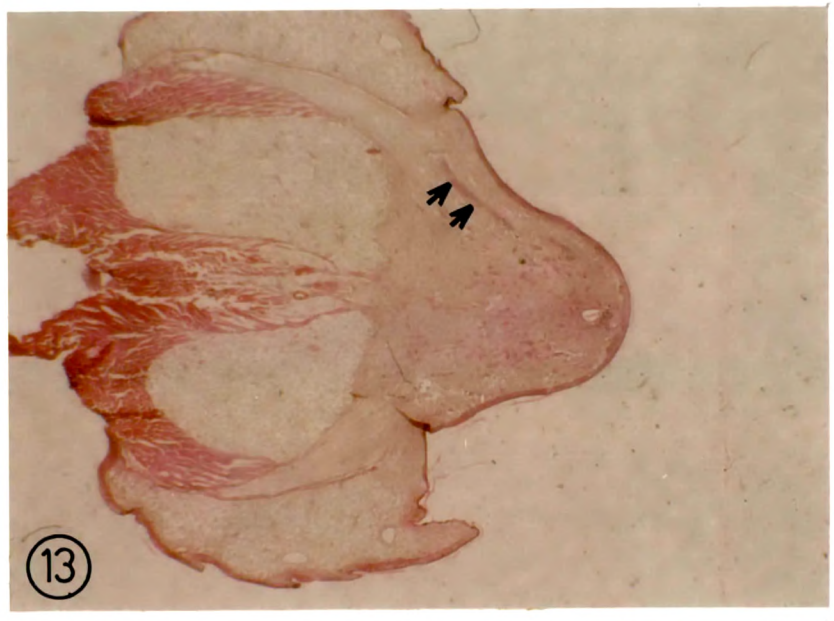

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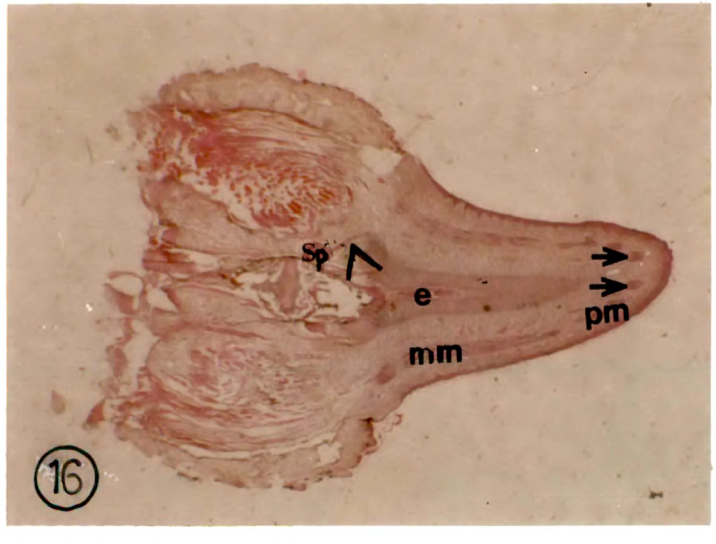

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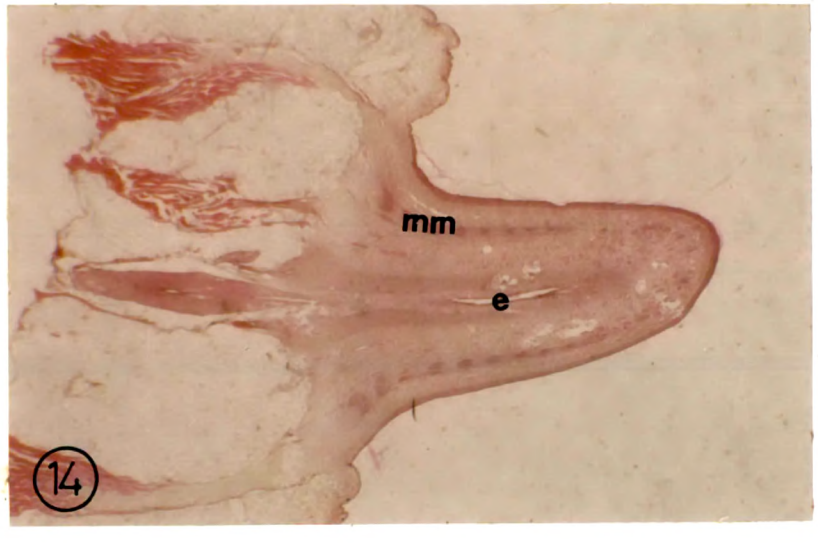

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Explanation of Figures

Fig.17. Distal area of the control(96 hrs) regenerate showing epidermis and mesenchyme. X 108.

Fig.18. Mesenchymal cells of the distal area, promuscle aggregates are visible (arrows). X 108.

