CHAPTER - V

ADRENERGIC AND CHOLINERGIC RECEPTOR FUNTIONS DURING TAIL REGENERATION IN THE LIZARD, *HEMIDACTYLUS FLAVIVIRIDIS*

Ahliquist in 1948 found that the catecholamine (CA) neurotransmitters act through specific receptors and termed them as α and ß receptors. His observations were further confirmed by the development of drugs which specifically antagonises α or ß-receptors. Later on it was identified that there are subtypes in these receptors (Lands *et al.*, 1967; Langer, 1974; Berthelsen and Pettinger, 1977). The mechanism involved in the activation of α-receptors by CA are not well understood while those of ß-receptors are studied in detail. The activation of ß-receptors leads to an increase in the cyclic AMP levels (Sutherland and Rall, 1960). In several developing systems it has been found that the agonists or antagonists of α- and ß-receptors evoke varying responses; either retard or enhance the process of development. In sea urchin embryos, Epinephrine(E) acts through the receptors and increase cyclic AMP levels while antagonists are found to inhibit these effects (Gustafson and Toneby, 1970; Thompson and Williams, 1970; Zurier *et al.*, 1973). Apart from CA neurotransmitters, acetylcholine is also found to influence certain developmental events. Cholinergic antagonists inhibited the developmental events of sea urchin embryo (Gustafson and Toneby, 1970), The role of CA has been studied in newt limb regeneration both *in vivo* and *in vitro* (Taban *et al.*, 1977, 1978; Taban and Cathieni, 1988). It has been found that NE specificity changes occur during limb regeneration in *Notophthalmus viridescens* with corresponding fluctuations in both cyclic AMP levels, acting through the ß-adrenoreceptors (Taban *et al.*, 1978). They hypothesized that fluctuations in both cyclic AMP and cyclic GMP levels are in accordance with trophic stimulations through nerves and implicated NE as trophic agent. Rathbone *et al.* (1980) opposed these views as the inability of NE to stimulate the mitotic index and protein synthesis in newt blastemata. However, a regulatory role for CA in the process of morphogenesis of the regenerating limb is suggestive. Acetylcholine supplementation or inhibition of cholinoreceptors does not affect the process of limb regeneration in newts (Singer, 1960). Sicard and DiNicola (1974) demonstrated a reduction in the growth of limb regenerate in *Notophthalmus viridescens* treated with cholinergic antagonist atropine.

The literature available on the adrenergic and cholinergic regulation of tail regeneration in lizards is relatively poor and particularly unattempted in the tropical lizard *Hemidactylus flaviviridis*. The present experiment is designed to elucidate both

adrenergic and cholinergic influence on crucial events (preblastemic and blastemic phases) of the regenerating tail of lizard *H.flaviviridis*. Specific α-receptor antagonist benextramine and ß-receptor blocker propranolol were used either alone, together or in combination with E or NE to study the adrenoreceptor mechanisms involved in the promotion of tail regeneration in lizards. The cholinergic mechanisms were evaluated by the use of cholinomimetic agent, carbachol and cholinergic muscarinic antagonist, atropine.

MATERIALS AND METHODS

As many as 275 adult wall lizards *H.flaviviridis* of both sexes (body wt. 10 ± 2 gms) were procured from animal dealer and caged in the laboratory for a week. The animals were maintained at temperature 30 ±2 °C fed with cockroaches 2-3 times a week and water was given daily.

Experiment-1. *Series-A* : Eighty lizards of both sexes were autotomised and allowed to regenerate. The animals which attained the preblastemic stage (wound epithelium) on the same day were selected and divided into 10 groups comprising 5-6 animals per group. The lizards in each group were treated as follows. Each drug was injected intraperitoneally in two equal doses daily at 9.00 hrs and 17.00 hrs for 4 days, 24 hrs apart. The drugs were prepared in 0.6% saline (details in chapter-I).

