CHAPTER - II

INFLUENCE OF NEUROTRANSMITTERS ON TAIL REGENERATION IN THE LIZARD, *HEMIDACTYLUS FLAVIVIRIDIS*

Considerable efforts have been made to identify the nature of 'trophic substance (s)' produced from nerve fibers during morphogenesis and regeneration. However, the identity of the neurotrophic factor is still unknown (Boilly and Albert, 1988). De Fazio (1968) suggested that the neural contribution to regeneration is related to adrenergic or cholinergic neurotransmitters at least in part. The functions of catecholamines (CA) are well studied in vertebrates (Usdin and Snyder, 1973). The CA neurotransmitters are modulators of a multitude of functions including endocrine regulation, stress and immune system functions (Usdin and Snyder, 1973; Frohman, 1980; Harvey *et al.*, 1984; Sanders and Munsen, 1985). Several lines of evidence support the premise that neurotransmitters have a role in morphogenetic movements and cell division (reviewed by McMohan, 1974). Epinephrine(E), Norepinephrine(NE) (Buznikov *et al.*, 1968), serotonin and acetylcholine (Buznikov *et al.*, 1964, 1972) have been detected in early sea urchin embryo and their concentrations change in association with various developmental stages. These molecules have been found to influence the regeneration of newt limbs both *in vivo* and *in vitro* (Taban *et al.*, 1977; Taban *et al.*, 1978) and it has been suggested that the action of CA neurotransmitters is through alterations in cyclic nucleotide metabolism. Fluctuations in cyclic AMP and cyclic GMP are recorded in cultured limb blastema of newts in response to NE stimulation (Taban *et al.*, 1977,1978). Histofluorescence studies have shown that CA are abundant within the regenerate (Taban *et al.*, 1978) and inhibition of tyrosine hydroxylase enzyme retards limb regeneration in the newt, *Notophthalmus viridescens* (Taban and Cathieni, 1988). The extent of cholinergic influence on limb regeneration in newts was investigated by Singer (1960) and found that cholinergic antagonists do not inhibit the process of limb regeneration at any stage.

Despite large number of reports available on neurotransmitter regulation during newt limb regeneration, such information is scanty on the tail regeneration in lizards. The neural influence on tail regeneration is well established. It is the spinal cord that supplies the trophic factor (s) during tail regeneration in lizards (Simpson, 1970). However, no experimental reports are available on the CA regulation during tail regeneration in lizards. The present experiment was designed to evaluate the effect of exogenous supply of catecholamine neurotransmitters - Epinephrine (E), Norepineph-

rine (NE) and cholinergic agent-acetylcholine (ACh) – on tail regeneration in the lizard, *Hemidactylus flaviviridis*.

MATERIALS AND METHODS

Experiment - 1. Adult *H.flaviviridis* weighing an average 10 ± 1 gm were procured locally and acclimated in the laboratory for a week. A total of 24 animals were used and they were divided into 4 groups of 6 each. Animals in each group were treated as follows:

Group-I : The animals received an ip. injection of epinephrine at a dosage of 500 µg/kg.body wt.

Group-II : The animals received an ip. injection of norepinephrine at a dosage of 500 µg/kg body wt.

Group-III : Animals were injected with 5 mg/kg body wt of acetylcholine chloride intraperitoneally.

Group-IV : These group of animals were served as control to the above groups and injected with 0.6% physiological saline only.

All drugs were prepared in 0.6% saline every day immediately before use and each drug was administered in a total volume of 0.05 ml per animal. The animals were fed with cockroaches 2-3 times a week and water was given daily. After 3 days of drug treatment autotomy was performed in all groups by pinching off the tail exerting mild thumb pressure keeping three segments intact from the vent. The growth in length of the tail was measured at fixed intervals and time taken to reach the different stages were recorded.

Experiment-2. Autotomy was performed in 70 lizards, *H.flaviviridis*(10 ± 1gm) and the regenerating animals were selected at two stages following the morphological criteria, (i) completion of wound healing and appearance of wound epithelium (WE stage) and (ii) animals at blastema stage (approximately 2 mm growth, BL stage). Only those animals which attained the above stages on the same day were selected and grouped.

