CHAPTER VI

IN LOCO AND SYSTEMIC ELECTROPHORETIC PROTEIN PROFILE DURING TAIL REGENERATION IN NORMAL AND HYPOTHYROIDIC LIZARDS, *HEMIDACTYLUS FLAVIVIRIDIS*

The most important biochemical process representing growth is the biosynthesis of proteins. Thornton and Bromley (1973) opined that protein metabolism gets extensively geared up in accordance with regressive (dedifferentiation) as much with the progressive phases (blastema formation, differentiation and growth) of regeneration. Remarkably similar fluctuations in protein content have been reported to occur during the course of wound repair in mammals as well as limb regeneration in adult newts (Reynolds *et al.*, 1963). Macromolecular synthesis constitutes an integral and essential aspect of epimorphic regeneration. Both quantitative and qualitative effects of this aspect deserve experimental evaluation. In this context, limb regeneration in Urodele amphibians has received considerable attention. Owing to the fact that a neurotrophic influence has been well established in urodelean limb regeneration, most of the quantitative and qualitative aspects of macromolecular synthesis during amphibian limb regeneration have been on a comparative basis between innervated and denervated limbs (Dresdon, 1969; Morzlock and Stocum, 1972; Singer and

Caston, 1972; Dresdon and Moses, 1973; Bantle and Tassava, 1974; Dearlove and Stocum, 1974; Donaldson et al., 1974; Manson et al., 1976; Singer, 1978; Bast et al., 1979; Slack, 1982; Garling and Tassava, 1984). Qualitative differences in protein profile during normal newt limb regeneration have been analysed by polyacrylamide gel electrophoresis (Schmidt, 1966a). Since the regenerative potency is highly restricted and represented only amongst poikilotherms, it is imperative that comparative studies be conducted in order to ascertain the unique mechanism(s) if any underlying vertebrate appendage regeneration. In this behest it is unfortunate that lacertilian tail regeneration has received scant attention. Whatever little that has been attempted in this context has emanated from this laboratory. Previous studies have concentrated mainly on quantitative alterations in macromolecular content (DNA, RNA and proteins) during tail regeneration in the Gekkonid lizard, Hemidactylus flaviviridis and the Scincid lizard, Mabuya carinata (Ramachandran et al., 1980; Shah et al., 1980a). In continuation the present study addresses to the electrophoretic separation of proteins to study the phase specific alterations during the process of lizard tail regeneration, so as to reveal the qualitative effects in terms of synthesis of regeneration specific protein(s).

Since there appears to be a co-ordinate involvement of systemic factors in concert with local factors during the process of regeneration, the operation of certain regulatory factors of systemic origin can well be considered. Many hormones are known to exert control over metabolic activities of animals especially the thyroid hormones. Previous studies from this laboratory had shown not only phase specific alterations in thyroid activity (Ramachandran et al., 1981a; Swamy et al., 1982a, 1983) but also the effect of hypothyroidism on regeneration specific metabolic modulations in Mabuya carinata (Swamy et al., 1982a, 1983). Moreover, retardation in regenerative outgrowth too has been reported under hypothyroidic conditions (Kothari et al., 1979; Ramachandran et al., 1984) as has been observed during the course of the present study (Chapter 4). The thyroid hormones have been reported to play a role in mitochondrial protein synthesis through the stimulation of synthesis of tissue specific protein synthetic modulators (Hashizume and Ichikawa, 1985). Hence it was deemed fit to study the protein profile of thyroid suppressed lizards also and compare the same with that of normal lizards during tail regeneration so as to reveal as to whether hypothyroidism per se can alter the protein banding pattern of unautotomised as well as autotomised tails. Together with the tail, electrophoretic separation of proteins of liver and skeletal muscle have also been undertaken during various periods of

tail regeneration as quantitative alterations in the protein content of liver and muscle have been previously shown to occur during the course of lizard tail regeneration (Shah et al., 1980a; Ramachandran et al., 1982; Ramachandran and Chacko, 1987b; Valsamma et al., 1987)

MATERIALS AND METHODS

The lizards H. flaviviridis procured from the local animal dealer were maintained in the laboratory on a diet of cockroaches. The animals were kept in the laboratory for a fortnight for acclimatisation to the laboratory conditions. Lizards weighing 10-12 gms and having a snout-vent length of 8-10 cms were taken for the study and tail autotomy was done by pinching off the tail two segments distal to the vent.

