CHAPTER - IV

GUANETHIDINE-INDUCED SYMPATHECTOMY RETARDS THE PROCESS OF TAIL REGENERATION IN THE LIZARD, *HEMIDACTYLUS FLAVIVIRIDIS*.

Peripheral sympathectomy with 6-Hydroxydopamine(6-OHDA) has been found to enhance the process of tail regeneration in the lizard *Hemidactylus flaviviridis* (chapter-III). The growth promotion has occurred during the early differentiating stages. The specificity of the neurotoxin 6-OHDA is well documented and the damage is found restricted only to the adrenergic nerve terminals leaving the cell bodies intact. Several other drugs are known to have anti-adrenergic properties viz., Vinblastine, Debrisoquine, Guanoxan, Bethanidine, Guanethidine etc. Many of these drugs vary in their mode of action and dosage required to achieve adrenergic denervation. Among the guanidine group of adrenergic blocking agents, guanethidine is known to produce long-lasting adrenergic blockade (Burnstock *et al.*, 1971). Guanethidine is widely used by clinicians as an anti-hypertensive agent, but in rats and in several other vertebrates it induces NE depletion (Eranko and Eranko,1971; Angeletti and Levi-Montalcini, 1972; Burnstock and Costa, 1975). Investigations have proved that this drug primarily influences the cell bodies rather than the axons (Heath and Burnstock, 1977). In rats, guanethidine induces permanent destruction of the adrenergic neurons in sympathetic ganglia. Besides this, guanethidine has been found to produce a pH dependent effect on cultured sympathetic neurons. At pH 7.0-7.2 guanethidine does not evoke any cytotoxicity, while at pH 7.4-7.6 and above it causes complete cell destruction (Johnson and Aloe, 1974).

Experiments in the newt, *Notophthalmus viridescens* by Sicard and DiNicola (1974) have reported guanethidine-induced adrenergic denervation and its effect on the limb regeneration. As the 6-OHDA-induced chemical sympathectomy has given interesting observations, another attempt has been made in the present experiment to study the guanethidine-induced adrenergic denervation in the process of tail regeneration in lizards. The animals were treated with guanethidine at two different pH and the effects on the process of tail regeneration were studied.

MATERIALS AND METHODS

Adult *Hemidactylus flaviviridis* weighing 10 ± 1 gms were obtained from local dealer and acclimated in the laboratory for 7 days on a diet of cockroaches; water was given

daily. A total of 45 lizards were used in the present experiments and they were divided into 3 groups of 15 each and caged separately.

Group-I : The animals were given daily ip.injection of guanethidine sulphate (Sigma) at a dosage of 50 mg/kg body wt . The drug was dissolved in 0.6% saline; pH of the drug was 10.0-10.20.

Group-II : This group of animals were injected with guanethidine at same dosage as above, but pH of the solution was adjusted to 7.4-7.6 with 0.1 N HCl.

Group-III : These animals served as controls to the above groups, received 0.6% saline only.

In all groups, each animals received 0.05 ml of drug or vehicle. The treatment started 5 days prior to autotomy and continued till 30 days from the date of autotomy. Autotomy was performed by pinching off the tail leaving three segments intact from the vent.

The extent of sympathectomy was assessed through the histofluorescence localization of CA in the cornea of lizards (details in chapter-I). The outgrowth of regenerate was measured with a millimeter scale at fixed intervals and evaluated histologically at specific stages. The time taken to reach different stages of the regeneration was also recorded.

Data analysis: The data were analysed statistically using Student's 't' test. $P < 0.05$ was taken as significant.

RESULTS

Histofluorescence localization of CA in the cornea of the guanethidine treated lizards showed a considerable reduction in the CA level after 5 days of treatment (the day of autonomy) as indicated by the fluorescence in the cornea (figs. 1 & 2). After 30 days of treatment the lizards exhibited a drastic reduction in the CA level (figs. 3 & 4).

Chemical sympathectomy with guanethidine retarded the tail regeneration in lizards and this growth impairment was found to be pH-dependent. The data obtained on the length of tail regenerated, average growth rate and percentage of growth inhibition are presented in tables:1 & 2. and figs. 5, 6 & 7.