Groups	**Treatment**	**Dosage mg/kg body wt**
I	Epinephrine	500 µg
II	Norepinephrine (NE)	500 µg
III	Propananol (PR) (ß-blocker)	5 mg
IV	Benextramine (BE) (_-blocker)	5 mg
V	Propranolol + Benextramine (PR + BE)	5mg + 5 mg
VI	Epinephrine + Propranolol (E + PR)	500µg + 5mg
VII	Epinephrine + Benextramine (E + BE)	500µg + 5mg
VIII	Norepinephrine + Propranolol (NE+PR)	500µg + 5mg
IX	Norepinephrine + Benextramine (NE+BE)	500µg + 5mg
X	Control (CON)	0.6% saline

All drugs were injected in a total volume of 0.05 ml/animal. The growth of the tail was measured at intervals 48 hrs, 96 hrs post-injection and evaluated histologically.

Series-B : 80 lizards of both sexes were autotomised and allowed to regenerate. The animals which attained the blastema stage (average length of regenerate 2 mm) only were selected and caged separately as 10 groups of 5-6 animals each. The animals were treated with ip.dosages of drugs or vehicle as in experiment-1 series-A given for 4 days, 24 hr apart. The length of the tail regenerated was measured at 48 hrs and 96 hrs.

Experiment. *Series-A*: Eighteen lizards at the preblastemic stage of regenertion were selected and divided into 3 groups of 6 each and treated as follows.

Group-I : The animals were injected with Atropine sulphate (National chemicals) at a dosage of 2 mg/kg body wt. intraperitonially for 4 days 24 hrs apart.

Group-II : Animals were given an ip. dosage of Carbachol (National Chemicals) at a dosage of 500 µg/kg body wt. for 4 days 24 hrs apart.

Group-III: The animals of this group served as control to the above groups receiving 0.6% saline.

Series-B : Twenty lizards which attained the blastema stage on the same day were divided into groups I, II (7 animals each) and III (6 animals). Each group was treated with atropine and carbachol in the same manner as in *series-A* for 4 days 24 hrs apart. The tail growth was measured at 48 hrs and 96 hrs in both groups.

Statistical Analysis : The data obtained on the tail length were subjected to one way analysis of variance followed by Duncan's multiple range test at 0.05 level of significance.

RESULTS

Experiment-1. *Series-A* : The length of tail regenerated in each treatment group and statistical analyses of the significance between each group are presented in table-1. and fig. 1. Epinephrine and NE supplementation significantly retarded the onset of blastema.

Table:1. Length of tail regenerated in lizards after treatment with adrenergic antagonists alone, together or in combination with agonists at preblastemic level (WE stage). The growth of the regenerates were measured at 48 hrs and 96 hrs after treatment and presented (in mm) as mean ± SD.

Treatment	Length of tail regenerate	
	48 hrs	96 hrs
Control	2.0 ± 0.00	5.30 ± 0.54
Norepinephrine	0.83 ± 0.68	2.92 ± 0.66
Epinephrine	0.17 ± 0.41	1.42 ± 0.58
Propranolol	1.7 ± 0.67	3.7 ± 0.44
Benextramine	2.3 ± 0.45	4.7 ± 0.65
Norepinephrine + Propranolol	3.08 ± 0.38	6.08 ± 0.66
Norepinephrine + Benextramine	3.33 ± 0.82	7.75 ± 1.77
Epinephrine + Propranolol	3.58 ± 0.49	7.08 ± 0.92
Epinephrine + Benextramine	3.42 ± 0.49	6.5 ± 1.05
Propranolol + Benextramine	2.2 ± 0.27	5.09 ± 1.97

Table:1 a & b. Analysis of growth rate in regenerating tail of lizards recorded at 48 hrs (a) and 96 hrs (b) after treatment with adrenergic antagonists alone,together or in combination with agonists at preblastemic level. The data were analysed by one way analysis of variance followed by Duncan's multiple range test at 0.050 significance level. * represents pair of groups significantly different at the 0.050 level.

(a) 48 hrs

	E	NE	PR	CON	PR+ BE	BE	NE+ PR	NE+ BE	E+ BE	E+ PR
E										
NE	*									
PR	*	*								
CON	*	*								
PR + BE	*	*								
BE	*	*								
NE + PR	*	*	*	*	*	*				
NE + BE	*	*	*	*	*	*				
E + BE	*	*	*	*	*	*				
E + PR	*	*	*	*	*	*				

(b) 96 hrs

	E	NE	PR	BE	PR+ BE	CON	NE+ PR	E+ BE	E+ PR	NE+ BE
E										
NE	*									
PR	*									
BE	*	*	*							
PR + BE	*	*	*							
CON	*	*	*							
NE + PR	*	*	*	*	*					
E + BE	*	*	*	*	*	*				
E + PR	*	*	*	*	*	*	*			
NE + BE	*	*	*	*	*	*	*	*		

E-Epinephrine; NE-Norepinephrine; PR-Propranolol;
CON - Control; BE-Benextramine.

