CHAPTER VII

CHOLINESTERASES DURING TAIL REGENERATION : A LOCAL AND SYSTEMIC ANALYSIS IN THE SCINCID LIZARD, MABUYA CARINATA

Attempts at unravelling the molecular mechanisms involved in the initiation of reparative regeneration in vertebrates, though manifold, have however not yet yielded any definite clue and still remains very much in the realm of speculations. Majority of the studies are restricted to unodelean limb regeneration. Dependence of the newt limb regeneration on the presence of an adequate nerve supply at the amputation site have been well recognized (Singer, 1952; Thornton, 1970). Though the precise mechanism by which the neuronal contribution affects regeneration is not known, neurotrophic influence on increase in volume and mitotic activity of the early regenerate (Singer and Craven, 1948) as well as on macromolecular synthesis (Dresden, 1969; Lebowitz and Singer, 1970; Singer and Caston, 1972; Morzlock and Stocum, 1972; Bantle and Tassava, 1974) have been shown. Besides, successful restoration of protein synthesis to the level of non-denervated regenerate by infusing nerve and brain homogenates into the denervated regenerates was also achieved by Lebowitz and Singer (1970) and Singer et al.(1976).

Unlike in the case of urodelean limb regeneration, lizard tail regeneration is considered to be mostly influenced by the spinal cord or the ependyma, rather than nerves (Kamrin and Singer, 1955; Simpson, 1964, 1965; Cox, 1969). Though a specific influence of peripheral nerves is apparently a non-entity, all the above studies do however, highlight some sort of neurotrophic influence in the initial quagmire of events associated with lizard tail regeneration. Evidently, detailed investigations of the type carried out in amphibian limb regeneration regarding the neurotrophic influence are lacking in reptilian tail regeneration. In recent years literature is replete with reports on the presence of cholinesterases even in tissues and sites where neurotransmitter mechanisms are inoperative (Silver, 1974), which have given currency to tentative speculations regarding the non-transmission functions of these enzymes.

In this light, since a neurochemical substance has been shown to be involved in the regenerative mechanics of newt limb regeneration (Jabaily and Singer, 1977) and as scant attention has been paid to these aspects in reptiles, a detailed analysis of cholinesterases viz., acetyl (AChE) and non-specific (NspChE) (pseudocholinesterase) has been undertaken in the present study, both in the regenerate as well as in the two visceral organs (liver and skeletal muscle), during various phases of tail regeneration in the

Scincid lizard, M. carinata. Apart from providing the quantitative alterations of cholinesterases at the local site during regeneration, the present study also aims to reveal the adaptive alterations of these enzymes in liver and skeletal muscle in conjunction with regeneration as part of the systemic metabolic and other responses.

MATERIALS AND METHODS

Healthy Mabuyas of both sexes obtained from Hyderabad, India and allowed to get acclimated to the laboratory conditions in Baroda were kept on insect diet. Tails were autotomized by pinching off at about 1-2 cms distal to the vent. Animals were then sacrificed at fixed intervals of 3, 5, 7, 10, 12, 15, 25, 40, 60 and 90 days post-autotomy. Liver and skeletal muscle along with the regenerating tail were quickly removed by sacrificing the animals under mild anaesthesia. Normal animals with intact unautotomized tails were also sacrificed to serve as controls. The tissues were homogenized in ice-cold distilled water and treated with 1% Triton X 100 (0.1 ml/ml of homogenate). After allowing it to stand for about 20 mins, at 4°C, the homogenates were centrifuged for 15 mins at 30000 X g at 4°C and the supernatants were used for assaying the activity of cholinesterases according to the method of Ellman et al. (1961). Acetyl cholinesterase activity was measured with Acetyl thiocholine

iodide (obtained from Sigma Cehmicals, U.S.A.) as the substrate and readings were obtained without and with Neostigmine sulphate (an inhibitor of AChE). The difference between the readings was then taken as that for AChE. Non-specific cholinesterase activity was assayed by using Butyryl thiocholine iodide (obtained from Sigma Chemicals, U.S.A.) as the substrate and Neostigmine sulphate as inhibitor of AChE. The conversion of 5,5'-dithio-bis-2-nitrobenzoate to the yellowish anion of 5-thio,2-nitrobenzoate was monitored by increase in the optical density at 412 mμ on a spectrophotometer with cuvettes having 1 cm light pathway. The specific activity of both the enzymes was expressed as μ moles of substrate hydrolyzed/mg protein/min.