Guanethidine at pH 7.4-7.6: Guanethidine at this pH had no adverse effect on blastema

Explanation for Figures

Figs. 1-4. Fluorescence localization of catecholamine(CA) in cornea of lizards. The bright yellow fluorescence indicates presence of catecholamines. All X 184.

Fig.1. Control lizards. Note the intense yellow fluorescence of CA in nerve fibres.

Fig.2. After 5 days of guanethidine-induced sympathectomy the CA levels are appreciably reduced.

Fig.3. Control-30 days of tail regeneration.

Fig.4. Guanethidine treatment(30 days). Note the drastic reduction of CA from nerve fibres.

Abbreviation

nf - nerve fibre

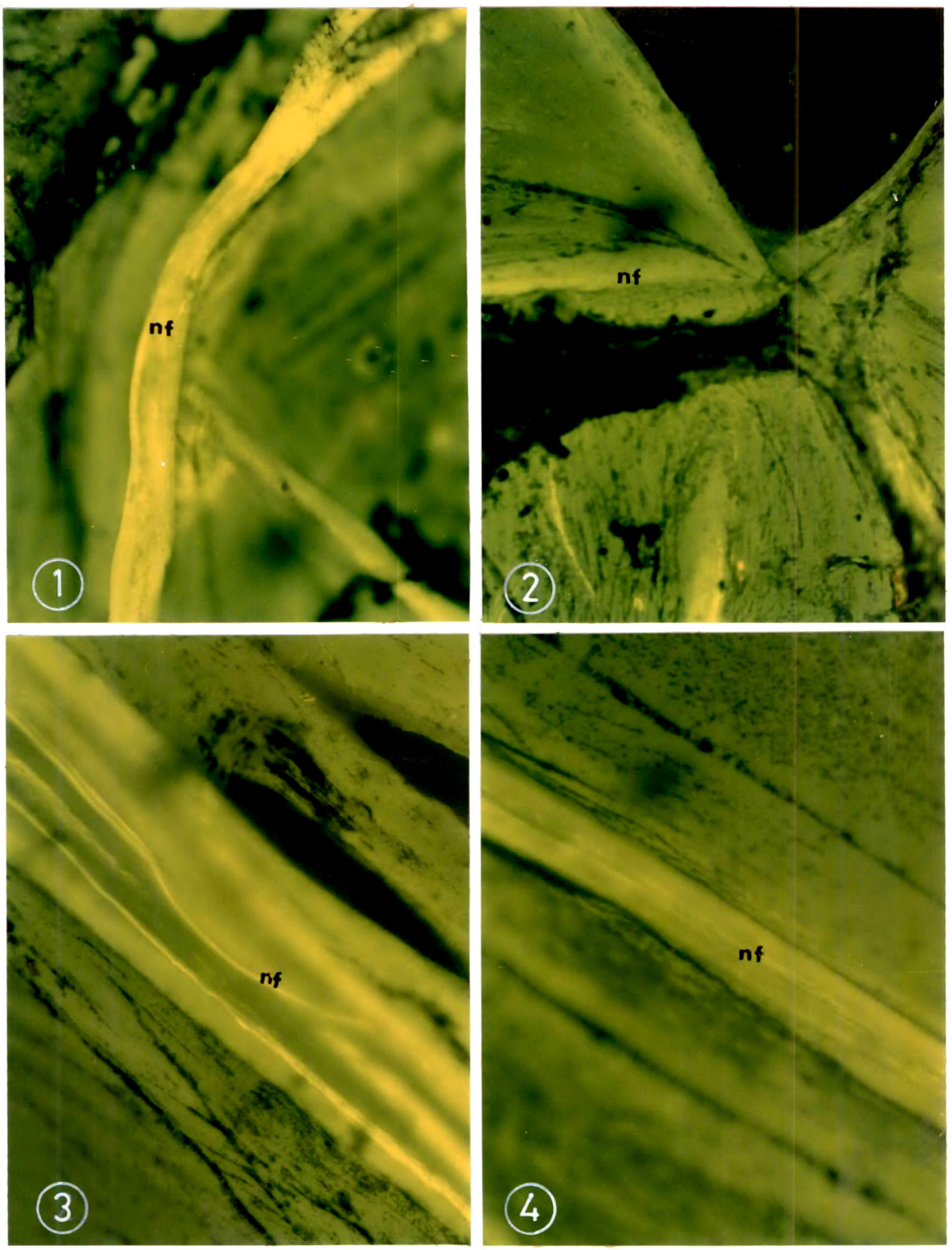
nf
1
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Table : 1. Number of days taken to reach various arbitrary stages of tail regeneration in lizards chemically sympathectomised with guanethidine at two different pH.