Series A. Injection of E, NE and ACh at WE stage: Twenty seven lizards at WE stage were selected and divided into 4 groups of 5-6 animals each. These groups were treated as follows:

Group-I : Epinephrine 500 µg/kg body wt.
Group-II : Norepinephrine 500 µg/kg body wt.
Group-III: Acetylcholinechloride 5 mg/kg body wt.
Group-IV : 0.6% saline alone.

The drugs were prepared fresh in 0.6 % saline and injected intraperitoneally once daily for four days.

Series B : Injection of E, NE and ACh at BL stage. Twenty four lizards which attained the blastema stage on the same day were selected for the experiment. They were divided into 4 groups and treated as in Series A. The tail growth rate was measured at 48 hrs and 96 hrs after the first injection. The regenerate was evaluated histologically (details in chapter-I).

Statistical analysis: The data of the length of the tail regenerates were analysed by Student's 't' test at 95 % confidence limits.

RESULTS

Experiment-1. Results of the exogenous administration of E, NE and ACh are presented in Tables 1 & 2 and graphically represented in figs. 1 & 2. Supplementation of E and NE did not evoke any positive influence on tail regeneration in lizards. Epinephrine administration significantly retarded the process of morphogenesis resulting in 50 % tail growth inhibition at the end of 30 days. Administration of NE, though decreased the length of the tail at early stages, had no effect on the process of differentiation or morphogenesis. At the end of the experiment the NE treated animals exhibited only 13 % inhibition in tail growth with respect to control (fig.2.). Exogenous supply of ACh failed to influence the regenerative process. No significant differences were noticed between the control and ACh treated animals at any stage of regeneration.

Experiment-2. (i) *WE stage*: The results obtained on the growth of the regenerating tail

Table:1. Number of days taken to reach various regenerative stages in lizards after administration of various neurotransmitters.

Treatment	WH	BL	ED	MD	LD	GR
Control	5-6	7-9	10-13	14-17	18-25	26 onwards
NE	5-6	7-9	10-14	15-20	21-26	26 onwards
E	6-7	8-10	11-15	16-20	21-30	delayed
ACh	5-6	7-9	10-13	14-17	18-25	26 onwards

Table:2. Length of tail regenerated in lizards after treatment with various neurotransmitters. The average tail length (in mm) is represented as mean ± SD.

Days	CON	NE	E	ACh
		*	***	NS
10	5.6 ± 0.2	4.3 ± 1.2	3.0 ± 1.0	5.0 ± 0.9
		***	***	NS
15	15.6 ± 0.5	12.2 ± 1.6	9.6 ± 1.0	15.6 ± 1.2
		***	***	NS
20	23.5 ± 2.0	16.6 ± 1.2	15.0 ± 1.5	21.5 ± 1.5
		***	***	NS
25	26.8 ± 1.6	21.6 ± 1.2	17.8 ± 2.5	24.5 ± 1.5
		***	***	NS
30	28.6 ± 1.6	24.6 ± 1.4	19.5 ± 2.8	27.3 ± 1.4