The protein content was measured by the method described by Lowry et al. (1951).

For each day and each tissue specified a total of five to seven determinations were made. The mean and standard deviation were obtained and students' 't' test was used to determine statistical significance.

RESULTS

AChE appears to be present in the decreasing order - muscle, tail and liver in the normal animals with intact tails while NspChE appears to show an activity order of

Table 1. Quantitative levels of Acetyl (AChE) and Non-specific Cholinesterases (NspChE) in the regenerate, liver and skeletal muscle during tail regeneration in M. carinata.

(Values are expressed as : μ moles of substrate hydrolized/mg protein/min.)

Periods of tail regeneration in days	Tail		Liver		Muscle	
	AChE	NspChE	AChE	NspChE	AChE	NspChE
N	22.79 ±1.50	0.68 ±0.09	4.52 ±0.18	1.29 ±0.26	25.03 ±1.34	2.32 ±0.44
3	14.01*@ ±1.10	6.68*@ ±1.01	4.31 ±0.06	1.28 ±0.45	15.47*@ ±1.26	0.93*@ ±0.09
5	12.50*@ ±1.10	2.31*@ ±0.73	3.96@ ±0.13	0.71*@ ±0.08	10.19*@ ±0.78	1.23*@ ±0.35
7	22.34*@ ±1.70	1.63@ ±0.54	4.67 ±0.21	0.38*@ ±0.08	15.79*@ ±0.98	0.78*@ ±0.14
10	44.77*@ ±1.04	2.93*@ ±0.89	5.24** ±0.23	0.72*@ ±0.09	21.49 ±1.09	1.73* ±0.26
12	58.88*@ ±1.94	3.36*@ ±0.76	5.61@ ±0.37	2.12*@ ±0.15	29.30*@ ±1.177	2.20 ±0.34
15	51.79*@ ±1.40	2. 16*@ ±0.55	5.14* ±0.25	1.56 ±0.18	23.14 ±0.78	2.80 ±0.33
25	50.88*@ ±1.26	2.73 *@ ±0.49	3.97@ ±0.34	1.14 ±0.26	25.69 ±0.96	3.58*@ ±0.56
40	42.18*@ ±1.50	2.40*@ ±0.67	3.85@ ±0.35	1.50 ±0.34	23.08 ±0.62	3.51*@ ±0.43
60	34.40*@ ±1.81	2.26*@ ±0.34	3.81@ ±0.35	1.29 ±0.44	24.95 ±0.62	2.54 ±0.36
90	22.00 ±4.10	0.70 ±0.10	4.40 ±0.26	1.20 ±0.36	26.50 ±3.40	2.45 ±0.33

N - Normal (Pre-autotomy state)

* $P < 0.01$; ** $P < 0.005$; @ $P < 0.0025$; @ $P < 0.001$; *@ $P < 0.0005$.