Groups	WH	BL	ED	MD	LD	GR
Control	5-6	7-8	8-10	11-15	16-23	23 onwards
Guanethidine (pH 7.4-7.6)	5-6	7-8	8-10	11-15	16-25	25 onwards
Guanethidine (pH 10.0-10.2)	6-7	8-9	10-13	14-18	retardation in morphogenesis	

Table : 2. Length of tail regenerated in control and chemically sympathectomized lizards with guanethidine at two different pH.

Days	Control	Guanethidine pH 7.4-7.6	Guanethidine pH 10.0-10.2
		***	***
10	6.35 ± 0.98	4.8 ± 0.63	2.40 ± 0.99
		***	***
15	14.50 ± 2.0	10.85 ± 1.75	8.83 ± 0.75
		***	***
20	20.62 ± 2.59	16.80 ± 2.78	12.83 ± 1.33
		***	***
25	26.90 ± 2.85	20.75 ± 3.02	13.66 ± 1.36
		***	***
30	29.40 ± 2.79	22.85 ± 3.19	14.16 ± 1.72

***P < 0.001

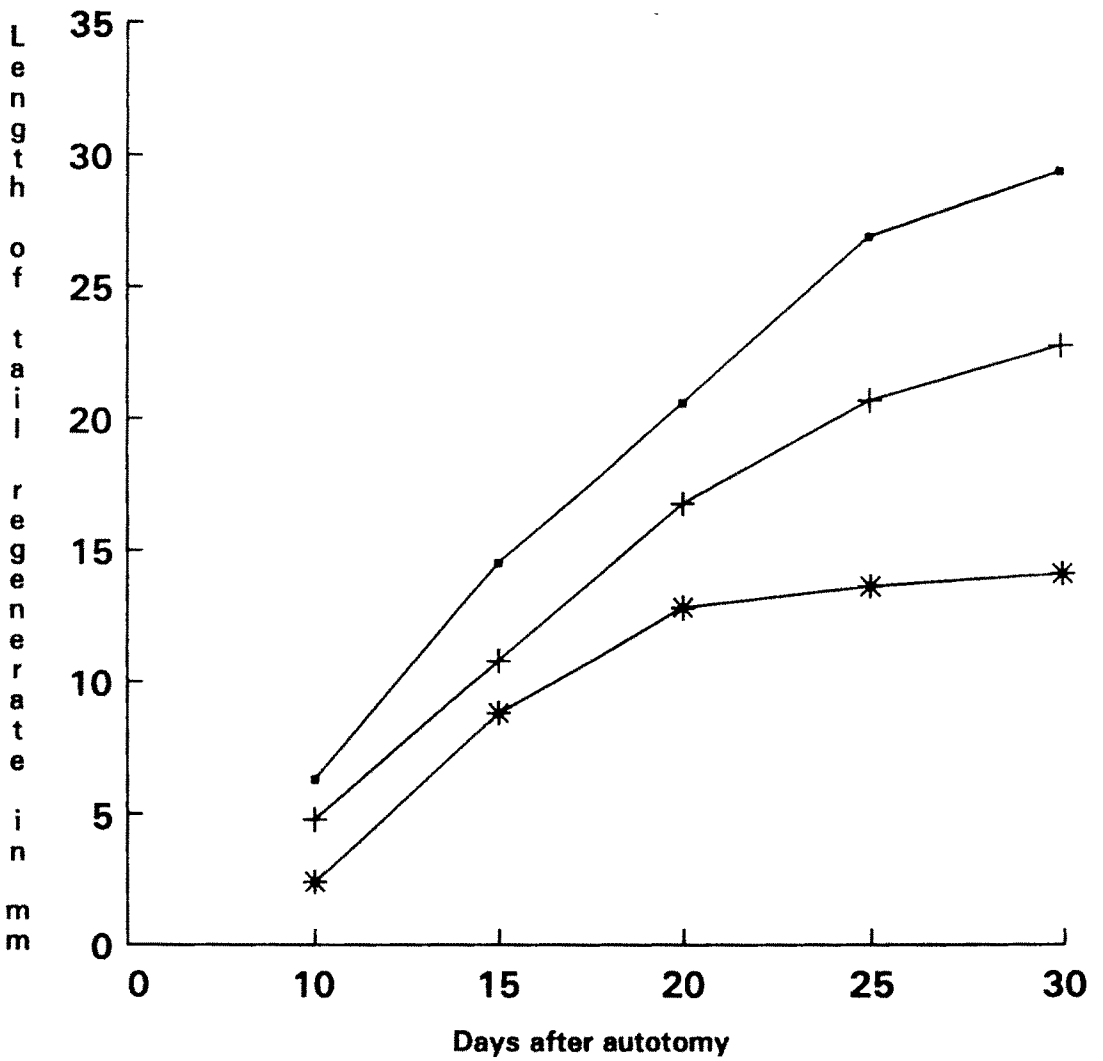

Fig.5. Length of tail regenerated in control and chemically sympathectomised lizards at 2 different pH. Tail length is mean +/- SD of 15 animals in each group.