Fig.19. 6-OHDA treatment (96 hrs). The apical epidermis is thickened, the promuscle aggregates fuse to form the myomeres(arrows). X 108.

Fig.20. Proximal epidermal area showing the signs of early differentiation. The epidermal invagination embark, the process of scale ridge formation. X 108.

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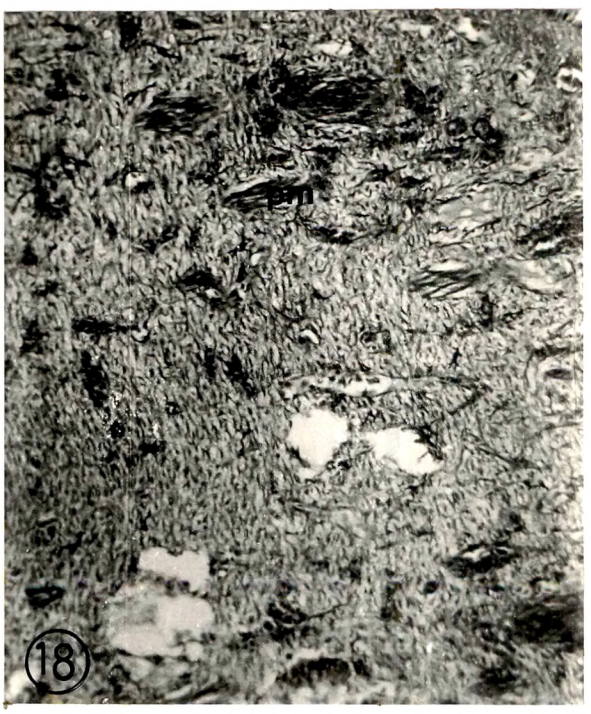

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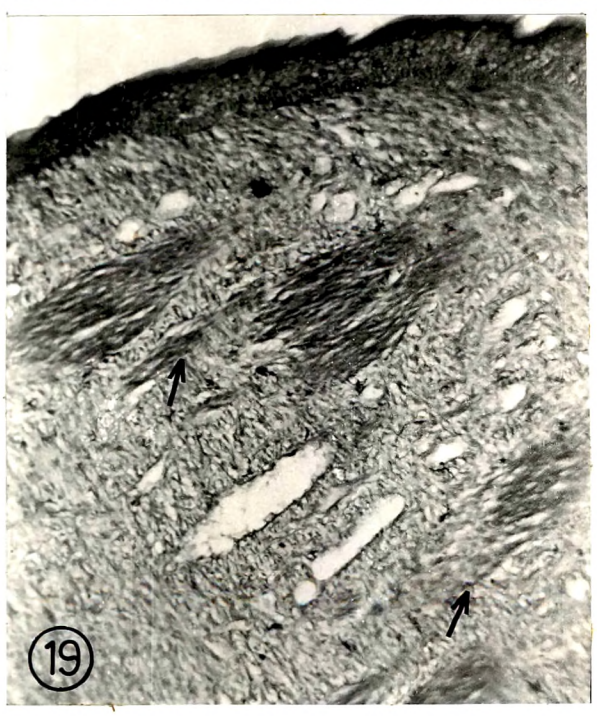

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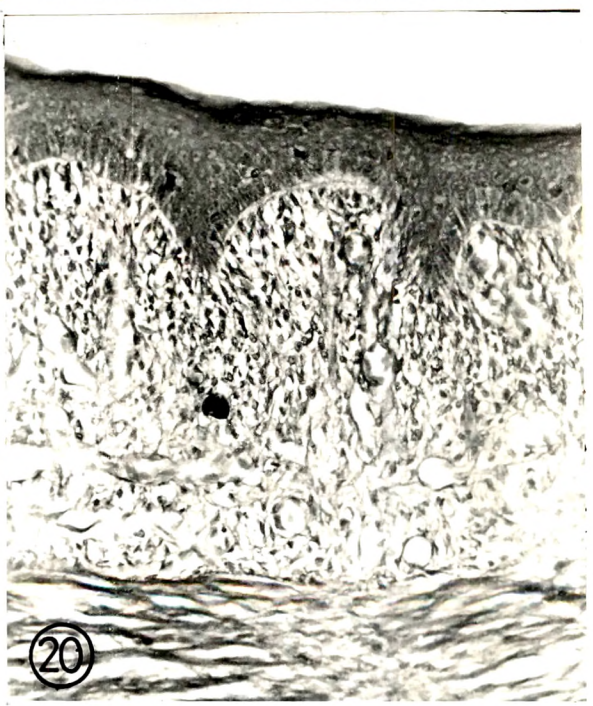