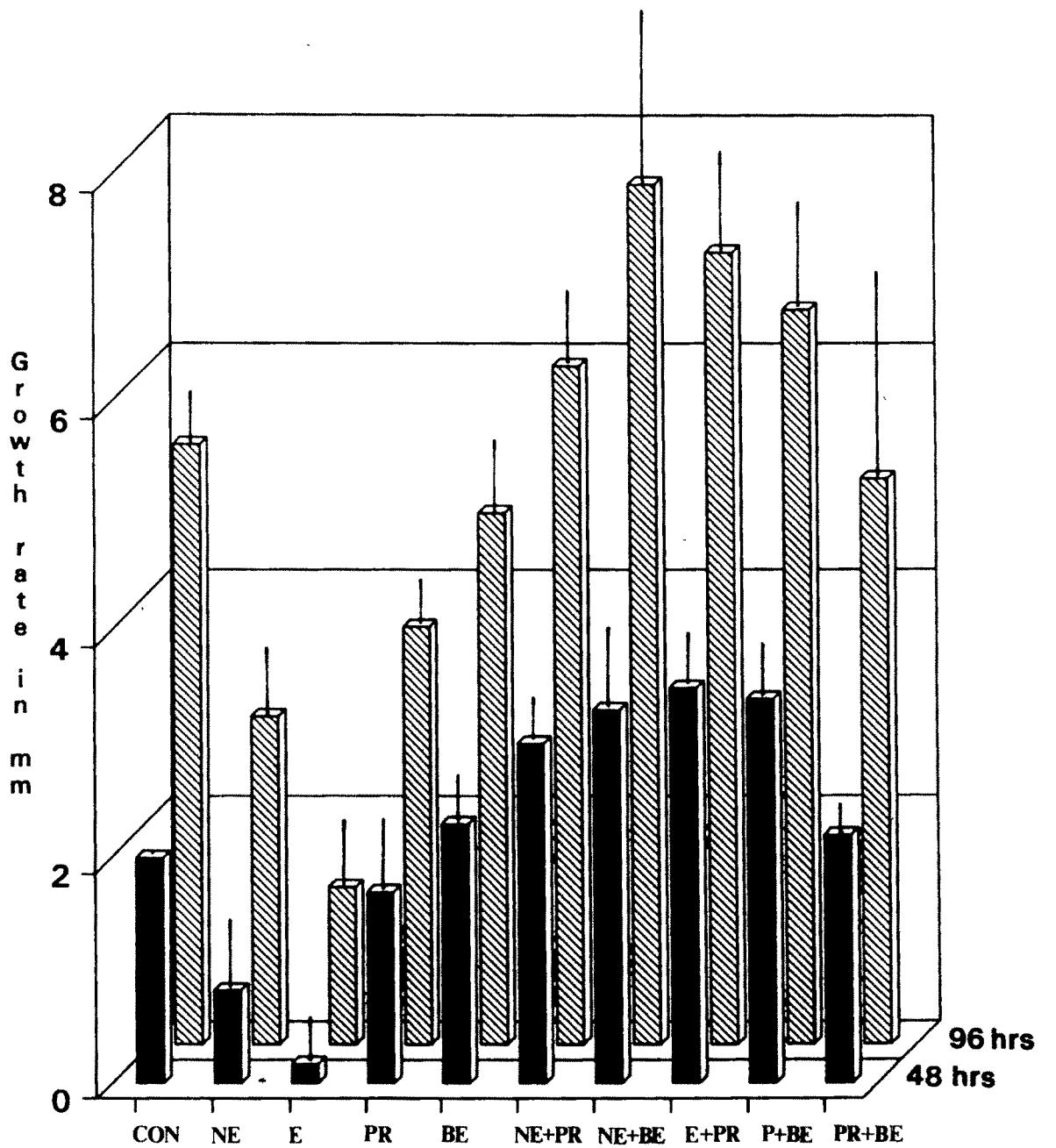

Fig.1. Length of tail regenerated in lizards after treatment with adrenergic antagonist alone, together or in combination with agonists at WE stage. N-5-6 animals per group.