A total of 120 animals were used for the experimental purpose. They were divided into two groups of 60 each. One group served as the euthyroidic control and the other group was thyroidectomised chemically by force feeding them with 0.1 ml of 0.2% 6-propyl,2-thiouracil (PTU) (obtained from Fluka Chemicals, Switzerland) pH adjusted to 8.0-8.2), every alternate day starting 15 days prior to autotomy. PTU feeding was continued even after tail autotomy (every alternate day) till the end of the experimentation.

The animals were killed at specific time intervals of 3, 5, 7, 10, 15, 25, 40 and 60 days post-autotomy as well as prior to autotomy. Tissues such as liver, muscle and tail were taken and the tissues homogenized in sample buffer (Tris-SDS, pH 6.8) and boiled for 2 min. The crude homogenate was centrifuged and the supernatant taken for electrophoretic analysis of the protein profile.

The protein profile was studied electrophoretically by the SDS-PAGE method with discontinuous buffer system as per the LKB laboratory manual (LKB 2001 Vertical electrophoresis). The buffers used were Tris-glycine as the electrode buffer (pH 8.3), Tris-SDS (pH 6.8 and 8.8) as the gel buffers and a sample buffer of Tris-SDS (pH 6.8). The gels of required thickness (1.5mm) was casted in glass plate moulds to form a gel sandwich of uniform and exact thickness. This disc gel system described by Laemmli (1970) includes a resolving gel (10% acrylamide) and a stacking gel (3% acrylamide). The resolving gel was poured to a height of 12 cm, overlayed with distilled water and left to polymerize before the stacking gel was prepared. Then the stacking gel was poured into the rest of the space and the slot mould was introduced and kept for polymerization.

Sample homogenate was prepared in the sample buffer with a concentration of 50 mg/ml. It was boiled for 2 mins., centrifuged and then filtered and cooled before loading.

100 µl of sample of each tissue was loaded in different slots of the gel. Then the gels were run for 4 hrs. using the LKB 2001 Vertical electrophoretic unit. A power adjustment of 400 volts, 45 milliamperes and 20 watts was given with an initial reduced current of 10 m. amps. for ten minutes. Molecular weight standards were run concurrently and included β-galactosidase (1,16000 daltons), phosphorylase b, (97400 daltons), Bovine albumin (66000 daltons), ovalbumin (45000 daltons), and carbonic anhydrase (29000 daltons). The proteins separated were stained by the Coomassie blue G 250 staining procedure.

RESULTS

Electrophoretically separated protein profile is represented in zymograms 1-6 and the various bands are numbered serially starting from the cathodic end. In general, the number of stainable protein bands was less in the tissues of hypothyroidic lizards.

Tail

A total of 41 stainable bands could be visualised in the unautotomised tail of euthyroidic lizards while only 27 could be seen in the hypothyroidic condition.

Apart from the reduction in number, there was also a perceptible decrease in the staining intensity of the bands. There were 4 prominent thicker bands (numbered 20, 26, 27, 28) with approximate molecular weights of 1,00000, 85000, 80000 and 76000 respectively. All these 4 bands were also represented in the hypothyroidic tails though with reduced staining intensity. In addition, there was 7 thinner but sharper bands (numbered 1,5,8,14,15,30,33) with approximate molecular weights of 1,40000, 1,32000, 1,26000, 1,16000, 1,14000, 70000 and 61000 respectively. In the hypothyroidic tail, six of the seven were present in milder intensity with the 7th one of molecular weight 61000 being lost. On the whole, 14 bands are lost in the hypothyroidic condition of which 6 are in the molecular weight range of 100000-130000(nos. 7,10,12,16,18,19), 4 are in the molecular weight range of 85000-1,00000 (nos. 21, 22, 24, 25) and 4 in the molecular weight range of 20000-75000 (nos. 32, 33, 36, 38). Interestingly, all these 14 bands remained unrepresented all throughout regeneration in the hypothyroidic tails. Qualitative alterations were well marked during regeneration in the euthyroidic control tails which involved both appearance of new bands as well as loss of some bands. In all about 4 new proteins appeared post-autotomy of which one was permanent and three were transitory. The permanent protein