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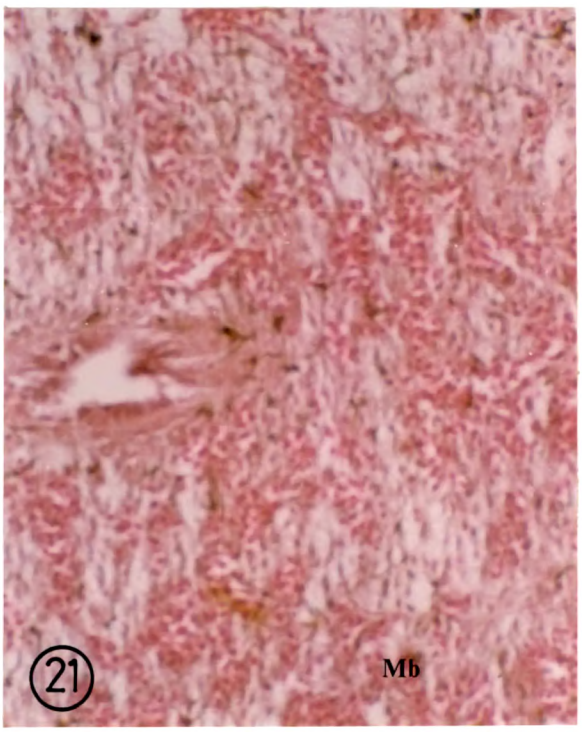
Explanation of Figures

Fig.21. Mesenchyme of the 6-OHDA treated regenerate. The highly eosinophilic cells are chiefly myoblast cells that gradually move towards the periphery. X 225.

Fig.22. Conversion of myoblast cells into promuscle cells, the cells lost their eosinophilia changed their shape and fuse to from the promuscle cells(arrows). X 144.

Fig.23. An advanced stage, myomeres area formed, some of the myoblast cells (arrows) differentiating to form the promuscles. X 144.

Fig.24. A spinal ganglia extending the nerve fibres into the mesenchyme of the regenerate. X 225.



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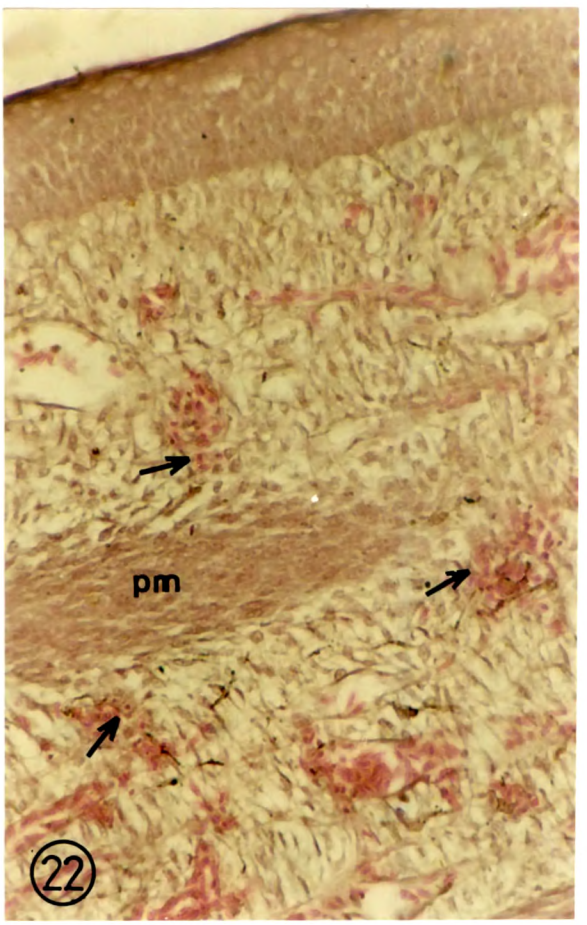

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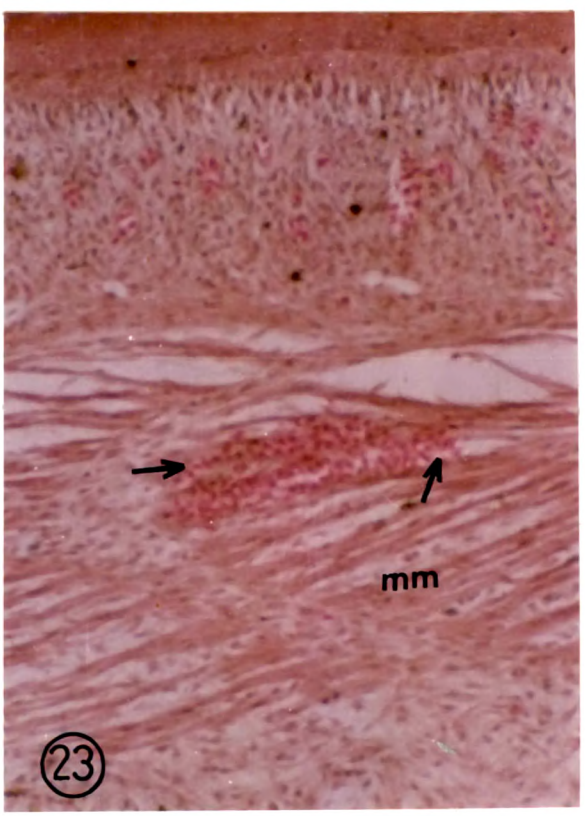