P <0.05 ; *** P<0.001 ; NS-Nonsignificant

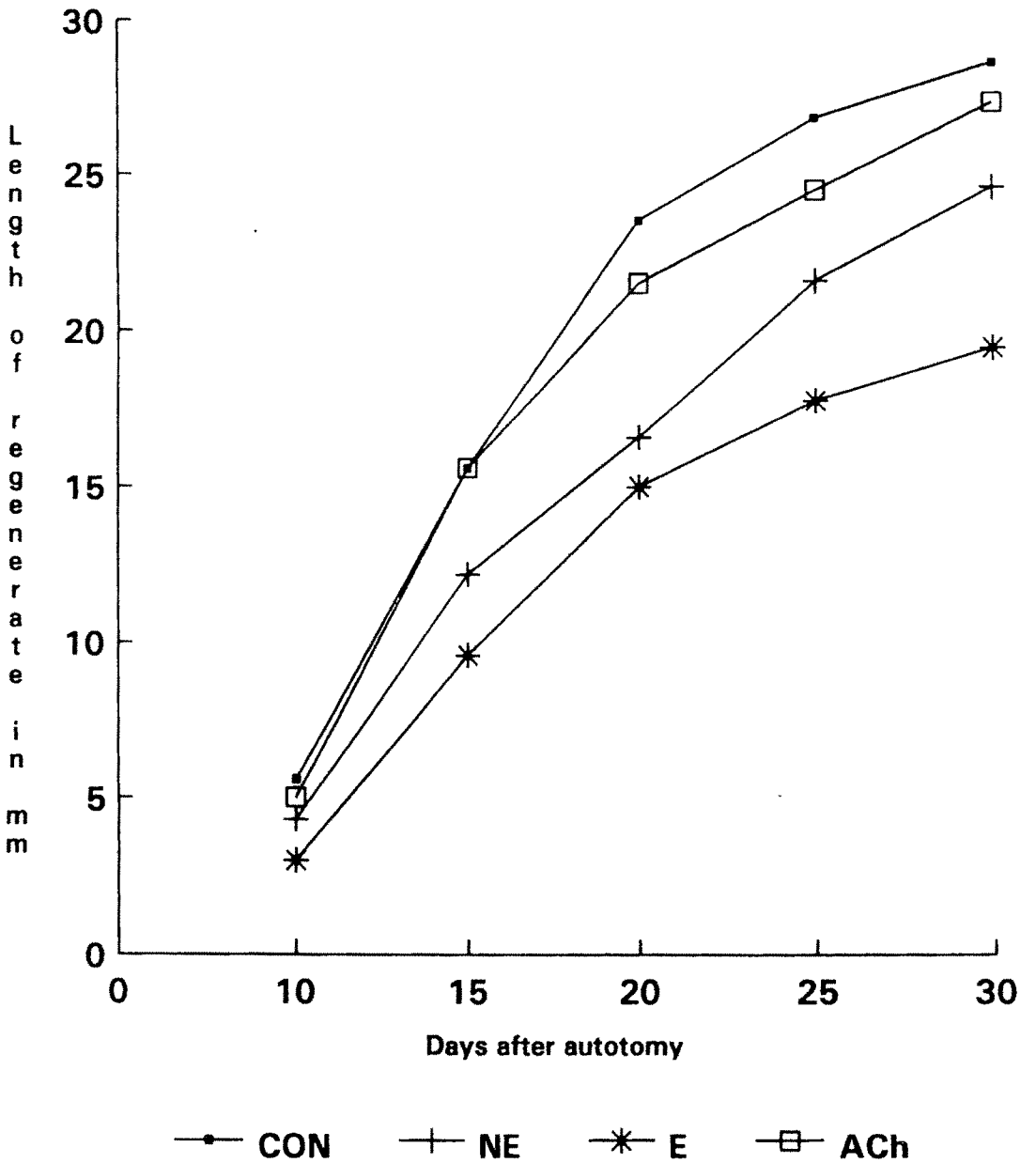

Fig.1. Length of tail regenerated in lizards after treatment with various neurotransmitters. The tail length is presented as mean +/- SD. N-6 animals in each group.