acquisition appeared as early as the 3rd day between band numbers 29 and 30 (approximate molecular weight between 70000-72000). Of the three transitory proteins, one was very short-living and was induced as early as the 3rd day and remained only till the 7th day appearing between band numbers 19 and 20 having an approximate molecular weight of 1,00000. The other two were comparatively long-living and made their appearance gradually one by one between the 3rd and 5th days and remained till the 25th day. They were represented between band numbers 37 and 38 with approximate molecular weight of 40000 and 42000. The latter two proteins appeared in autotomised hypothyroidic tails also in an exactly identical fashion to that of the euthyroidic tails. However, of the former two proteins i.e. the short living transitory one and the permanently induced one, while the latter could never be discerned, the former could be very faintly seen in the hypothyroidic condition. Regeneration also involved permanent loss of as many as 6 proteins and were lost at different periods during regeneration. The first two to disappear were the 7th one (mol. wt. 1,30000) and the 32nd one (mol.wt.65,000) as early as the 5th day. The next to disappear was band number 22 (mol. wt. 96,000) on the 7th day and band nos. 19 and 24 (with approximate mol. wts. of 1,02000 and 90000) on the 10th day. The last one to disappear was

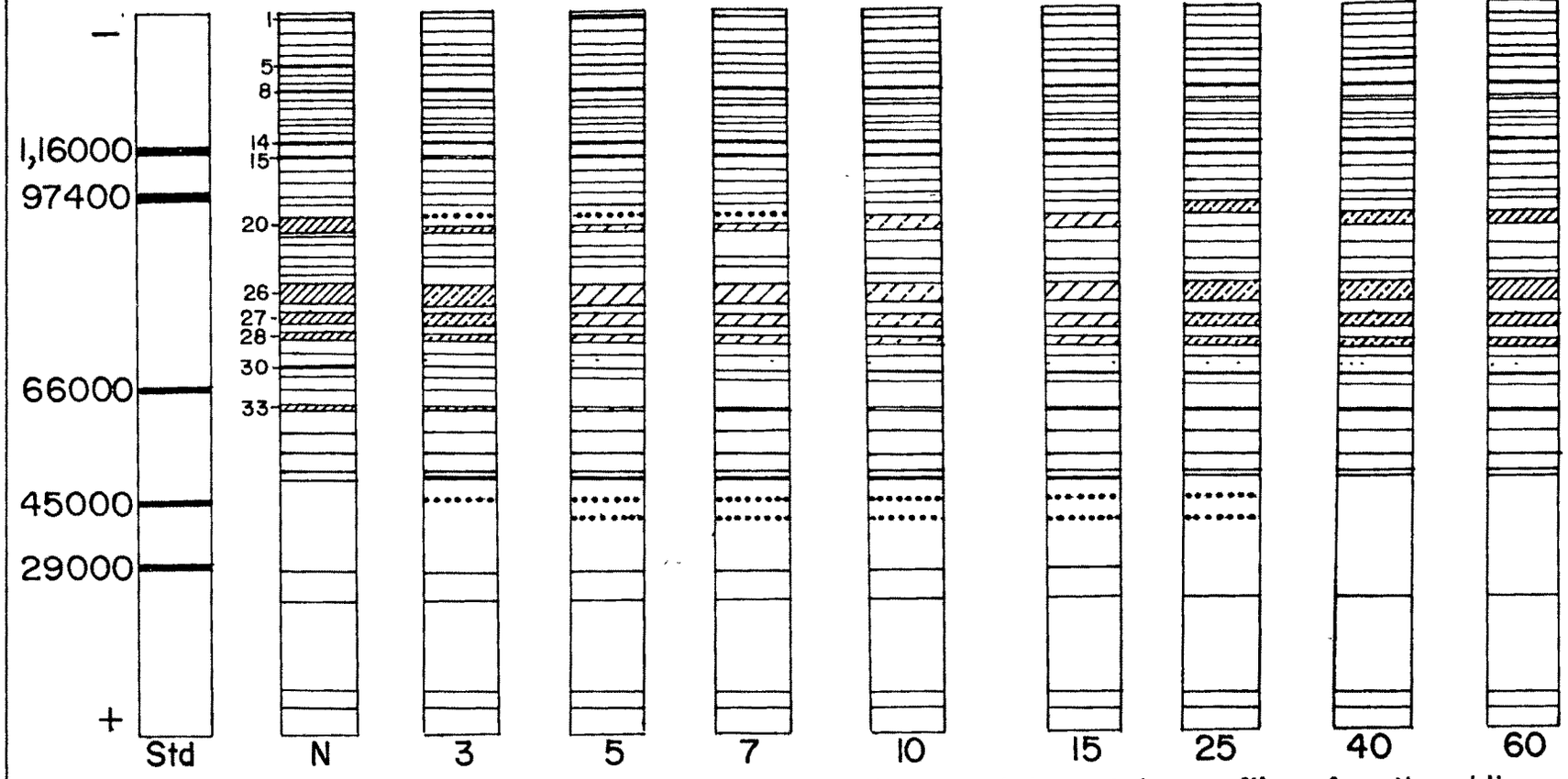

Fig. 1 Schematic electrophoretogram depicting caudal protein profile of euthyroidic H. flaviviridis.

Lanes 2–10 represent various periods of regeneration in days.

Lane 1 is of standard proteins. Figures left of lane 1 represent molecular weights of standard proteins. Dotted bands indicate the newly appearing proteins.