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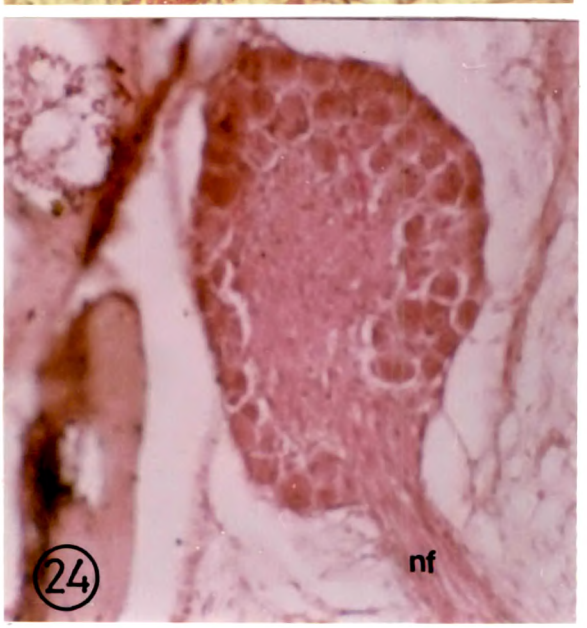

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Explanation of Figures

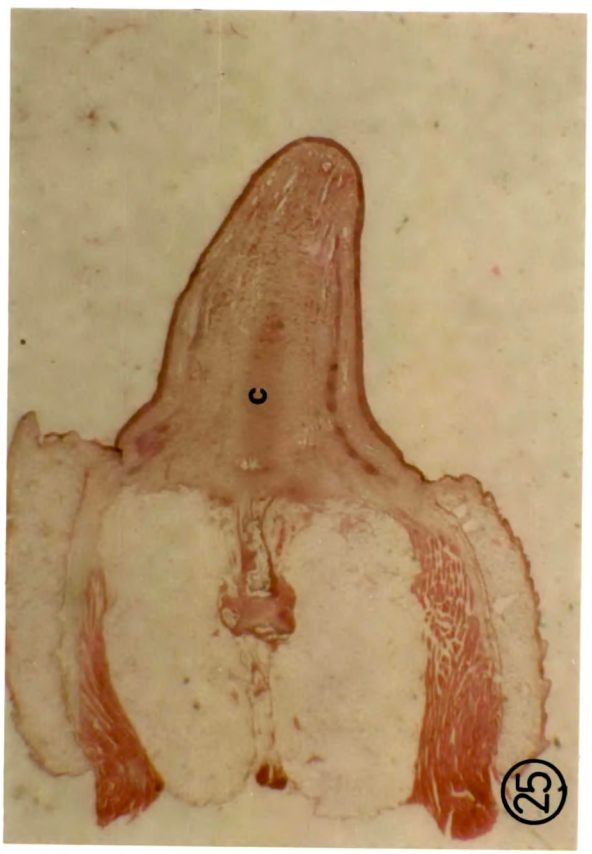
Fig.25-32. Histological features of tail regenerate in control and chemically sympathectomised lizards at blastema(BL) stage.

Fig.25. Control (48 hrs). Early signs of differentiation are evident. Chondrogenesis sets in, promuscle aggregates are visible in proximal area. X 11.5.

Fig.26. 6-OHDA treatment (48 hrs). Cluster of myoblast cells (circle) are seen in the stump area, that are derived from the connective tissue septum. X 11.5.

Fig.27. The process of myogenesis in chemically sympathectomised animals. X 108.