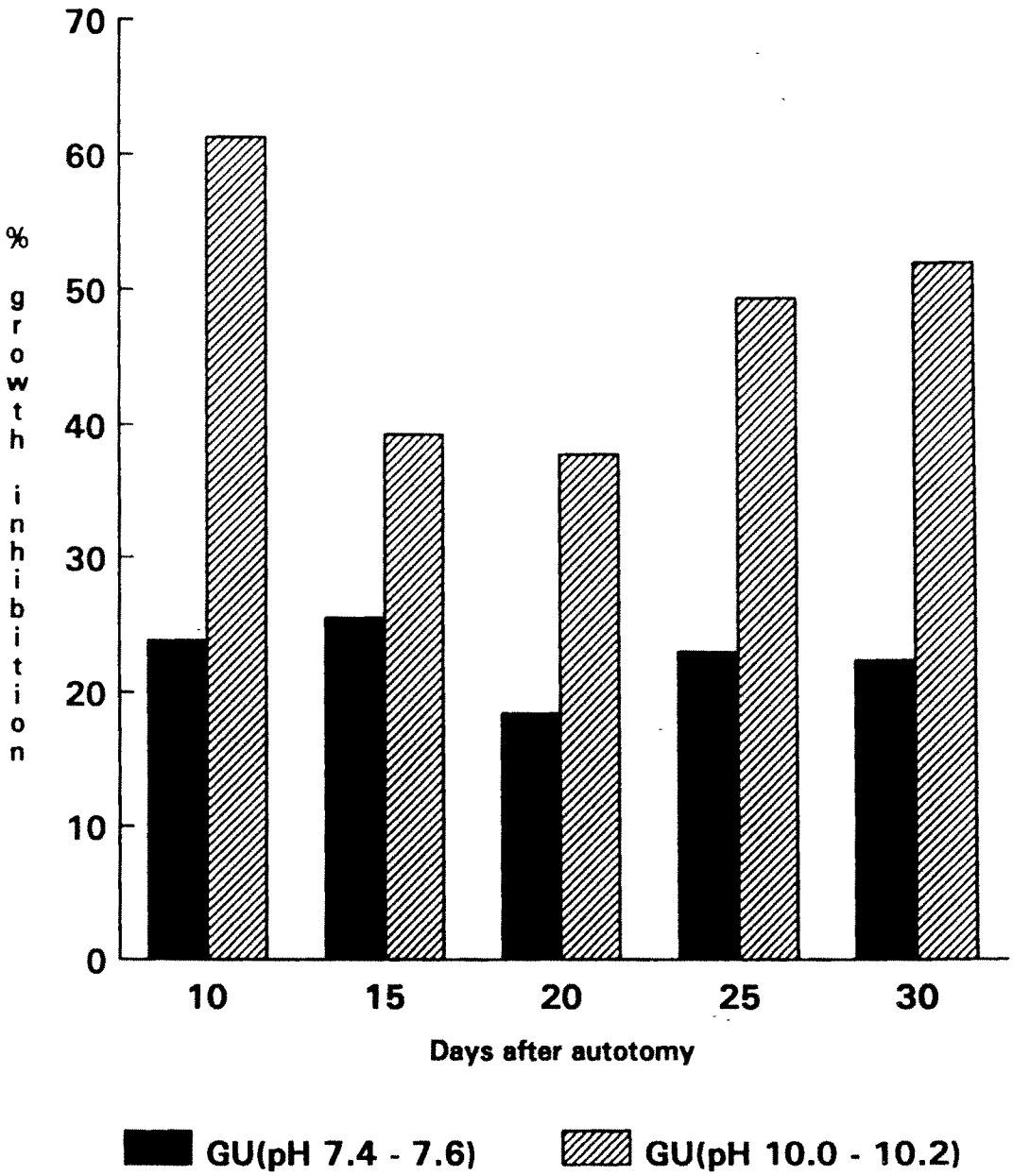

Fig.6. % of growth inhibition in regenerating tail of lizards treated with guanethidine at two different pH