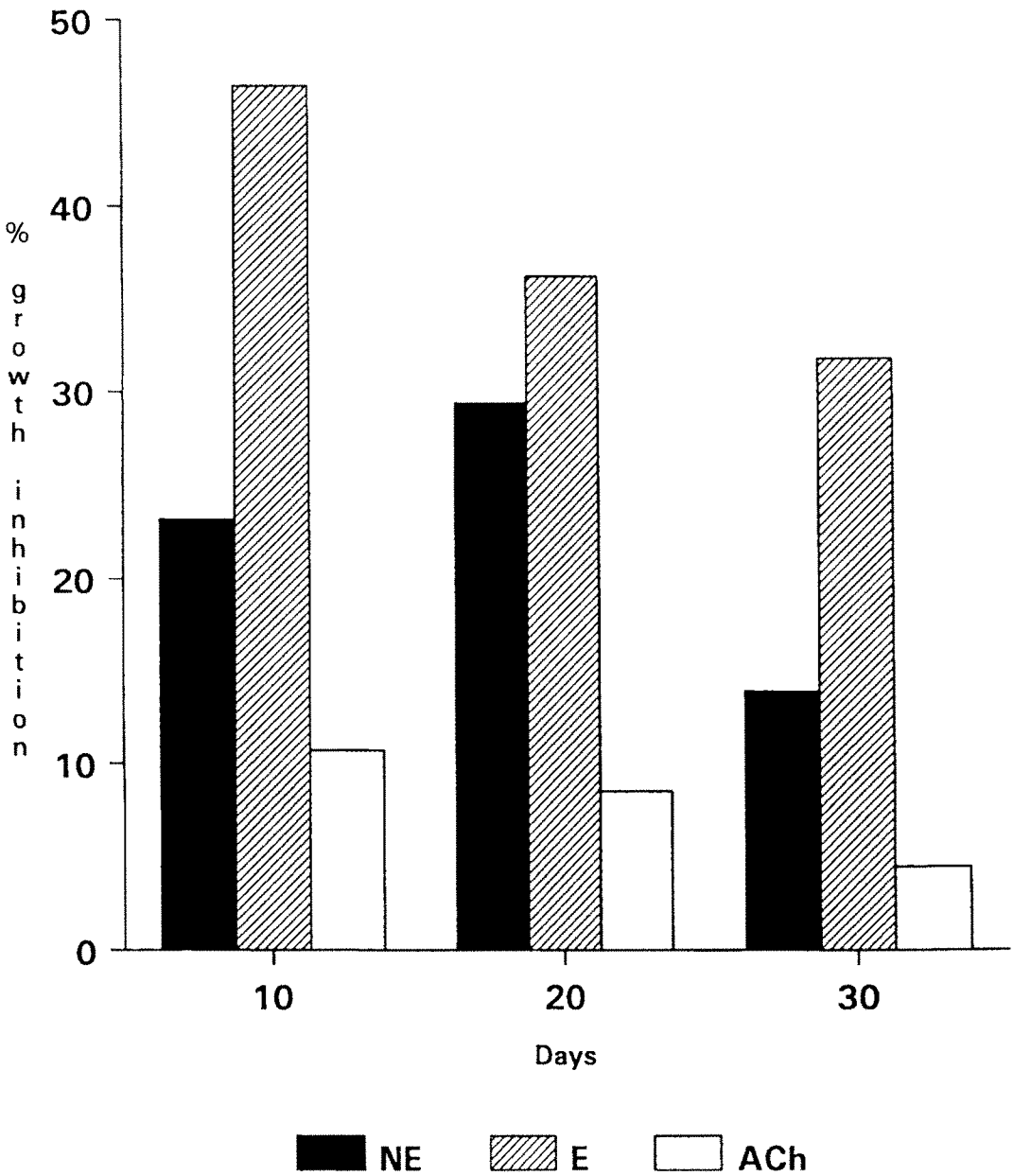

Fig.2. % of growth inhibition observed in tail regenerate in different treatment groups at 10 days of intervals.

Table :3. Effect of cholinomimetic agent Carbachol and cholinergic muscarinic antagonist Atropine on preblastemic (WE stage) and blastemic (BL) stages of tail regeneration in lizards. The tail growth was measured at 48 hrs and 96 hrs after treatments. The values are (in mm) represented as mean ± SD.