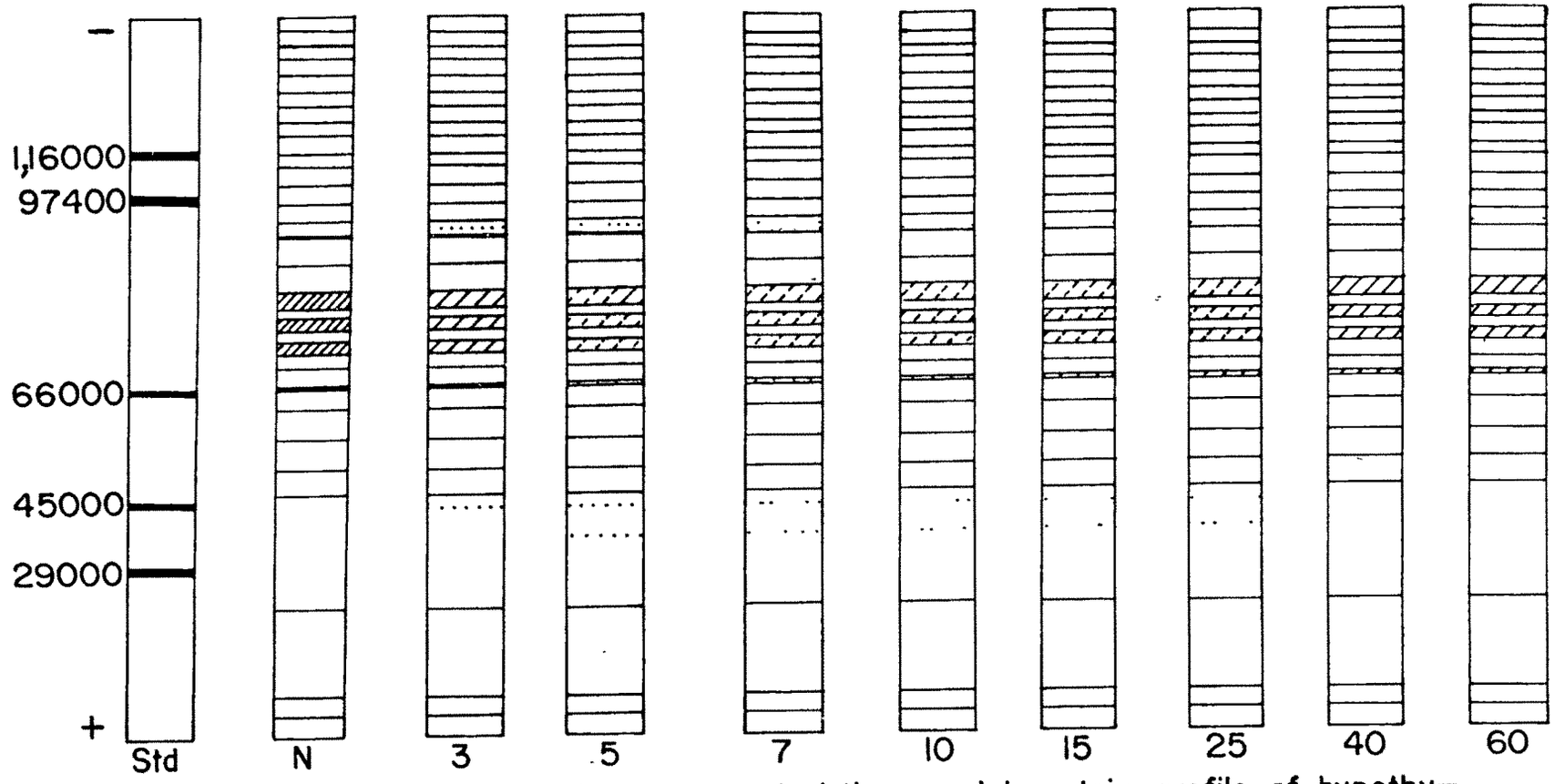

Fig. 2 Schematic electrophoretogram depicting caudal protein profile of hypothyroidic H. flaviviridis.

Lanes 2–10 represent various periods of regeneration in days.
Lane 1 is of standard proteins. Figures left of lane 1 represent molecular weights of standard proteins. Dotted bands indicate the newly appearing proteins.

protein number 38 as late as the 25th day of approximate molecular weight 30000. None of these protein losses could however be visualised in the autotomised hypothyroidic tails. Another noteworthy change was with reference to band number 20 which got reduced to half its thickness on 3rd, 5th and 7th days. Other minor variations observable were with reference to band numbers 1 and 5 which became lighter on 7th and 10th days and were prominent on 5th and 15th. Similarly band number 8 was also lighter on 7th and 10th days. Protein number 15 became lighter by the 3rd day and ramined so till the end. Band numbers 21 and 25 were lost on 3rd, 5th and 7th days and regained from 10th day though with lighter intensity. The prominent band numbers 26-28 became lighter during the first ten days. Band numbers 30 and 33 were also weaker during the first 10 days and remained lightly stained from 15 to 60 days. Finally, band number 37 became slightly darker from day 7 to 15 and remained lighter from days 25 to 60.

Muscle