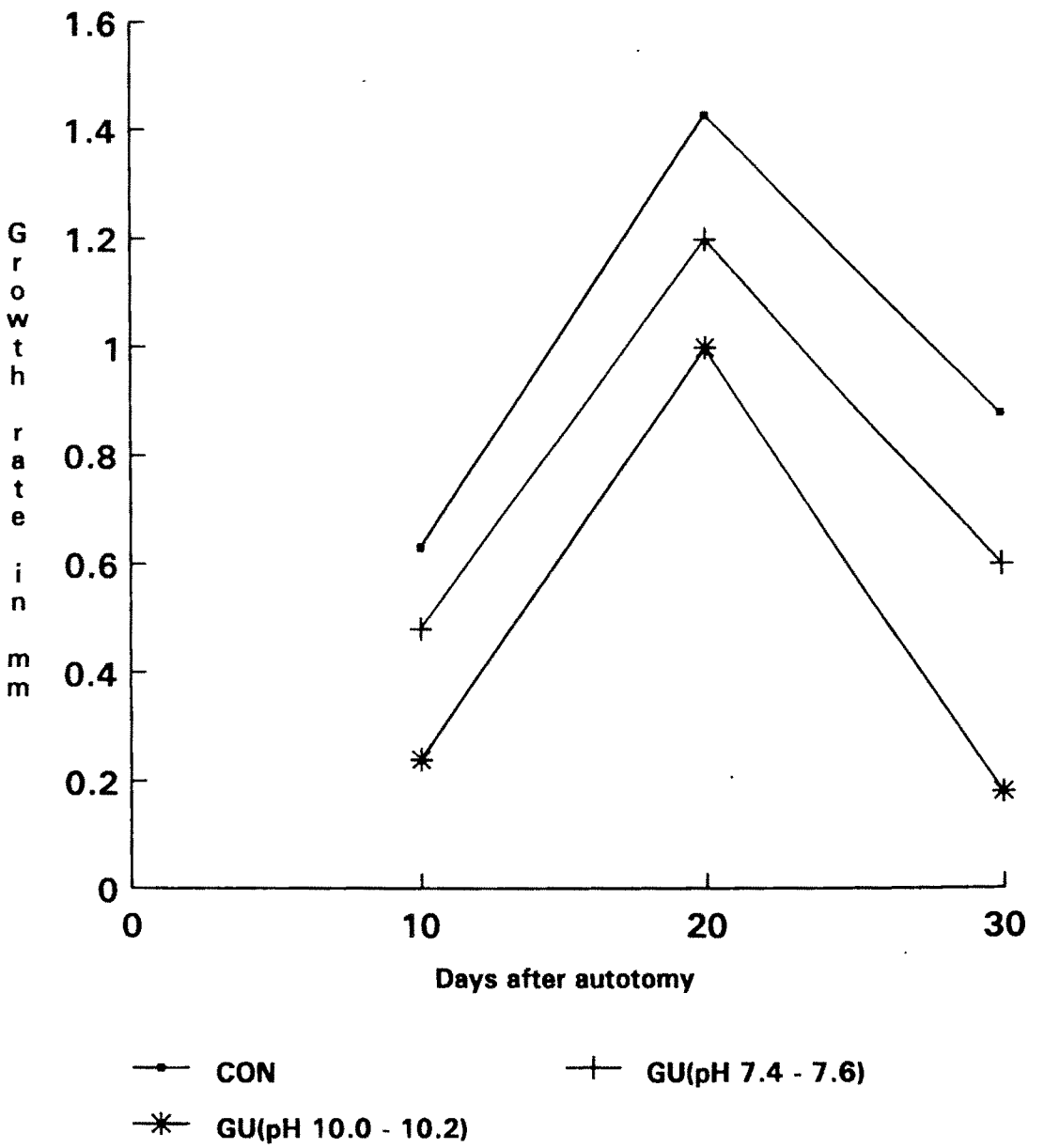

Fig.7. Per day growth rate of regenerating tail in control and guanethidine treated lizards at intervals of 10 days.

formation or differentiation. The total length of the tail regenerated and average growth rate of the tail were significantly decreased in comparison to the controls (fig.5). Guanethidine treatment caused 23%, 18% and 22% of growth inhibition with respect to controls at 10, 20, and 30 days respectively (fig.6). The average per day growth rate of the tail (fig.7) was initially low in sympathectomised animals but later on, the growth rate found to be increased. Histological observations have shown that the progress of morphogenesis of the tail was retarded as the treatment continued.

Guanethidine at pH 10.0-10.20: At this pH, guanethidine treatment adversely affected the tail regeneration in lizards. The guanethidine treated lizards showed reduction in body weight, ptosis and reduced skin pigmentation. The animals became pale in colour and the mortality rate was increased to 40% by day 15. The process of wound healing and blastema formation were delayed in guanethidine treated animals. Though the process of differentiation was observed, the morphogenesis of the tail greatly hampered as the treatment continued. This was evident by the reduction in average growth rate per day (fig.7).

DISCUSSION

Guanethidine is known to destroy the cell bodies and axons of the adrenergic post-ganglionic fibers and causes long-lasting CA depletion in several species of animals (Burnstock and Costa, 1975). The mechanism by which guanethidine destroys the sympathetic ganglia has extensively studied in sympathetic ganglia of rats both *in vivo* and *in vitro* (Hill *et al.*,1973; Heath *et al.*,1973,74; Johnson and Aloe, 1974; Johnson *et al.*, 1979; Manning *et al.*, 1982). However, cytotoxic mechanisms of this drug still remain unknown. In the present experiment guanethidine-induced adrenergic denervation retarded the process of tail regeneration in lizards in a pH-dependent manner. These observations are in contrast to that noted in the previous experiment (chapter-III). Histofluorescence localization of CA in the cornea of guanethidine treated lizards showed a modest decrease in the neurotransmitter content. Guanethidine treated animals exhibited decrease in body weight, pigmentation and ptosis as the treatment prolonged. This implies that the drug affects both adrenergic nerves and general body metabolism at higher pH levels. Similar observations have been reported in guanethidine treated rats (Zochodone *et al.*, 1988).