Treatment	Preblastemic stage		Blastemic stage	
	48 hrs	96hrs	48 hrs	96 hrs
Control	1.92 ± 0.20	3.92 ± 0.20	3.79 ± 0.56	5.64 ± 0.46
Carbachol	1.50 ± 0.44	3.66 ± 0.60	3.58 ± 0.49	5.41 ± 0.49
Atropine	0.83* ± 0.68	4.00 ± 0.63	3.14 ± 0.37	5.57 ± 0.98

* $P < 0.05$

Only Atropine treatment at 48 hrs in WE stage showed significant difference between the control values. All other treatment groups were nonsignificant (Analysis of Variance).

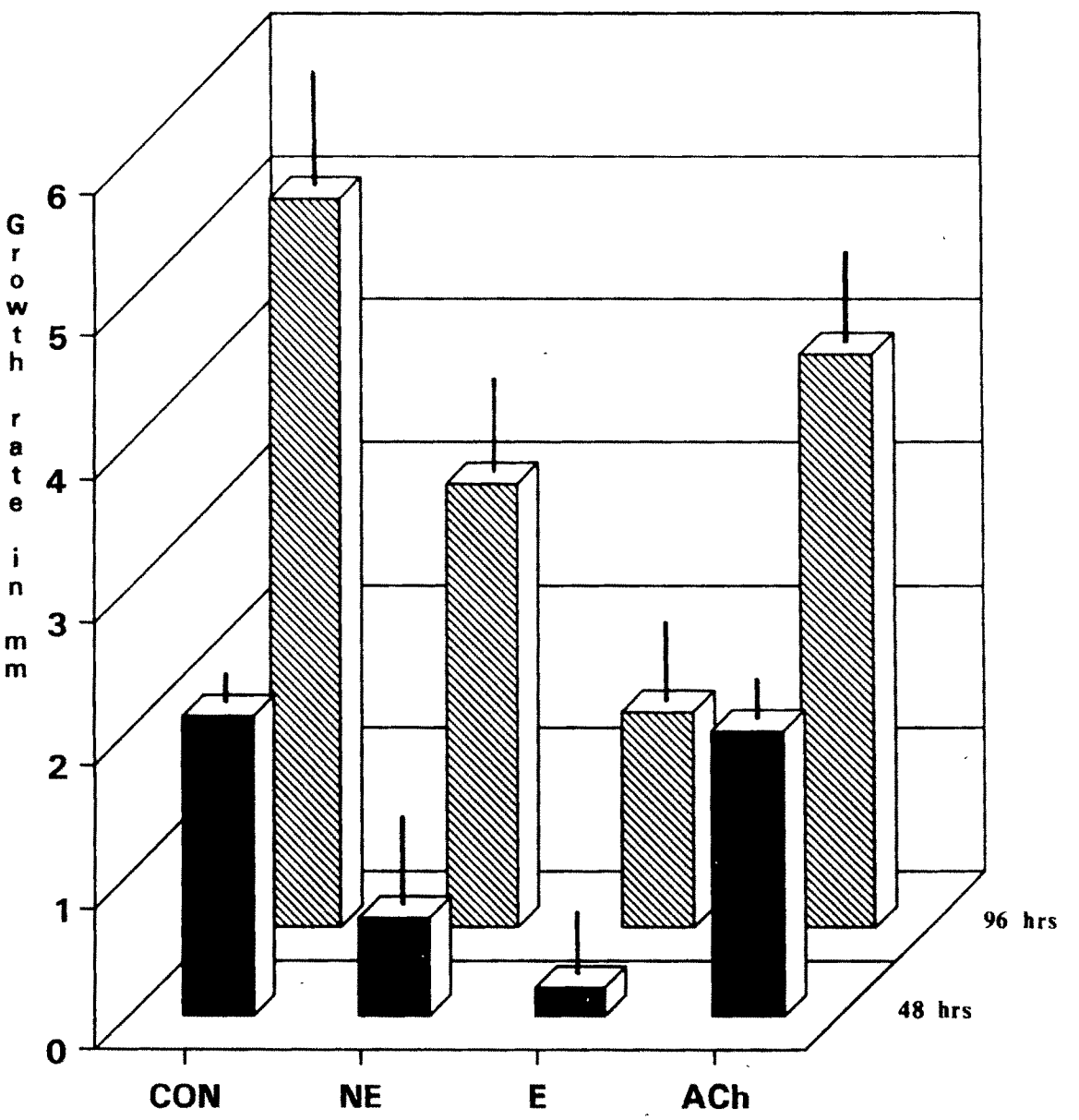

Fig.3a. Length of tail regenerated in lizards after treatment with NE, E and ACh at WE stage. Tail length was measured at 48 and 96 hrs. N-5-6 animals per group.