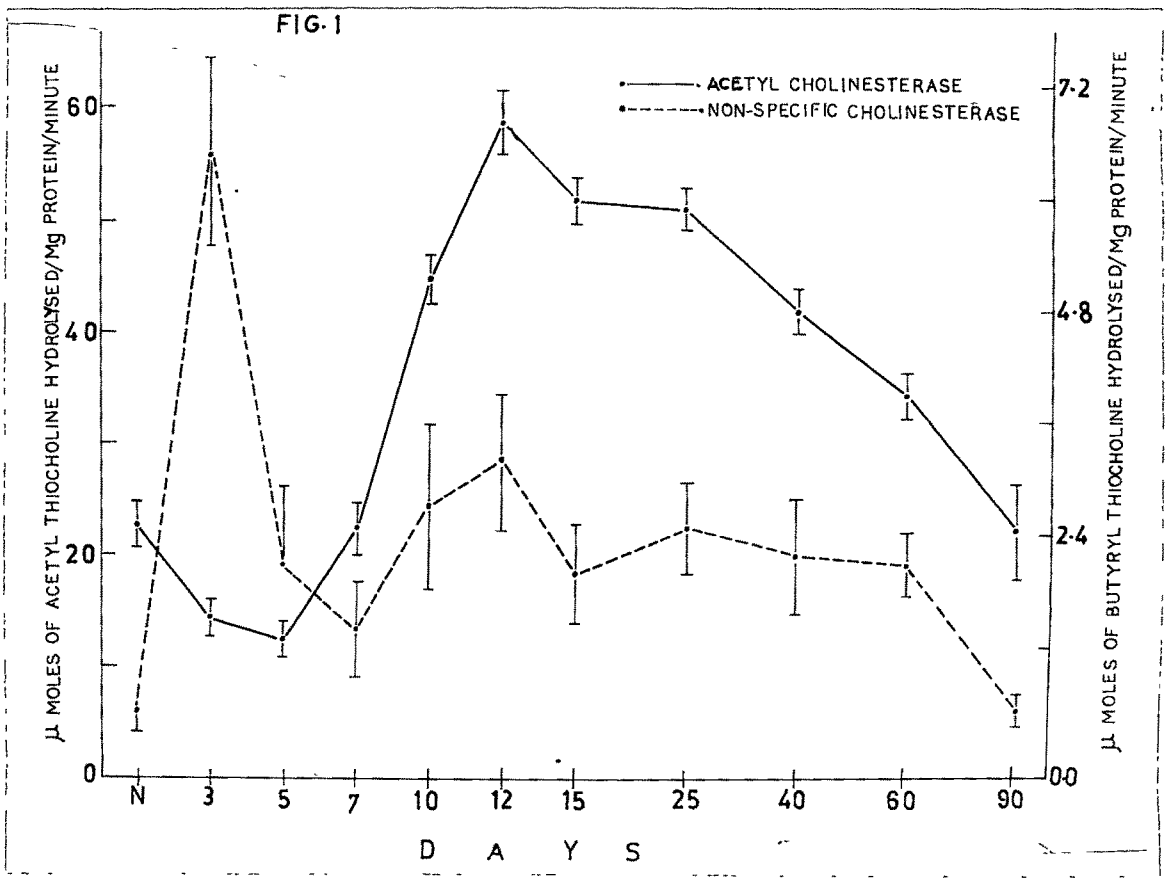

Fig. 1. Changes in acetyl (AChE) and non-specific cholinesterase (NspChE) activity in the regenerate during tail regeneration in M. carinata.