Fig.28. Circled area in fig.22 is magnified showing the myogenic cells. X 108.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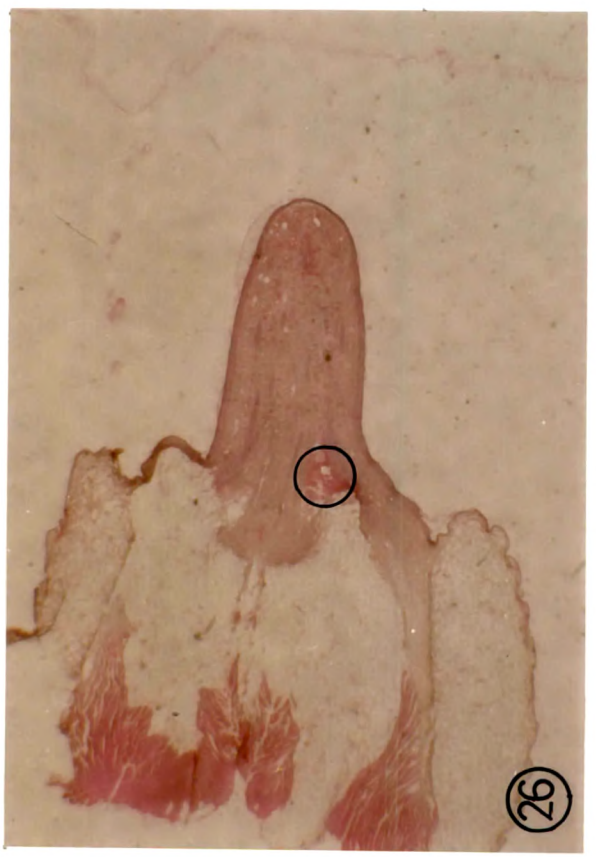
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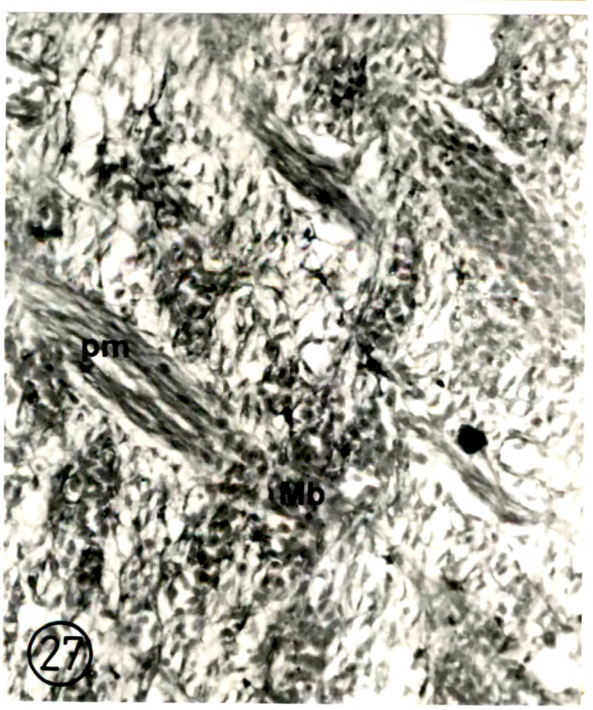
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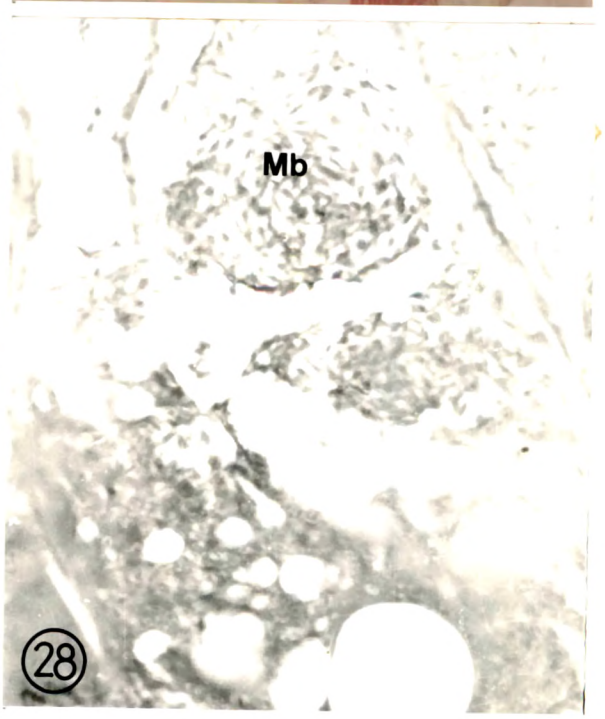
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Explanation for Figures

Fig.29. 96 hrs. The process of differentiation proceeds proximodistally. Myomeres are visible at proximal area, cartilage tube extends towards the tip of the regenerate. X 9.0.

Fig.30. 6-OHDA treatment (96 hrs). The process of differentiation is enhanced as evidenced by the increase in myogenesis and chondrogenesis. Myomeres are visible at the tip of the regen erate(arrows). X 9.0.