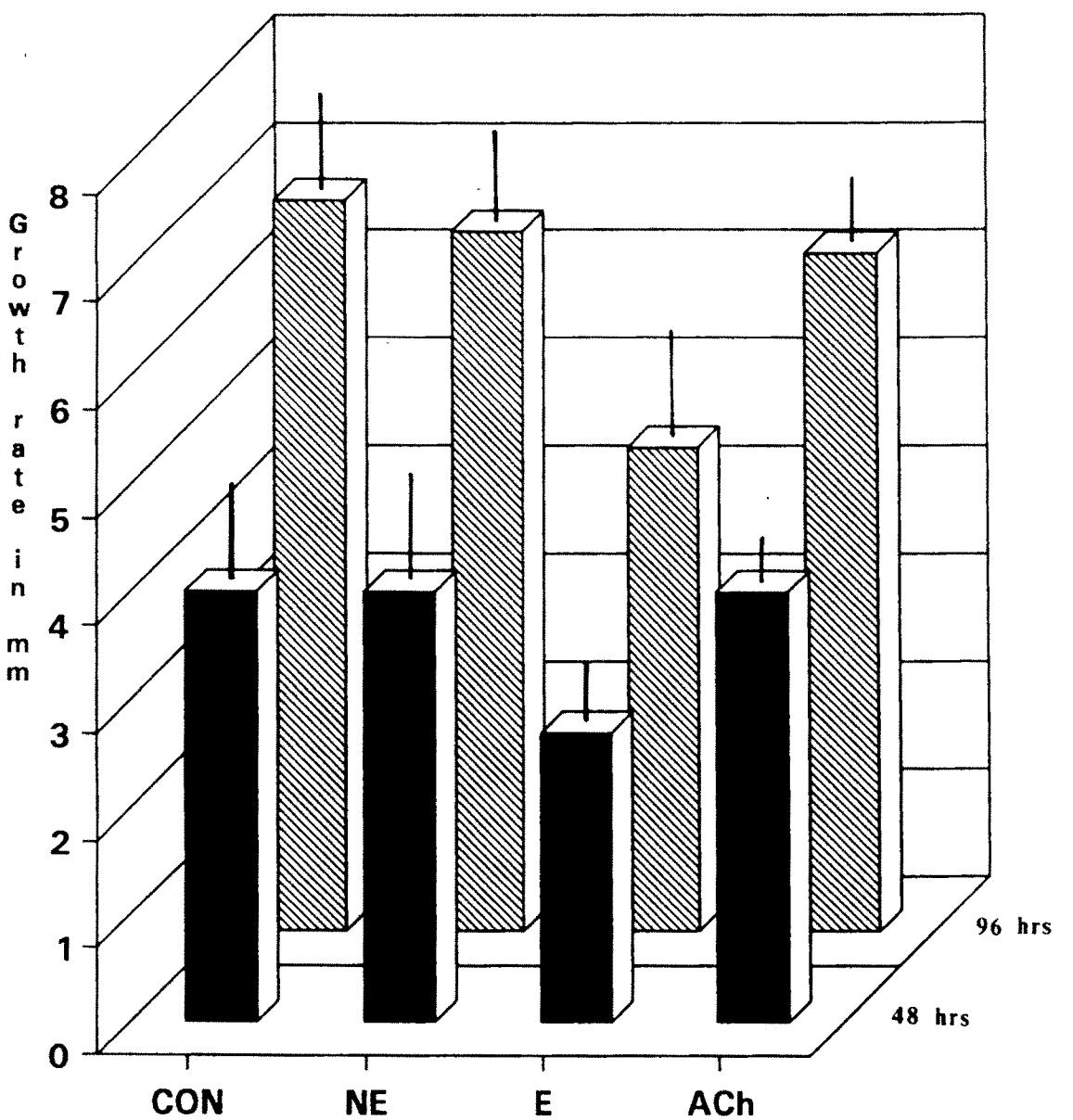

Fig.3b. Length of tail regenerated in lizards treated with NE, E and ACh. The tail length was measured at 48 and 96 hrs N-5-6 animals per group.