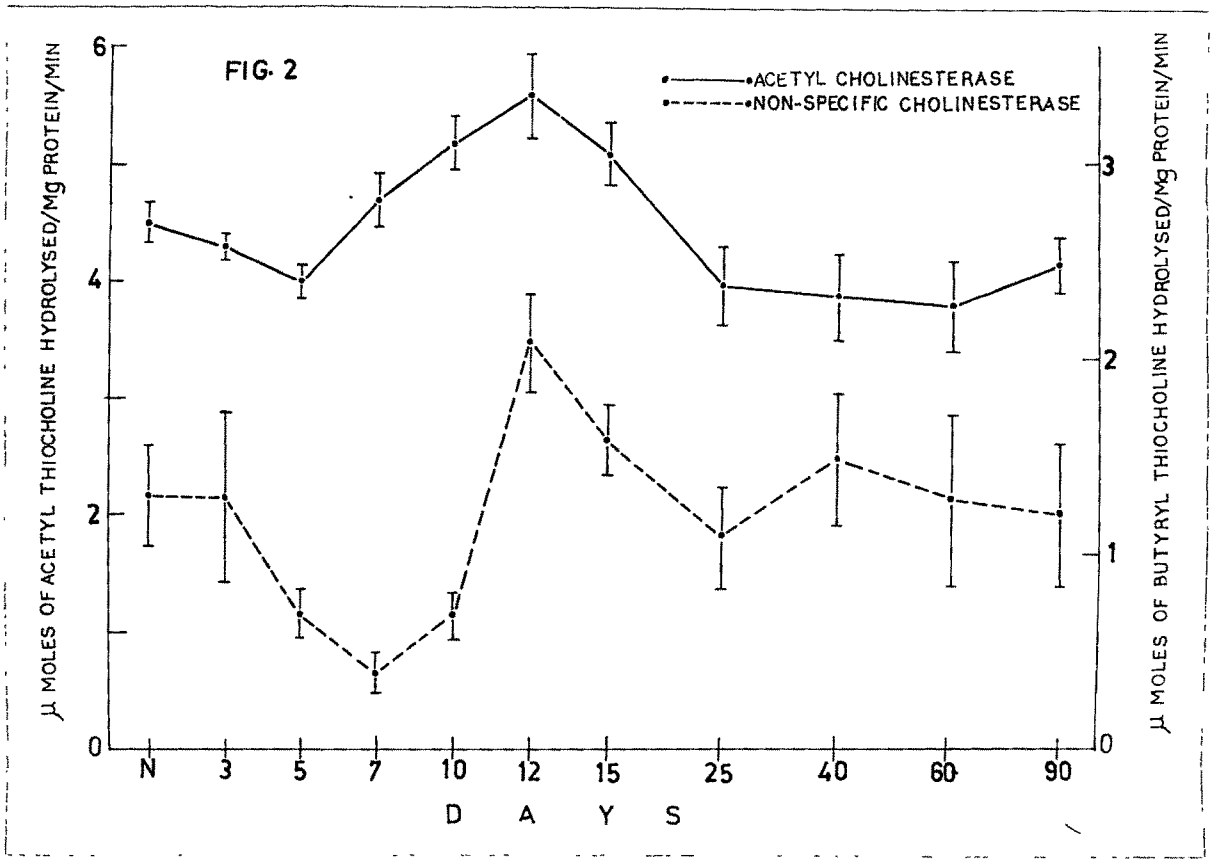

Fig. 2. Changes in acetyl (AChE) and non-specific cholinesterase (NspChE) activity in liver during tail regeneration in M. carinata.

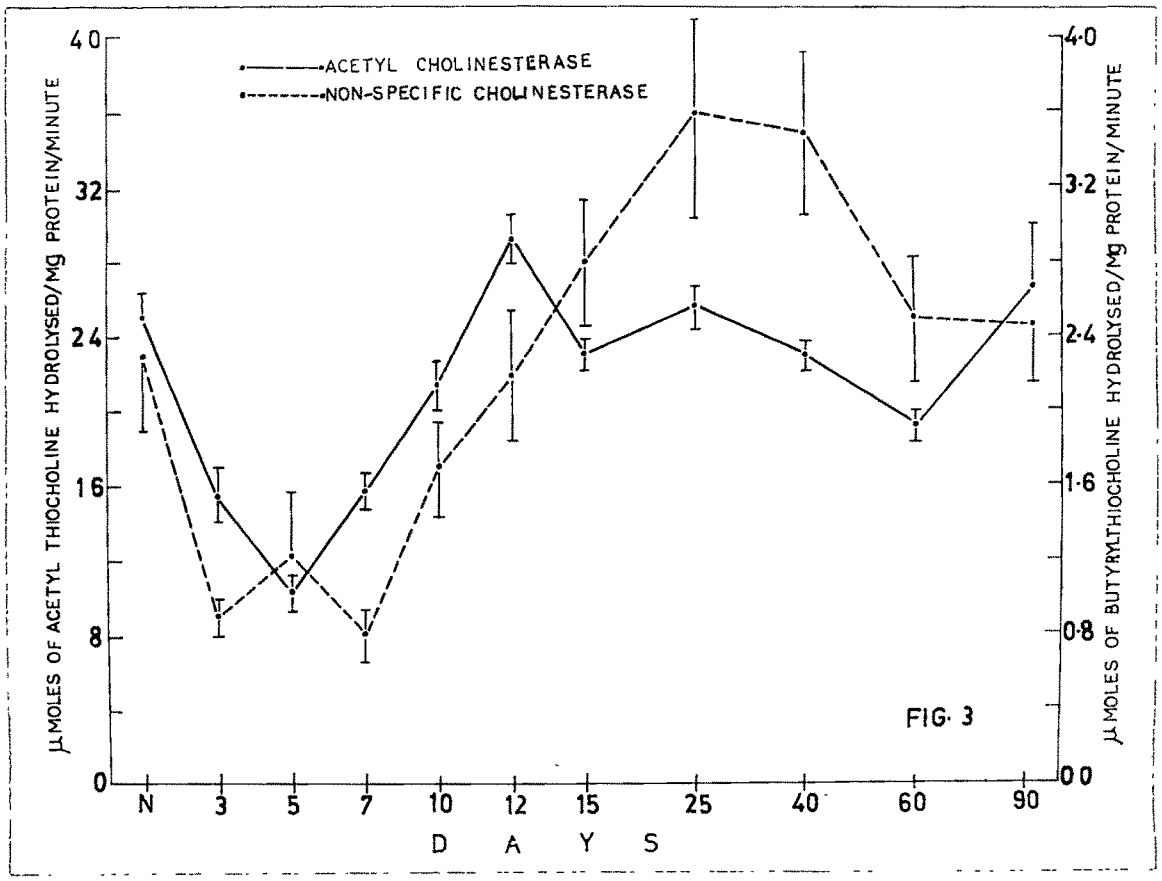

Fig. 3. Changes in acetyl (AChE) and non-specific cholinesterase (NspChE) activity in muscle during tail regeneration in M. carinata.