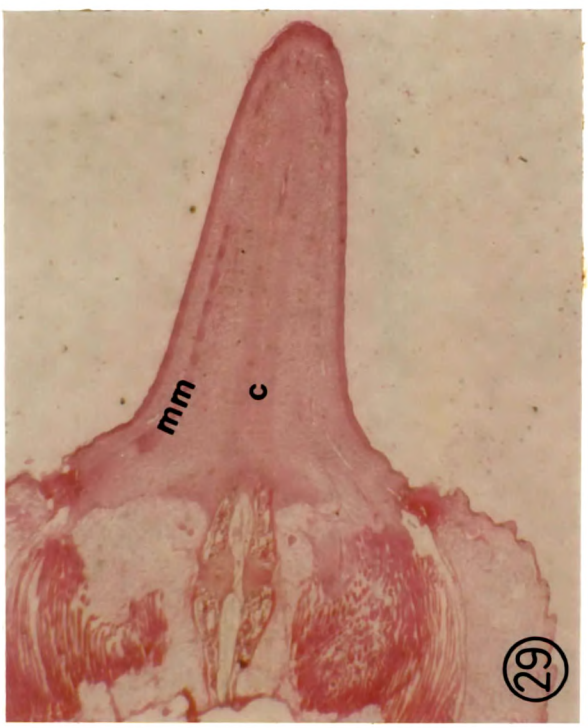
Figs. 31-32. Acridine orange-induced fluorescence of nucleic acids in control and 6-OHDA treated regenerate at 96 hrs. DNA emits yellow fluorescence and RNA as flame red emission. Both X 72.

Fig.31. Control - myoblast cells are sparse and emits yellow fluorescence (arrows).

Fig.32. 6-OHDA treatment. Promuscle aggregates(arrows) are emitting intense yellow fluorescence and their number increased.

Abbreviations

aec - apical epidermal cap
c - cartilage
ct - connective tissue septum
cf - collagen fibres
e - ependyma
mb - myoblasts
mm - myomeres
pm - promuscles
sp - spinal ganglia
nf - nerve fibre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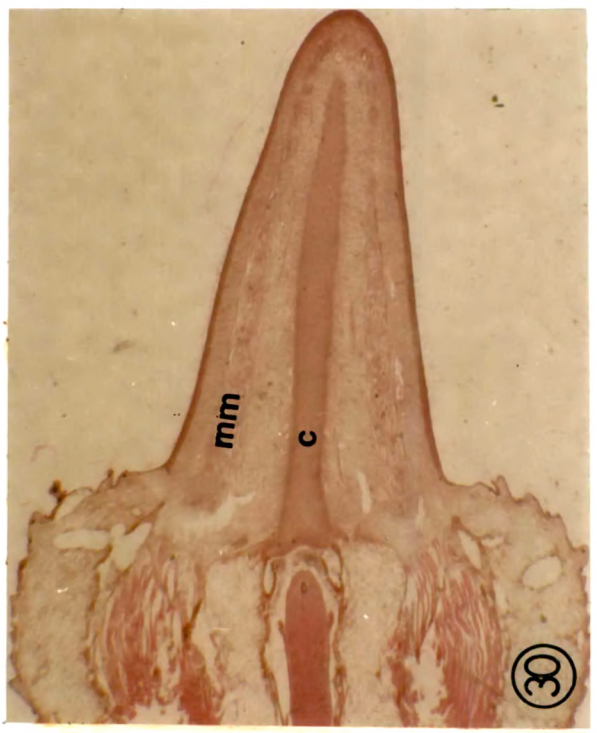
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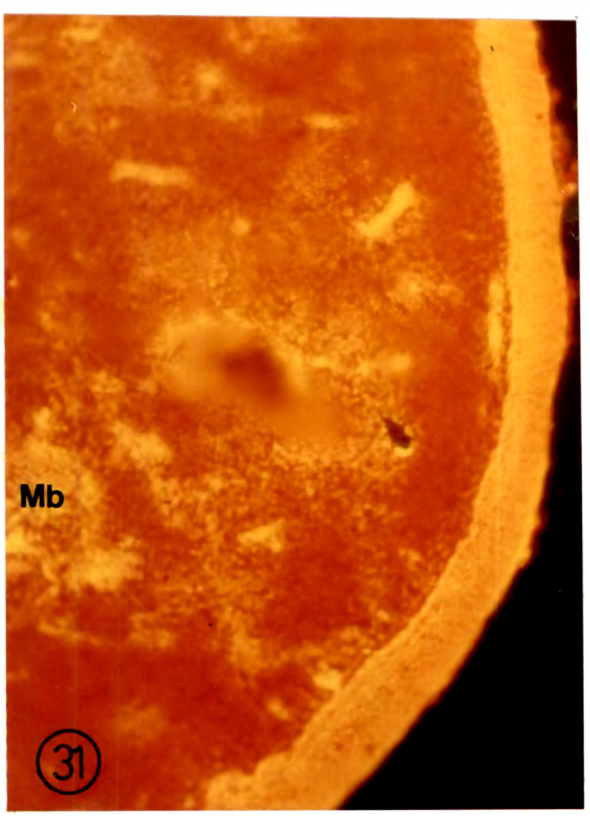
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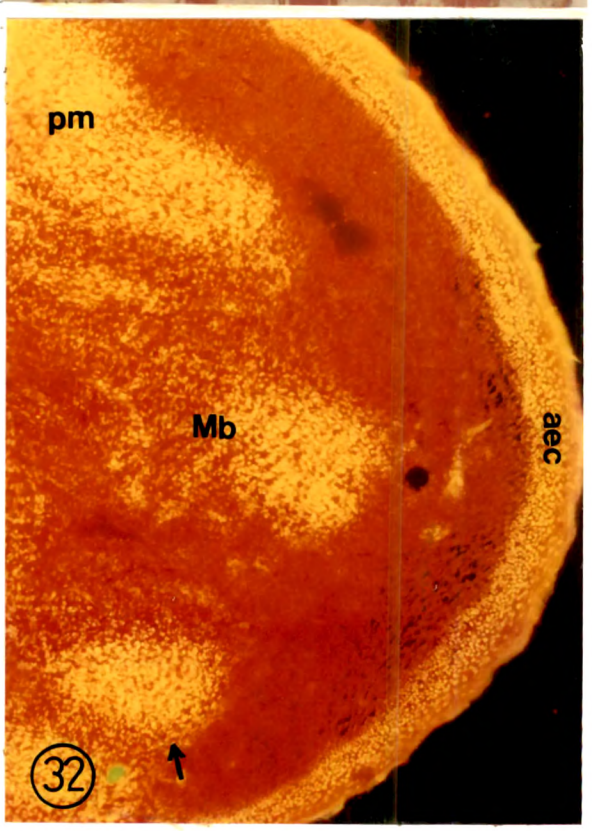
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revealed an advanced regenerate especially in the differentiation of the myogenic cells. The distal area of the regenerate also showed cells with myogenic traits which differentiated fast to form myomeres.