Table:2. Length of tail regenerated in lizards after treatment with adrenergic antagonists alone, together or in combination with agonists at blastemic phase (BL stage). The growth of the regenerates were measured at 48 hrs and 96 hrs after treatment. The tail growth is presented (in mm) as mean ± SD.

Treatment	Length of tail regenerate	
	48 hrs	96 hrs
Control	5.0 ± 0.53	7.60 ± 0.69
Norepinephrine	4.00 ± 1.00	6.80 ± 1.30
Epinephrine	2.30 ± 0.57	4.60 ± 1.14
Propranolol	3.70 ± 0.67	5.80 ± 0.57
Benextramine	3.90 ± 0.89	6.10 ± 0.89
Norepinephrine + Propranolol	5.08 ± 0.80	7.33 ± 1.21
Norepinephrine + Benextramine	5.08 ± 0.80	8.33 ± 0.82
Epinephrine + Propranolol	4.58 ± 0.49	8.16 ± 1.32
Epinephrine + Benextramine	5.00 ± 0.32	8.08 ± 0.80
Propranolol + Benextramine	3.50 ± 0.50	6.60 ± 0.89

Table:2 a & b. Analysis of growth rate in regenerating tail of lizards recorded at 48 hrs (a) and 96 hrs (b) after treatment with adrenergic antagonists alone, together or in combination with agonists at blastemic stage. The data were analysed by one way analysis of variance follwed by Duncan's multiple range test at 0.050 level. * represents pair of groups significantly different at the 0.050 level.

(a) 48 hrs

	E	PR+ BE	PR	BE	NE	E+ PR	CON	E+ BE	NE+ PR	NE+ BE
E										
PR + BE	*									
PR	*									
BE	*									
NE	*									
E + PR	*	*								
CON	*	*	*	*	*					
E + BE	*	*	*	*	*					
NE + PR	*	*	*	*	*					
NE + BE	*	*	*	*	*					

(b) 96 hrs

	E	PR	BE	PR+ BE	NE	NE+ PR	CON	E+ BE	E+ PR	NE+ BE
E										
PR										
BE	*									
PR + BE	*									
NE	*									
NE + PR	*	*								
CON	*	*	*							
E + BE	*	*	*	*						
E + PR	*	*	*	*	*					
NE + BE	*	*	*	*	*					

E-Epinephrine; NE-Norepinephrine; PR-Propranolol;
CON - Control; BE-Benextramine.