About 30 electrophoretically separable protein bands could be discerned in the femoral muscle of Hemidactylus prior to caudal autotomy which got reduced to 22 in the hypothyroidic condition. The 8 bands which were lost in the unautotomised condition and which never

reappeared at any stage post-caudal autotomy were band numbers 2 (1,38000), 7 (1,25000), 10 (1,15000), 11 (1,14000), 20 (85000), 23 (70000), 25 (58000) and 30 (20000). The most prominent protein bands of skeletal muscle were the 3rd, 22nd, 24th, 26th, 29th and 30th. The major bands 22, 24 and 26 became lighter and thinner during 3rd, 7th and 15th days. By day 3rd post-autotomy, all the bands had become lighter by staining intensity with loss of 4 proteins (nos. 11, 13, 29 and 30). Except for the 5th, 25th and 60th days, the staining intensity of all the bands remained weak during the rest of the periods of study. Three of the 4 lost bands reappeared gradually with the 29th appearing on the 5th day, the 11th on the 7th day and the 13th on the 10th day. Band number 30 was however lost permanently. Two protein bands were lost once again on the 15th day (13 and 15) which reappeared by the 25th day. Day 40 post-autotomy depicted loss of band numbers 7, 8 and 9 which were regained by day 60. The hypothyroid skeletal muscle did not depict the above changes post-caudal autotomy. The only changes that could be discerned post-autotomy were the loss of three proteins (27, 28, 29) on the 3rd day and the reappearance of 28 and 29 by day 5 itself and the 27th by the 40th day.