At 48 hrs after 6-OHDA administration, the proximal areas showed increase in the number of myoblasts (figs. 25-28). These myoblast cells were found to originate either from the satellite cells of stump muscles or from the connective septum. These cells are characterised by large oval nuclei and highly eosinophilic cytoplasm. During differentiation these cells move towards the periphery, lose their eosinophilia, change the shape and get converted into myotubules. These myotubules subsequently fuse to form the myomeres and later into muscle bundles. By 96 hrs post-sympathectomy, the regenerate showed an early differentiation with enhanced myogeneis (Figs. 29-32). At this stage also, the promuscles were seen distally.

DISCUSSION

The results of the present experiments clearly showed that CA depletion through chemical sympathectomy enhanced the process of tail regeneration in the lizard *Hemidactylus flaviviridis*. Histoflurescence observations have shown that the depletion of CA was at near total level.

The tail growth enhanced in lizards in both experiment 1 and 2. This finding is in contrast to that reported in urodele; in the newt, *Notophthalmus viridescens*, 6-OHDA administration inhibited the growth of the limb regenerate(Taban and Cathieni, 1988). Results of the present experiments demonstrate that chemical sympathectomy at pre-autotomy level and in the critical stages enhances the growth rate through an increase in the process of differentiation. A comparison of the regenerating stages between 6-OHDA treated and control animals revealed that the process of wound healing occurs simultaneously in both groups while blastema formation and differentiation are enhanced in sympathectomised lizards. This points to the fact that, dedifferentiation, cell proliferation and differentiation are the stages which have been positively influenced by decreased CA levels. The histological features of the regenerate support this view. It has been observed that in sympathectomised animals the proportion of the myogenic cells increased, differentiated at fast rate and then contributed to the enhancement in tail growth. Considerable metabolic and hormonal variations have occurred during 6-

OHDA- induced sympathectomy. The effects of sympathetic denervation were primarly reflected in the altered glycaemic levels. An elevation in the glycaemic levels was found in sympathectomised lizards. Similar observations have been reported in sympathectomised pigeons (Oommen, 1992). 6-OHDA-induced sympathectomy also changed the levels of hormones such as growth hormone, corticosteroids and thyroid hormone. Growth hormone and corticosteroids have been found to be increased while thyroid levels decreased in sympathectomised pigeons (Rintamaki, 1986). Another aspect which has to be considered is the insulin to glucagon molar ratio. Sympathectomy abolishes the sympathetic tone upon the glucagon secreting cells of the pancreas which in turn depresses the glucagon secretion. In the case of saurians, it is the A-cells which are predominant within the islets (Rhoten, 1973). The glucagon exerts a regulatory role in controlling the glycaemic levels. Similar conditions have already been reported in birds (Pilo and Verma, 1985). Sympathectomy does not alter the insulin secretion, as it is under the vagal control. However, an imbalance in the counterregulatory mechanisms as a result of sympathetic denervation might elevate the insulin secretion. This could primarily affect the insulin to glucagon molar ratio through an increase in insulin secretion. The basal release of insulin in response to a glucose load in saurians has been found to be very high compared to mammals (Rhoten, 1973). Increase in the corticosterone levels can further aggravate the glycaemia. Similar observations are reported in vagotomised (John *et al.*,1985) and chemically sympathectomised birds (Rintamaki, 1986). Thus it is presumable that elevated glycaemic levels resulted after sympathectomy could be due to a combined action of corticosterone and depressed glucagon secretion which reduces gradually due to increased insulin secretion.