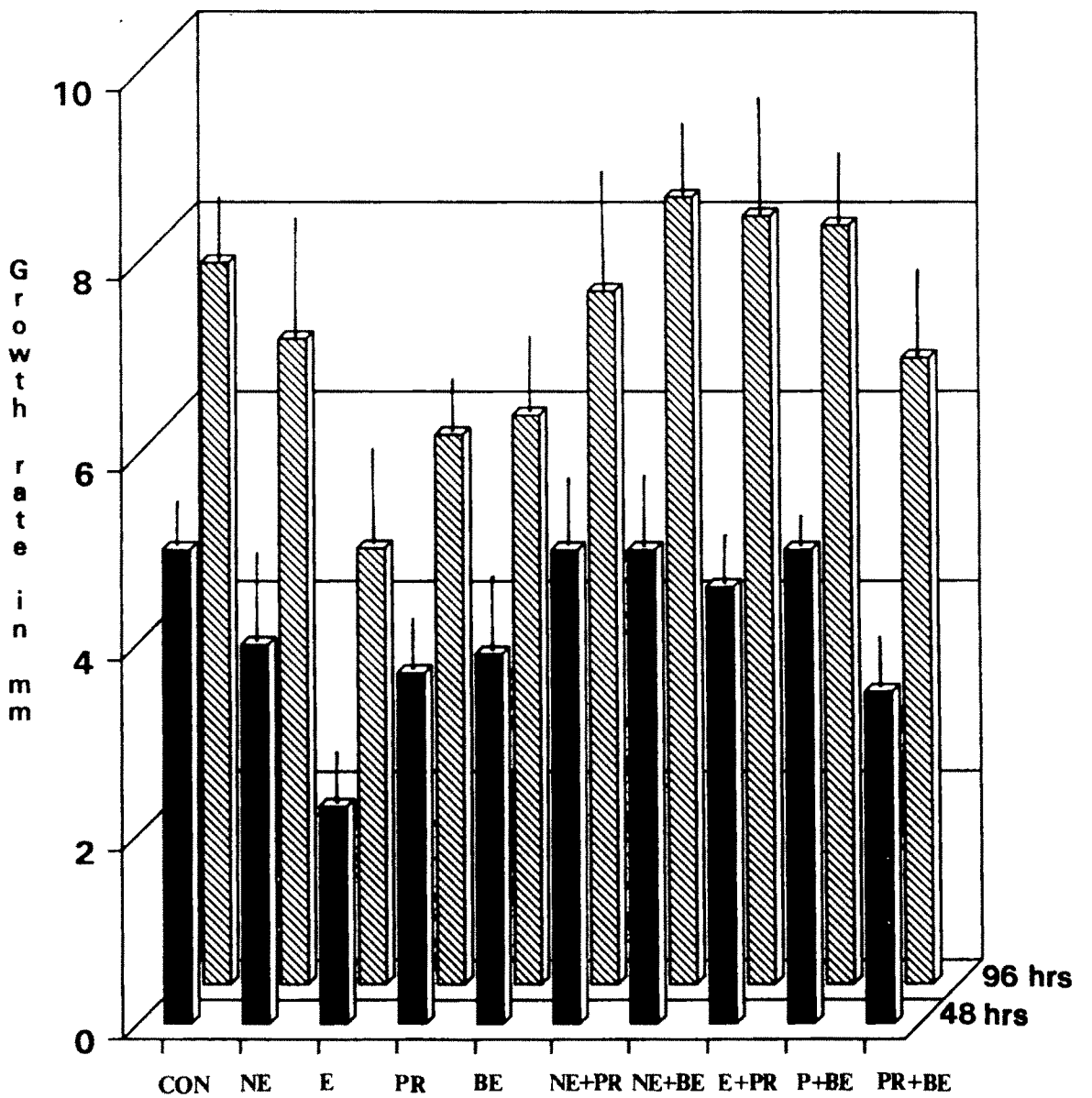

Fig.2. Length of tail regenerated in lizards treated with adrenergic antagonists alone, together or in combination with agonists in BL stage. N-5-6 animals per group.

Table:3. Length of tail regenerated in lizards after administration of neurotransmitters at two specific stages (preblastemic and blastemic) of regeneration. The tail length was measured at 48 hrs and 96 hrs after treatment in each group. The average tail length (in mm) is represented as mean ± SD.

Treatment	Preblastemic stage (WE stage)		Blastemic stage (BL stage)	
	48 hrs	96hrs	48 hrs	96 hrs
Control	2.1 ± 0.22	5.1 ± 0.89	4.0 ± 0.89	6.8 ± 0.81
	***	***	NS	NS
NE	0.7 ± 0.67	3.1 ± 0.74	4.0 ± 1.0	6.5 ± 0.89
	***	***	***	NS
E	0.2 ± 0.45	1.5 ± 0.61	2.75 ± 0.61	4.5 ± 1.04
	NS	NS	NS	NS
ACh	2.0 ± 0.31	4.0 ± 0.70	4.08 ± 0.37	6.3 ± 0.61