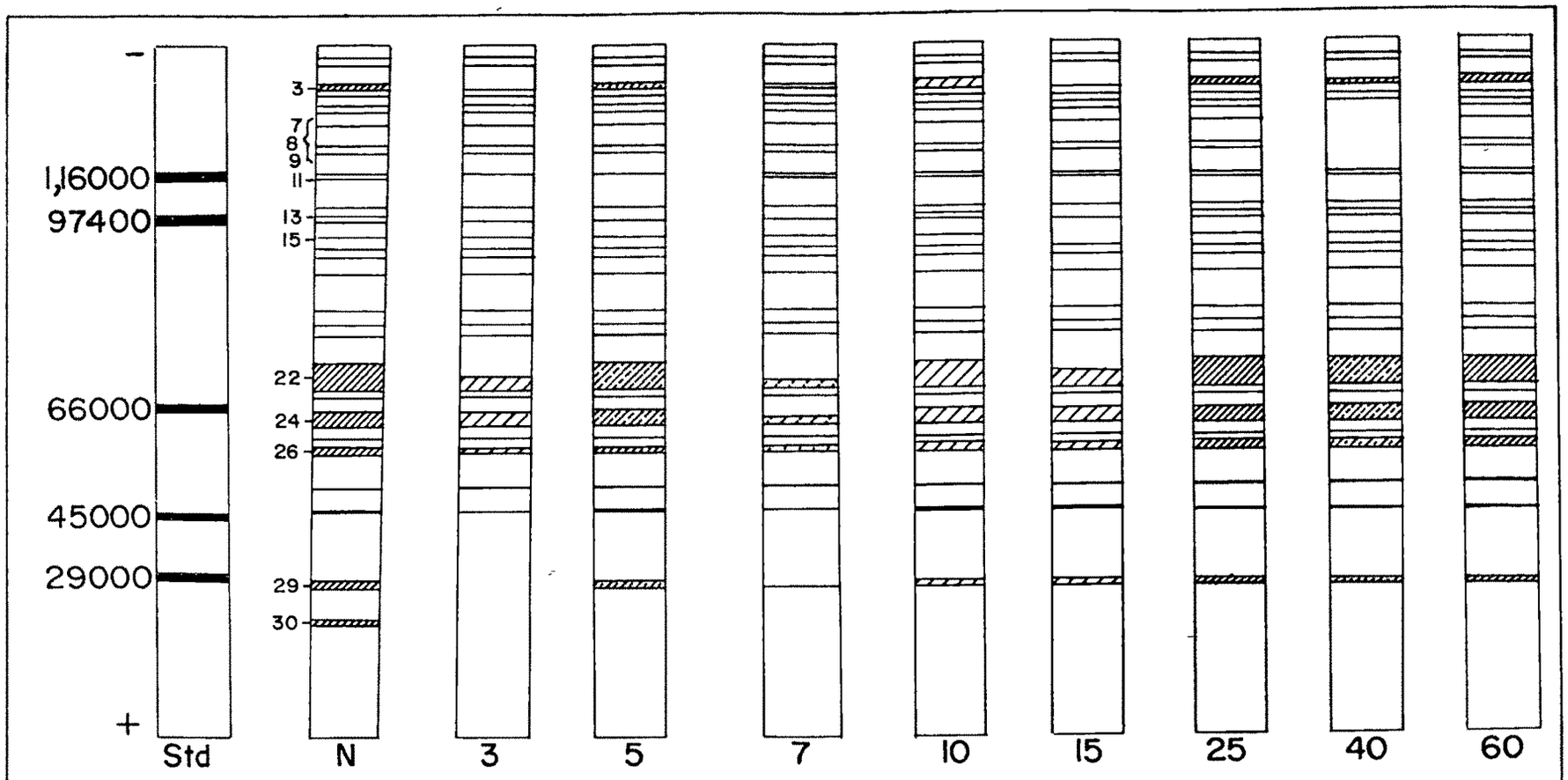

Fig. 3 Schematic electrophoretogram depicting muscle protein profile of euthyroidic H. flaviviridis.

Lanes 2–10 represent various periods of regenerafion in days. Lane I is of standard proteins. Figs. left of lane I represent molecular weights of standard proteins.