Elevation of insulin and growth hormone results in several growth promoting actions. The importance of insulin and its multiple response upon cell proliferation and differentiation in newt limb regeneration is experimentally established both *in vivo* and *in vitro* (review, Globus and Vethamany-Globus, 1985). Though the exact mechanisms by which growth hormone increase occurs after sympathectomy is not understood, the actions of growth hormone are well illustrated in all vertebrate groups. In lizards there are ample experimental evidences that support the hormonal influence during tail regeneration, which is expressed maximally during differentiation (Lichet and Howe, 1969; Turner and Tipton, 1971). An elevated GH can itself release insulin from the islets (Tepperman, 1965). In hypophysectomised ducks, replacement therapy with bovine GH elevated plasma concentrations of insulin (Foltzer and Mialhe, 1976). Hypophysectomised newts had undergone extensive atrophy of the pancreatic islets (Vethamany-Globus and

Liversage, 1973). Replacement therapy with GH or amino acid mixture to hypophysectomised newts promoted limb regeneration which is primarily attributed to a direct stimulus of insulin secretion (Sato and Inoue, 1973). Based on the above observations it could be suggested that the enhancement in tail growth rate observed in the sympathectomised lizards could be related to an increase in secretion of GH, corticosteroids and insulin which operated in concert to promote the cell proliferation and differentiation. The necessity of a multiple hormone background for cell proliferation and differentiation for newt limb regeneration is established (review, Globus and Vethamany-Globus, 1985). However, the precise manner in which these hormones co-ordinate and integrate the proliferation and differentiation phases remains unknown (Globus and Vethamany-Globus, 1985).

Sympathectomy at preblastemic(WE) stage (Experiment-2; *Series-A*) resulted in an inhibition of growth rate of the tail regenerate. However, the histological features revealed an advancement in the process of regeneration. During tail regeneration, formation of functional wound epithelium initiates the process of dedifferentiation which eventually gives rise to accumulation of embryonic like blastemal cells. Sympathectomy at this level considerably reduced the cell proliferation and the cells instead of accumulating into a critical mass of blastemal cells, immediately channelized into a differentiating pathway. Thus it is speculative that a massive depletion of CA might reprogramme the regenerative events. Other possibilities exist which are related to the hormonal variations. It has been found that as the glycaemic level increases, concomitant increase in the level of GH, corticosteroids and insulin occurs in sympathectomised birds. In regenerating systems increased sensitivity to the hormones are associated with the proliferation-differentiation pathway. It is speculative that the responses to specific hormone, varies according to specific regenerating stages and alteration above the specific threshold level at these crucial events might alter the programmed regenerative events and reprogramme them into another pathway.

Histological features of the tail regenerate show an increased rate of differentiation of the tail which was evident in both experiments. Significant among the features is the increase in the myogenic potential. It has been noted that within the mesenchyme, cells with myogenic trait increased which fused and differentiated at a faster rate . Though sympathectomy at preblastemic level could decrease the length of the regenerate, the early signs of differentiation was observable with an increase in myogenesis which has given an unusual proportion of promuscle cells. The epidermal cells also showed signs

of early differentiation and thickening of the apical epidermis.

The regulation of regenerative events by cyclic nucleotides has been established. Fluctuations in the level of cyclic AMP and cyclic GMP are recorded both in stump and within the regenerate during regeneration (Taban *et al.*, 1978; Taban and Cathieni, 1989). The fluctuations of these nucleotides are primarily controlled by catecholamine neurotransmitters acting through ß-adrenergic receptors (Taban *et al.*, 1978). Since sympathectomy depletes the peripheral CA levels, corresponding decrease in cAMP and cGMP might occur. It has been suggested that the concentration of cyclic nucleotides determine the pathway chosen by the mesodermal cells in embryonic chick leg. In developing chick limbs decreased intracellular levels of cAMP stimulated the myoblast terminal differentiation (Reporter, 1973; Wahrman *et al.*, 1973a,b; Ravdin and Podleski, 1975). Addition of exogenous cAMP inhibited differentiation of chick myoblast *in vitro* (Zalin, 1973). It is suggestive that cAMP plays an antagonistic role to cGMP (Makman *et al.*, 1974). It has been reported that insulin can reverse the increase in cAMP levels by virtue of its ability to increase cGMP levels; also it is known to stimulate myogenesis in myoblast cell line (Mandel and Pearson, 1974). The evidence taken together seems to suggest that a decreased ratio of cAMP to cGMP may favour myoblast differentiation. These evidences are in support to the present findings.