*** P<0.001 ; NS-Nonsignificant

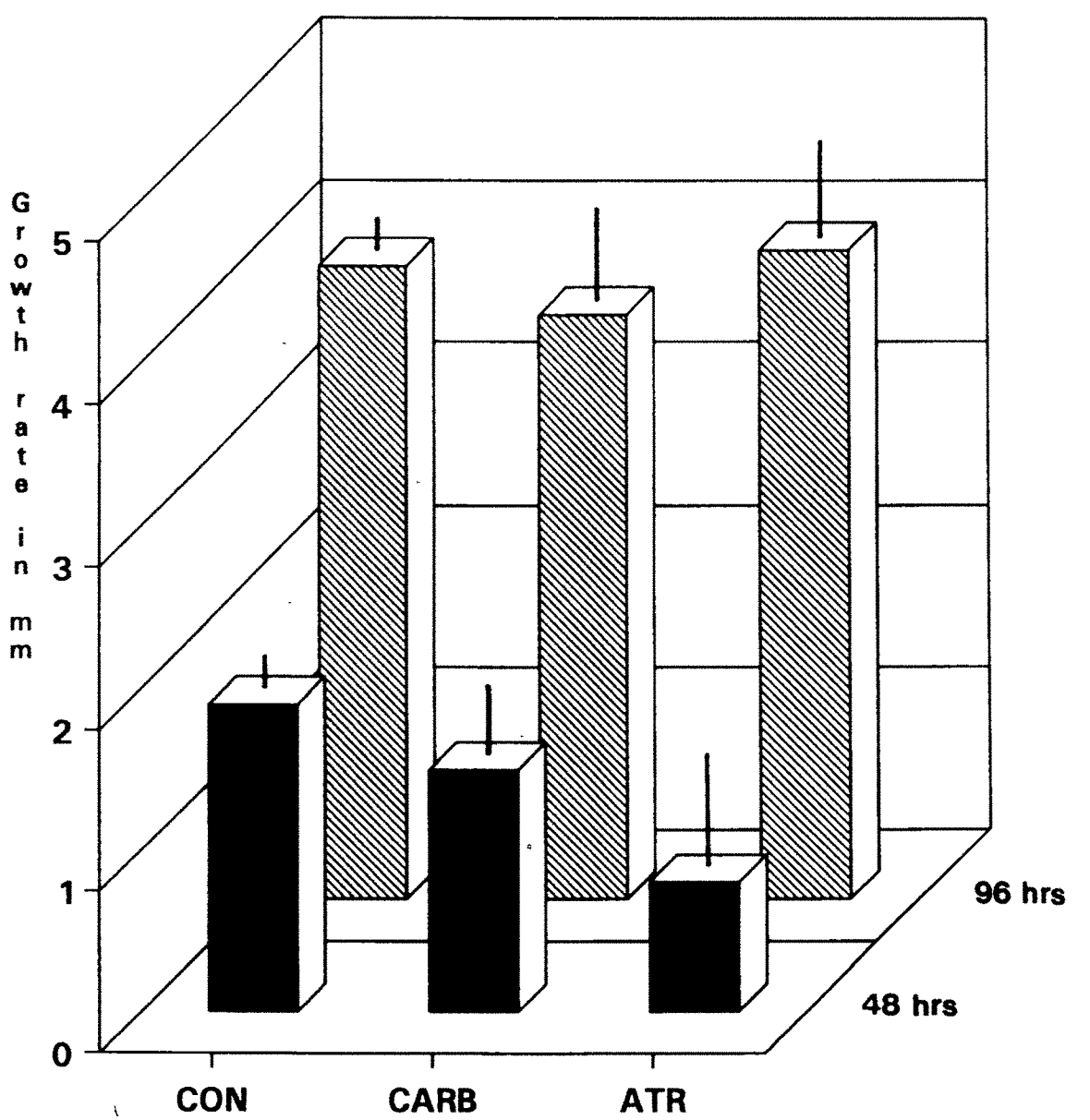

Fig.3a. Length of tail regenerated in lizards treated with carbachol and atropine at WE stage. N-groupI & II-7 each, groupIII-6animals.