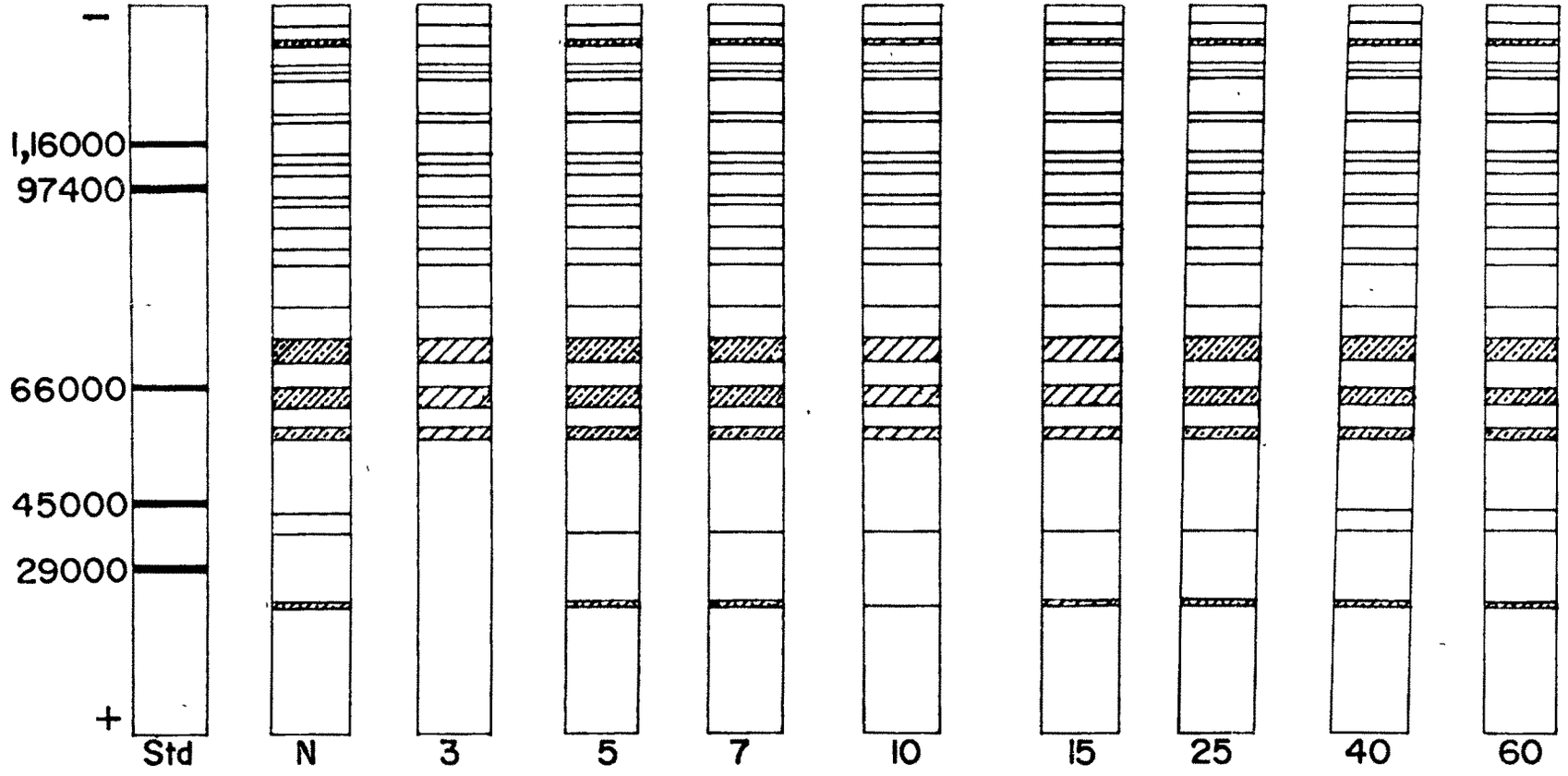

Fig. 4 Schematic electrophoretogram depicting muscle protein profile of hypothyroidic H. flaviviridis.

Lanes 2-10 represent various periods of regeneration in days.

Lane 1 is of standard proteins. Figures left of lane 1 represent molecular weights of standard proteins.

Liver

In the unautotomised condition, whereas the euthyroidic liver showed 35 stainable bands, the hypothyroidic liver showed only 21 with a loss of 14 bands. The biggest bands were the 19th and 27th with band number 34 also being quite prominent. Of the 14 bands that were lost permanently due to hypothyroidism, 5 were from the first 18 bands (in the molecular weight range 90000 to 1,14000), 3 from in between 19 and 27 (21, 22 and 23 - mol. wt. 80000, 75000 and 70000) and 6 from in between 27 and 34 (29 to 33 in the range of 30000-40000 and, the 35th - mol. wt. 20000). Moreover all the three prominent bands (19, 27 and 34) had become lighter in staining intensity. Post-caudal autotomy, there was no change whatsoever in the hypothyroidic liver except for the prominent bands 19 and 27, which remained lighter till the 15th day and then became darker from the 25th day onwards. However, the euthyroid liver did show qualitative alterations all restricted to the region of the first 18 bands corresponding to a molecular weight range 9000-1,40000. The major bands (19 and 27) were lighter on 3rd, 7th, 10th and 40th days while they were intensely stained on days 5, 15 and 25. Similarly, the 34th became lighter during the 3rd and 5th days. All the qualitative alterations post-caudal autotomy in euthyroid liver involved appearance