are presented in table-3 and fig 3. Supplementation of E at this stage significantly retarded the regeneration (fig.3). In E-treated animals, the process of dedifferentiation and blastema formation were greatly delayed. Norepinephrine treatment also retarded the growth of the regenerate; however, the regenerate showed signs of differentiation by 96 hrs. Injection of Ach had no effect on the growth.

(ii) *BL stage*: Epinephrine administration at BL stage adversely affected the regeneration. The rate of cell proliferation and differentiation were decreased in E-treated regenerate. In contrast, NE supplementation had no significant effect on the tail growth rate which reached nearer to the control value by 96 hrs. Acetylcholine chloride injection had no effect on the growth of the blastema.

DISCUSSION

Experiment-1. Exogenous administration of catecholamines and cholinergic agent does not have any positive influence on the tail regeneration in the gekkonid lizard, *H.flaviviridis*. Epinephrine supplementation resulted in a delay in wound healing, blastema formation and subsequently retarded the process of morphogenesis. Contrary to this, injection of NE had no such adverse effects, despite a small decrease in length of the tail at early stages. considering various metabolic effects of CA, the evaluation and interpretations of its *in vivo* effects needs utmost carefulness. However, some speculations can be advanced on the basis of known effects of E and NE. It has been found that long-term infusion of E in rats causes hyperglycaemia and increase in serum T_3 levels while NE infusion increases the serum insulin levels (Radu *et al.*, 1986). In the lizards *H.flaviviridis*, it has been noted that CA administration causes a persisting hyperglycaemia (*personal observation*). Similar observations were made in the anole lizard, *Anolis carolinensis* which always maintains a hyperglycaemia in blood (Rhoten, 1973). A persisting hyperglycaemia is known to cause several metabolic and hormonal variations in all vertebrate groups. The saurian pancreas is unique, which possesses relatively few islets with predominant number of α cells that secrete glucagon (Rhoten, 1973). A prolonged stimulation can exhaust α cells of the islets on one hand and suppress ß cells on the other hand. Such diabetogenic actions have also been induced by hormones such as thyroxine, prolactin, growth hormone and corticosteroids (Gorbman, 1958; Haist, 1959; Lazurus and Volk, 1962; Tepperman, 1965). In reptiles, the glycaemic level remained elevated for 1-3 days after glucose administration concomitantly, a biphasic

release of insulin also occurs similar to mammals (Rhoten, 1973). This is attributed to the difference in metabolism of glucose in reptiles. Another hormone which alters in hyperglycaemia is the growth hormone. Neuropharmacological studies have shown that infusion of NE and E into domestic fowl depresses the plasma GH levels through peripheral actions (Buonomo *et al.*, 1984).

The results obtained in the present experiments can be interpreted in the light of above observations. Considering the long-term administration of CA and the resultant increase in glycaemic levels, it is apparent that both insulin and growth hormone levels were altered. These hormonal insufficiency became more evident in the differentiating stages of the regeneration. In lizards, it has been observed that the hormonal dependence of the regenerate is pronounced during the differentiating stages (Licht and Howe, 1969). The observed delay in wound healing and blastema formation could also be due to the diabetogenic actions. The process of wound healing is known to be delayed in skin (Shah *et al.*, 1974a) and regenerating liver (Shah *et al.*, 1974b) of diabetic rats. A direct local action of E and NE in feather regeneration of pigeon is reported recently (Pilo and Patil, 1992). Topical application of E in regenerating feather tracts completely inhibited the regeneration while NE had no such inhibitory effects. These results are in direct agreement with the current observations. The divergence in the action of E and NE could be due to the difference in density of receptor population.