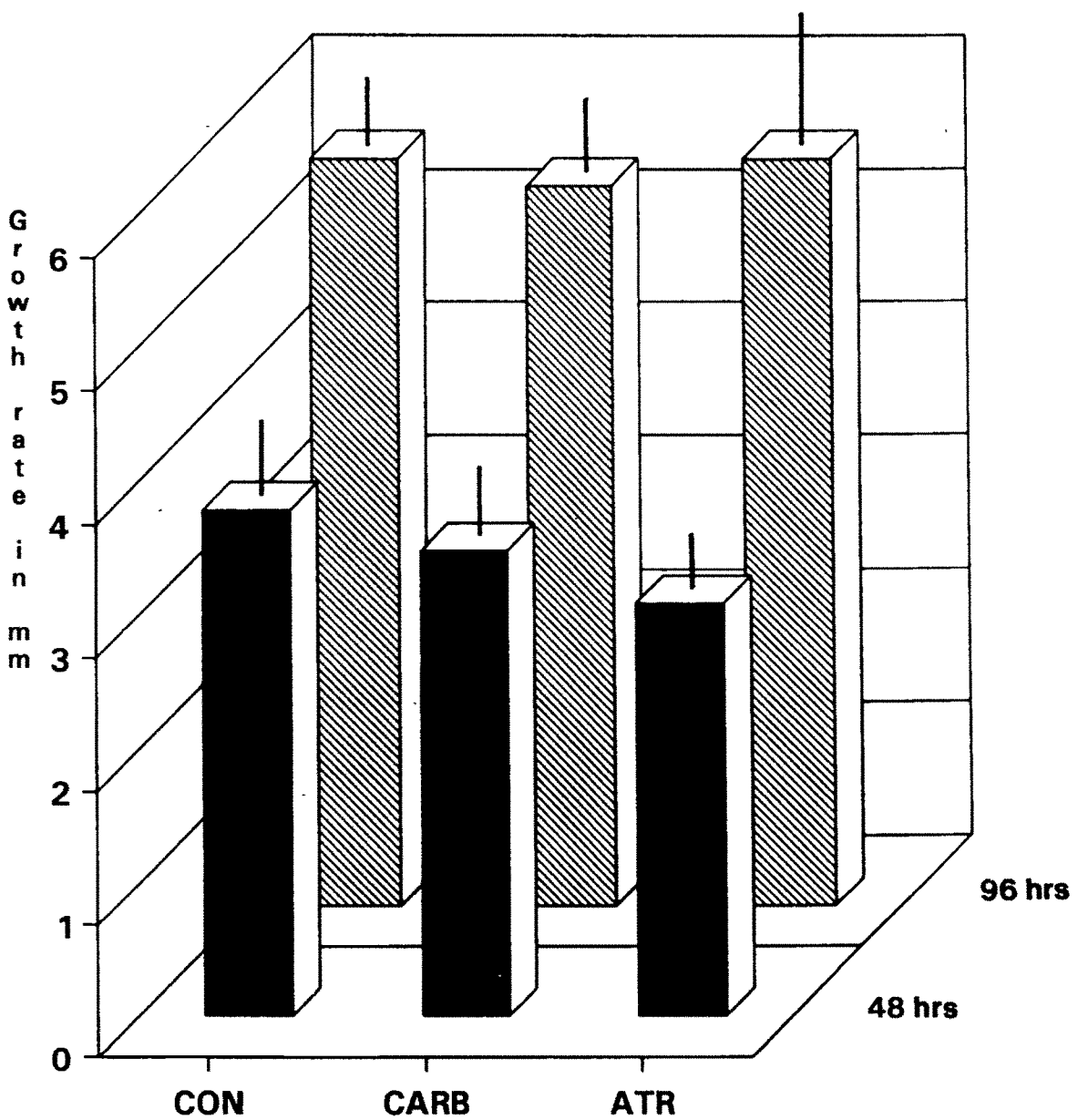

Fig.3b. Length of tail regenerated in lizards treated with carbachol and atropine at BL stage. N-groupI & II-7 each, groupIII-6 animals.

Propranonol (PR) and Benextramine (BE) had no apparent effect on blastema formation and further growth. Treatment with Norepinephine + Propranonol, Norepinephrine + Benextramine, Epinephrine + Propranonol and Epinephrine + Benextramine (groups 6,7,8 and 9) all increased the growth rate of the regenerate at marginal or significant level with respect to control when measured at 48 hrs and 96 hrs. α and ß blockers together had no significant effect on blastema formation. Histological features revealed inhibition of cell proliferation and differentiation of the regenerate in E and NE treated animals. All other treatment produced a normal viable regenerate.

Series-B: The data on the length of the tail regenerated at 48 hrs and 96 hrs are given in table - 2 and graphically depicted in fig.2. Treatment with epinephrine, norepinephrine, propranonol and benextramine (groups 2,3,4 and 5) significantly decreased the growth of the blastema till 48 hrs. By 96 hrs only E, PR and BE treated animals showed significant decrease in growth compared to control. Histological examination of tail revealed a normal regenerate in all groups except with E treatment. In E treated animals the process of differentiation was found to be retarded. Supplementation of NE or E along with α or ß-receptor blockers were nonsignificantly increased the tail growth (groups 6,7 8 and 9).