muscle, liver and tail. During the first 12 days post-autotomy, while AChE levels showed a similar pattern of changes in all the three tissues, NspChE levels showed a differential pattern in the three tissues under scrutiny. In all the three tissues AChE depicted a fall during the 3rd and 5th days and gradual increase through 7th and 10th days to reach maximal levels on the 12th day. Though the level of enzyme activity found in skeletal muscle and regenerate on the 5th day post-autotomy was the lowest, in the case of liver similar low level was recorded even during the 25th, 40th and 60th days of regeneration. From the low levels on the 5th day, AChE activity appeared to show increase on 7th day to reach an above-normal level on the 10th day in liver and tail and a near-normal level in skeletal muscle, and a maximal level in all the three tissues on the 12th day. In the regenerate AChE activity appeared to show a decline on the 15th day with apparently same level being maintained even on the 25th day. However, in the case of skeletal muscle the enzyme activity depicted a fall on the 15th day and again an increase on 25th day; while in the hepatic tissue the AChE showed continuous fall to reach a sub-normal level on the 25th day. Since then AChE activity showed a sharp decline during 40th and 60th days of regeneration in the tail to reach the near-normal level on the 90th day. In the skeletal muscle the activity of AChE which was more or less comparable

to normal did show less significant, nevertheless, gradual decline during 40th and 60th days post-autotomy. At the same time in the liver the activity of AChE which was already sub-normal on 25th day, continued to maintain the same low level even on 40th and 60th days and then by 90th day the enzyme level rose to attain a near-normal level.

NspChE appeared to show a tremendous increase in the tail on the 3rd day post-autotomy. Correspondingly at this stage the hepatic tissue showed no alteration, while the muscular tissue depicted a drop. Since then on the 5th and 7th days whereas the enzyme activity showed a fall in the regenerate (though still very much above normal), in the liver and skeletal muscle the enzyme activity decreased further to reach the lowest subnormal levels. Though there was a gradual increase in NspChE activity in both the visceral organs with a maximal level being attained on 12th day in the case of liver, in the muscle this ascending order was continued to attain a maximal level on 25th day. In the regenerating tail the enzyme activity again increased on 10th day and attained a second smaller peak on the 12th day. On the 15th and 25th days post-autotomy NspChE activity, by a gradual fall, touched the normal level in the liver, while in the regenerate an above-normal plateau was maintained during 15th through 60th days post-autotomy after a slight decrement from the 12th day level. Thereafter, there was a significant drop in

NspChE activity in the regenerate to register ultimately normal level on the 90th day of regeneration. The skeletal muscle maintained a plateau of peak level on the 25th and 40th days of tail regeneration with a subsequent drop back to the normal level on the 60th day. These changes in AChE and NspChE activities during tail regeneration are depicted in Table 1 and Figs. 1-3.

DISCUSSION

From the results (see Table 1 and Figs. 1-3) it is apparent that the skeletal muscle and tail have maximal AChE activity in comparison to the hepatic tissue. NspChE activity is higher in the muscle and liver and least in the tail. However, during caudal regeneration, the tail tends to depict maximal level of NspChE activity as compared to that in the muscle and liver. An interesting aspect of the present study is the supra-normal levels of NspChE activity found in the tail almost from the time of tail autotomy (3rd day) till the completion of the process of regeneration (i.e. 90 days). AChE too depicts elevated levels during the progressive phases of regeneration (i.e. from 10th through 60th days post-autotomy). Though the changes in the levels of cholinesterases in the two visceral organs (liver and muscle) appeared to be less significant as compared to those in the regenerate during tail regeneration, the skeletal muscle tended to be slightly

more responsive. These generalizations tend to indicate an active involvement of cholinesterases during regenerative ontogeny.