Guanethidine treatment at pH 7.4-7.6 did not show any delay in attaining the different stages of the tail regeneration though the growth in length remained low. The retardation of tail length was observable only in the late differentiating stages. On the contrary, at elevated pH levels (10.0-10.2) the onset of blastema and further differentiation were considerably delayed. Prolonged treatment hampered the morphogenesis of the tail. Previous studies have shown that guanethidine treatment retards limb regeneration in the newt, *Notophthalmus viridescens* (Sicard and DiNicola, 1974). Sicard and DiNicola suggested that adrenergic neurotransmitters may play a role in the process of morphogenesis. However, various properties and the pH of guanethidine have to be considered to explain the mode of action of this drug. At pH 7.4 - 7.6, guanethidine causes total destruction of the sympathetic ganglia and depletes CA levels in rats (Zochodone *et al.*, 1988). In the present experiment, it has been found that guanethidine (pH 7.4 - 7.6), though depleted the CA levels appreciably, could not evoke any influence on the process of tail regeneration. At this point it is not clear whether the destruction of sympathetic ganglia had occurred. When the pH of guanethidine increased, the cytotoxic action was clearly observable. Chronic guanethidine treatment destroys the sympathetic neurons depriving it of NGF primarily obtained by retrograde axoplasmic transport (Johnson *et al.*, 1979). Immune mediated mechanisms are suggested for the nerve destruction; several immunosuppressive agents prevented guanethidine-induced destruction of rat sympathetic neurons (Manning *et al.*, 1982).

The pH-dependent cytotoxicity of sympathetic ganglia to guanethidine has been demonstrated in *in vitro* studies. At pH 7.0 - 7.2 guanethidine did not evoke any cytotoxicity to cultured sympathetic ganglia, whereas in sealed plasma clot cultures (pH 7.8 - 8.0) it caused complete cell destruction even in the presence of NGF (Johnson and Aloe, 1974). Addition of insulin or glucose had also not altered the pH-dependent cytotoxicity in cultured chick sympathetic ganglia (Levi-Montalcini *et al.*, 1954). Thus it is apparent that elevating the pH of guanethidine can completely destroy the sympathetic ganglia. The mechanism by which sympathetic destruction occurs has been found to be primarily due to inhibition on mitochondrial respiration. Guanethidine adrenergic blockade has been found to inhibit oxidative phosphorylation in isolated liver mitochondria in rats (Malmiquist and Oates, 1968). The guanethidine treated cells were unable to generate ATP and sequester Ca^{2+} into mitochondria thereby elevating the Ca^{2+} into cytotoxic levels (Juul and Sand, 1973). It is reasonable to believe that at elevated pH levels, guanethidine causes complete cell destruction of the sympathetic neurons. Another possibility suggested is the increased uptake of guanethidine at elevated pH.

Gripenberg(1973) has shown that guanethidine incorporation into mast cells at pH 8.0 is 3-4 times greater than that at pH 7.2. The pH dependent cytotoxicity of guanethidine was clearly observable in the lizards treated at different pH levels. On the basis of available evidences it seems that, at higher pH levels guanethidine causes complete destruction of sympathetic ganglia as well as the inhibition of body metabolism through inhibition of mitochondrial respiration.

Previous experiment has been shown that adrenergic denervation positively influences the tail regeneration in lizards (chapter-III). 6-Hydroxydopamine-induced sympathectomy destroys only the terminal varicocities of the adrenergic fibers sparing the neurons intact, while guanethidine destroys neurons also. The pH-dependent effects further support these findings. Considering these antagonistic actions of the two drugs, it is likely that certain trophic molecules derived from the sympathetic ganglia may also contribute to the process of tail regeneration. Several neuropeptides have been localized within the sympathetic ganglia and their coexistence with the classical neurotransmitters are well known. Sympathetic ganglia has been found to contain networks of peptide immunoreactive fibers (reviewed by Hokfelt *et al.*, 1980). The role of several low molecular weight peptides in trophic activities during limb regeneration is postulated (Jabaily and Singer, 1978; Singer, 1978; review, Globus and Vethamany-Globus, 1985). The results of the present study is suggestive of the fact that sympathetic ganglia-derived trophic agents might be involved in promoting the process of tail regeneration.