Experiment-2. The length of the tail regenerated in different groups of animals in both series A & B are presented in table-3 and graphically shown in figs. 3a & b. Cholinergic antagonist atropine suppressed the process of dedifferentiation and blastema formation. This inhibition in cell proliferation was observable till the blastema formation. Once the critical mass of blastemal cells accumulated, no inhibitory effects were observable on growth and differentiation of the regenerate. Administration of carbachol had no significant effects on the regenerative process. Inhibiting cholinergic effects or enhancing the cholinergic actions during blastema stage did not affect the tail growth or the process of differentiation.

DISCUSSION

The results from the present experiments confirm the previous observation (chapter-II) that CA have adverse influence on many of the process of tail regeneration in lizards. Epinephrine administration decreased the cell proliferation and differentiation. Al-

though NE supplement delayed the blastema formation it did not evoke any inhibitory effect on the differentiating stages. These evidences overrule the possibility of NE as modulators of cell proliferation and differentiation during tail regeneration.

α- and ß- adrenoreceptor blocker alone or together with agonists had no inhibitory actions in dedifferentiation and blastema formation. However, addition of NE or E with α- and ß- receptor blockers at preblastemic level significantly increased the growth of the regenerate while addition of these agents in blastemic phase evoked only a marginal increase in the growth rate. It is unlikely that α- and ß- adrenergic effects act alone in any stages of regeneration (Rathbone *et al.*, 1980). The noted inhibitory effect of NE and E may be primarily due to the inhibitory effects of CA neurotransmitters acting through both α- and ß- adrenoreceptors.

Altogether different responses are obtained by blockade of adrenoreceptors in blastemic stage. Both α- and ß-adrenergic antagonists significantly decreased the growth of the regenerate but the process of differentiation was unaffected. Also NE and E together with adrenoreceptor blockers increased the growth rate of the regenerate at non-significant level. Addition of NE with ß- receptor antagonist significantly increased the protein synthesis in cultured newt blastema which is attributed to increase in adrenergic effect; when adrenergic actions are blocked, protein synthesis is not inhibited (Rathbone *et al.*, 1980). The present observations are in line with above findings. No appreciable difference could be observed after administration of α- or ß-adrenoreceptors either with NE or E in any stages of regeneration.

It is highly likely that a change in receptor sensitivity occurs during various stages of tail regeneration. This is observable from the fact that blockage of both adrenoreceptors had no effect on dedifferentiation and blastema formation while the adrenoreceptor antagonists decreased the growth rate from blastemic stage. The observed decrease in the growth rate could be a more general metabolic alteration within the regenerate as the differentiating stages are more influenced by neuronal and hormonal factors (Licht and Howe, 1969; Turner and Tipton, 1971; Liversage *et al.*, 1985). The adrenoreceptor mechanisms involved in mediating the tail regeneration require further emphasize and detailed investigation.

The cholinergic influence in limb regeneration of newts has been studied by several investigators. Experiments have shown that inhibition of cholinergic actions by atropine

or botulinum toxin does not inhibit the process of limb regeneration in newts (Singer, 1960; Drachman and Singer, 1971). However, Sicard and DiNicola(1974) observed impairment in morphogenesis of newt limbs treated with atropine. The previous experiment (chapter-II) has shown that acetylcholine administration does not enhance the process of tail regeneration in lizards. In the present experiment also, cholinomimetic agent carbachol failed to elicit positive influence in any of the crucial events in tail regeneration. Thus it can be concluded that cholinergic influence is negligible in the process of tail regeneration in *Hemidactylus flaviviridis*. However, cholinergic inhibition with atropine produced a notable delay in the early events (preblastemic phase) of cell dedifferentiation and blastema formation. This decrease in the rate of cell proliferation has observed precisely in the didefferentiation-proliferation phase. It is known that muscarinic cholinergic actions increase cellular concentration of cyclic GMP. In sea urchin embryos atropine inhibited acetylcholine-stimulated increase in cyclic GMP levels and impaired the development (Gustafson and Toneby, 1970). It is well established that the proliferation-differentiation phases of regeneration are marked by fluctuations in cyclic nucleotides; maximum of cyclic GMP during dedifferentation with a diminished cyclic AMP levels (Liversage *et al.*, 1977; Taban and Cathieni, 1989; Cathieni and Taban, 1992). The cholinergic antagonist- induced inhibition in dedifferentiation during tail regeneration is suggestive that this critical event might be mediated by cyclic nucleotides.