Subsequent to tail autotomy there was a significant decrement in AChE activity in both the regenerate as well as the skeletal muscle which by the 5th day had reached its lowest point; while in the liver the enzyme activity was not much altered. This fall in AChE activity in the early period of regeneration could lead to an increased content of acetylcholine (ACh) at this stage. This contention stands justified by the earlier reports of an increased ACh content (Singer, 1959) as well as low AChE level (Singer et al., 1960) during the early periods of limb regeneration in the newt, Triturus viridiscens. Rasmussen (1975) had opined that ACh can induce alterations in membrane permeability, bring about release of membrane bound Ca^{+2} as well as increase of cAMP level by inhibiting phosphodiesterase activity. Such a set of events occurring in tail and muscle of M. carinata during the first 5 days post-autotomy can be easily deduced by the observed concurrent reduction in phosphodiesterase activity (Chapter VI). Interestingly, liver AChE too depicted a statistically significant ($P < 0.0025$) drop on the 5th day. Similar drop in enzyme activity in the regenerating system as well as in the two visceral organs indicate a common regulatory influence. Since then through 7th and 10th to 12 days (which corresponds

to dedifferentiation, and blastema to early differentiation) there was a gradual increase in AChE activity in all the three tissues reaching the peak levels on the 12th day. From Table 1 and Figs. 1-3, it becomes clear that near normal levels were reached by 15th day itself in the muscle and by 25th day in the case of liver, while in the regenerate near-normal AChE activity was attained only by 90th day. Apparently, while supra-normal levels of AChE activity was maintained in the regenerate from 10th to 60th days of tail regeneration, such was the case during 10th, 12th and 15th days in liver and only during the 12th day in muscle. The changes outlined above indicate a predominant involvement of AChE in the regenerating system especially during the early to late differentiation phases (10th to 25th days). Similar high incidence of AChE during early differentiation phase of regenerating newt limbs has been reported by Singer et al. (1960). The cholinergic system, specifically ACh-AChE system has been shown to influence carbohydrate metabolism and tissue assimilation in birds and mammals (Shimazu, 1967; Bergmen and Miller, 1973; Chieri et al., 1975; Szabo and Szabo, 1975 a, b; Wilbrandt, 1975; Pilo et al. 1976; Pilo and Patel, 1978). It is interesting that maximal levels of AChE activity is present during the early progressive phase of regeneration when neurotransmission activities can be considered to be practically nonexistent. This fact together with the

substantial reports available on association of AChE activity with membrane permeability, ionic transport and metabolic activities related to differentiation and development (Burt, 1968; Filogamo and Marchisio, 1971; Silver, 1974), lead to the surmise that AChE is somehow involved in the metabolic transformations occurring during the period of transition between blastema and differentiation, whence the undifferentiated pluripotential mesenchymal cells of blastema get committed to unidirectional channels of differentiation. Incidentally this period of regeneration is marked by increasing levels of glycogen (Chapter II), glycogen synthetase (Shah and Hiradhar, 1975a) and potassium ions (Shah and Hiradhar, 1974b). Moreover, Shah et al. (1977b) have shown an elevated blood glucose level on and around 12th day of tail regeneration in M. carinata. All these observations tend to implicate AChE either directly or indirectly in either interrelated or independent events like ion transport, glucose uptake, glycogen synthesis etc., during late blastemic and differentiation phases of tail regeneration in M. carinata, and ample literature cited above in this connection justify this inference. Concomitant elevated activity of AChE in the liver too might indicate similar influences on carbohydrate metabolism. Further, the high levels of AChE in the regenerate also indicate low levels of ACh which may again be of adaptive significance in promoting protein synthesis associated with

differentiation, as an inhibitory influence of ACh on protein synthesis in cultured limb regenerates has been shown by Foret and Babich (1973).

The noteworthy observation of the present study is the nine-fold increase of NspChE activity almost immediately after the tail autotomy on the 3rd day. This when viewed in the light of significant and continuous fall in NspChE activity depicted by the visceral organs (liver and muscle) during the 3rd, 5th and 7th days post-autotomy, is very striking. This observation gains added importance, as changes in AChE activity in the regenerate subsequent to tail autotomy were found to be parallel to those of the two visceral organs. Conceivably, the 880% increase in NspChE activity occurring in the tail on the 3rd day after autotomy may form part of the chemical contribution made by the cut end of the spinal cord. Though the activity of NspChE in the regenerate did show a decrement on the 5th and 7th days, even the lowest level of this enzyme found on the 7th day (see Table 1 and Figs. 1-3) was about 140% above the normal level. Some of the earlier references cited in the introductory part of the present chapter have indicated the involvement of a neurotrophic factor in the initial protein synthetic activities associated with amphibian limb regeneration. Singer et al. (1976) and Jabaily and Singer (1977) had been successful in restoring both protein as well as DNA and RNA