Increasing the cholinergic influence with administration of acetylcholine had no effect on the process of tail regeneration in lizards. In regenerating newt limbs, it has been found that anticholinergic drugs do not inhibit the regeneration (Singer, 1960; Drachman and Singer, 1971). Thus it can be concluded that cholinergic influence is negligible during tail regeneration in lizards.

Experiment-2. The second series of experiments were conducted to elucidate the mechanism of action of neurotransmitters at certain crucial events of tail regeneration in lizards. The appearance of wound epithelium initiates the remarkable process of dedifferentiation which results in accumulation of critical mass of blastemal cells. These blastemal cells further proliferate to express the differentiated phenotypes. Augmenting or depressing the neurotransmitter levels at these stages can delineate some of their functions. Epinephrine supplementation both at WE and BL stages produced inhibitory effects on the regeneration process. The anti-cell-proliferative effect of E was more visible at the WE stage. Injection of E at BL stage resulted in decreased cell proliferation

and impairment in differentiation. Contrary to this, NE administration at BL stage had no adverse effect on cell proliferation or differentiation.

It is suggestive that the proliferation-differentiation stages of the limb regeneration is determined by neurotransmitters in the form of CA and fluctuations in cyclic nucleotide levels (Sicard,1983). In addition, endocrine system contributes to cell proliferation during regeneration (Tassava and Mescher, 1975; Vethamany-Globus *et al.*, 1978). Catecholamine neurotransmitters are known to elevate the cyclic AMP levels and in several developing and regenerating systems, cyclic AMP enhances the process of differentiation (Globus and Vethamany-Globus *et al.*, 1978; Solurosh *et al.*, 1978). Increased cyclic AMP levels are reported in differentiating newt limbs (Sicard, 1975). Thus it appears that increasing NE levels during tail regeneration influences the proliferation of blastemal cells and their differentiation. Norepinephrine is also known to enhance protein synthesis in cultured newt blastemata (Rathbone *et al.*, 1980). Another metabolic effect of NE is on the glycaemic level and a corollary increase in the release of insulin from the ß cells of islets. An increase in insulin level highly favours cell proliferation and differentiation. In newt limb regeneration, insulin is proposed as one among the 'tripartiate control system' which regulate cell proliferation and differentiation (Liversage *et al.*, 1985). Addition of insulin both *in vivo* and *in vitro* augmented DNA synthesis (Vethamany-Globus *et al.*, 1978). Insulin augments the RNA polymerase activity in cultured mammary epithelial cells (Lockwood *et al.*, 1967) and liver of diabetic rats (Younger *et al.*, 1966). It is highly likely that increase in insulin coupled with the cyclic AMP levels promoted the proliferation and differentiation in NE-supplemented tail regenerates. However, increasing the NE levels during WE stage had an inhibitory effect, which may be due to the change in sensitivity of regenerating cells to neurotransmitters. Sicard (1983) hypothesized that CA desensitization takes place during early stages of regeneration and thus dedifferentiation occurs independent of CA. The current observations are in support of this view. It is suggestive that the sensitivity to CA neurotransmitters changes in accordance with the different regenerating stages. Dedifferentiation proceeds in a low CA milieu and independent of hormonal influence while further proliferation and differentiation depend upon increasing CA levels coupled with the hormones during tail regeneration. Eventhough E could increase the cyclic AMP levels and glycaemia at all stages, the regeneration found to be inhibited. Similar inhibitory action of E is reported in feather regeneration in pigeons (Pilo and Patil, 1992). It is speculative that the inhibitory effects are result of a direct action of E on cell

proliferation. The intriguing mechanisms of E action remain unresolved. Increasing the cholinergic actions at these critical stages of regeneration had no effect on tail regeneration. These findings overrule the possibility of cholinergic influence on the tail regeneration in lizards.