synthetic capacities by denervated newt limbs to the corresponding innervated levels by infusing brain extracts. In one of the recent studies, Bast et al. (1979) in an attempt to gain insight into the mechanism responsible for the reduced macromolecular synthesis in the denervated limb regenerate have by their ^{35}S Methionine and ^{3}H Uridine labelled studies on the effects of denervation on the size, distribution and quantity of regenerate polysomes, suggested a net reduction in the amount of ribosomes as well as a coordinate decrease in translatable mRNA concentration. Presumably cellular ribosome content could be regulated either by an alteration in rRNA production at the level of rDNA transcription or by controlling the turnover of ribosomes in the cytoplasm (Perry, 1973). It is conceivable, therefore, that the neurotrophic factor, a basic protein (Singer et al., 1976) might have some influence in either or both the above molecular events. As alterations in nuclear acidic and basic proteins are known to be involved in gene action and transcription, the above concept gains relevance by the reported increase of both these classes of proteins in 48 hr denervated regenerates by infusion of the soluble brain protein extract (Singer et al., 1976). In the light of these facts and reports, the currently obtained high NspChE activity by about 72 hrs post-autotomy is rather interesting. With the purported role of NspChE in permeability alterations and ionic changes, it

may be assumed that NspChE by inducing such molecular alterations might be directly or indirectly contributing to increased rRNA production and functional ribosomes and hence to increased macromolecular synthesis characteristic of the early phase of regeneration. Since K^{+2} and Mg^{+2} ions are known to be involved in ribosomal assembly and as NspChE has been shown to control ion transport (Marx and Corter, 1963; Silver, 1974), the highly elevated level of NspChE activity by inducing membrane permeability and by maintaining a conducive ionic environment, might not only control ribosome number but also bring about increased rate of rDNA transcription and hence protein synthesis. It is to be resolved as to whether the NspChE is elaborated by the spinal cord or the brain. Once resolved, it may be pertinent to direct scientific attention towards understanding of the relation of NspChE with the neurotrophic principles.

Supra-normal levels of NspChE during the progressive phases of regeneration in all the three tissues denote an active involvement of this enzyme in the metabolic alterations occurring in association with differentiative events of the regenerate. The 200-300% above-normal level of NspChE in the tail regenerate during the progressive phases of regeneration could be construed to be involved in intricate metabolic events involving lipids and other esters as lipid metabolism

is noted to be an active feature during differentiation as per our previous studies (Radhakrishnan, 1972; Shah and Ramachandran, 1973, 1975, 1976). In fact such an involvement of NspChE in lipid metabolism has been hinted at by many workers (see Silver, 1974).

Comparatively high levels of NspChE in skeletal muscle during the later half of regeneration as compared to AChE activity is definitely indicative of the significance of this enzyme in metabolic responses of the skeletal muscle in relation to tail regeneration. Incidentally this period is also marked by a gradual recovery in the glycogen store of the skeletal muscle which was depleted in the earlier periods in response to autotomy (Chapter II). Again, the increased activities of ChEs in hepatic tissue too during 10th to 40th days of regeneration might also be considered to have similar significance. These enzymes may be considered to have some functional significance in the phagocytic activity of blood cells as well as lymphocytopoisis noted to occur in liver during tail regeneration in M. carinata (Ramachandran et al., 1981). Pertinent to quote in this connection are the reports of Savensmark (1963), Ballantyne and Burwell (1965), Ballantyne (1966) wherein they have suggested involvement of cholinesterases in the inactivation of any potentially toxic esters produced as a result of lipid metabolism during mitosis. Further, they have also hinted that ChEs can act on

a wide spectrum of choline and non-choline ester substrates, and in hepatic reticulo-endothelial cells, they could play a role in lipid and ester metabolism generally or in detoxification following phagocytosis (Stuart et al., 1960; Clitherow et al., 1963). Two recent reports very pertinent to the conclusions reached in the present report are that of Vardy et al.(1981) and Kelly and Singer (1981), who have shown the involvement of a neuronal factor in limb morphogenesis and, dependence of early limb regenerate on phosphate uptake mediated by nerve dependent changes in the properties of cell membrane, respectively.

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