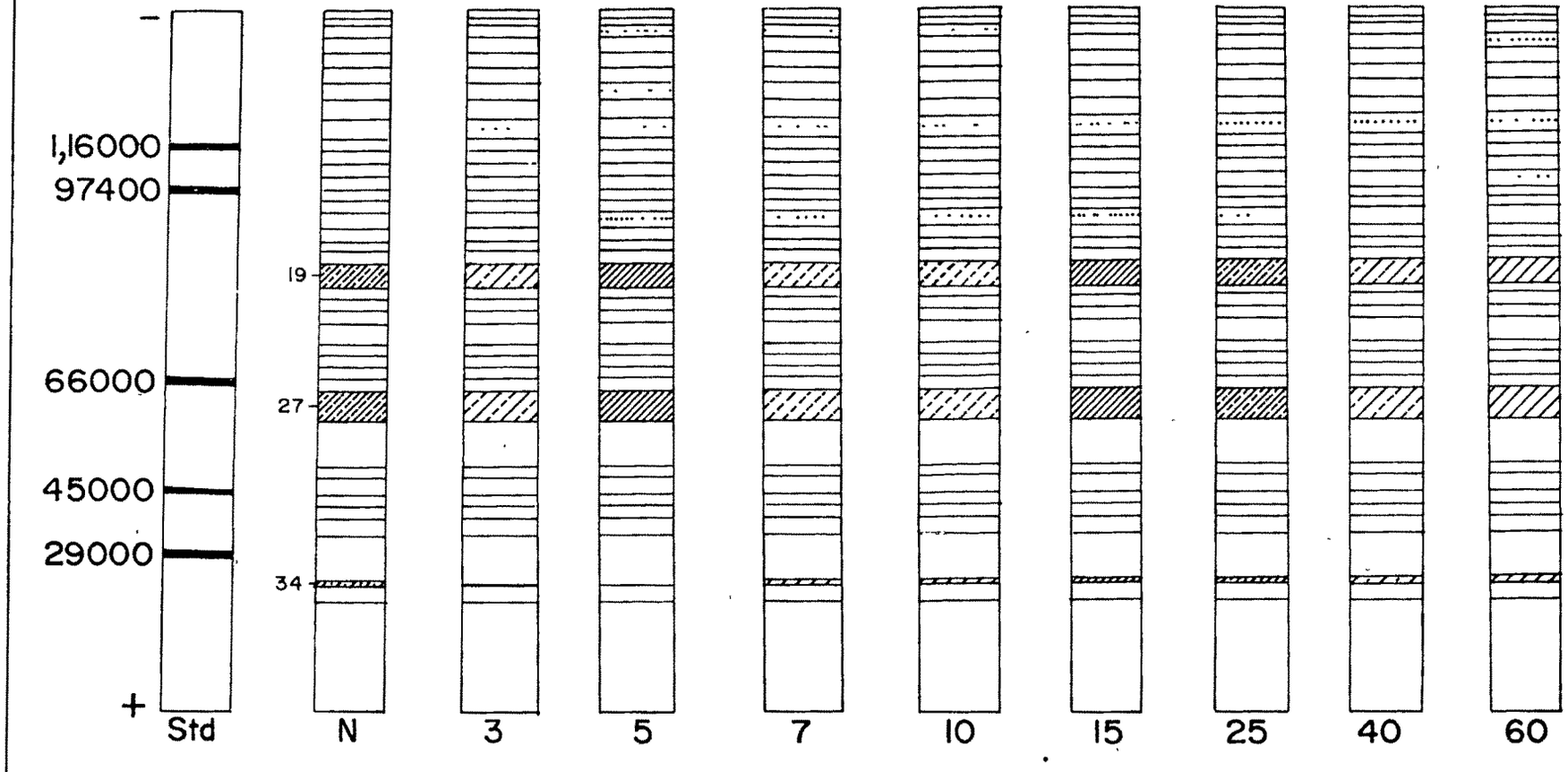

Fig. 5 Schematic electrophoretogram depicting hepatic protein profile of euthyroidic H. flaviviridis.

Lanes 2-10 represent various periods of regeneration in days. Lane 1 is of standard proteins. Figures left of lane 1 represent molecular weights of standard proteins. Dotted bands indicate the newly appearing proteins.

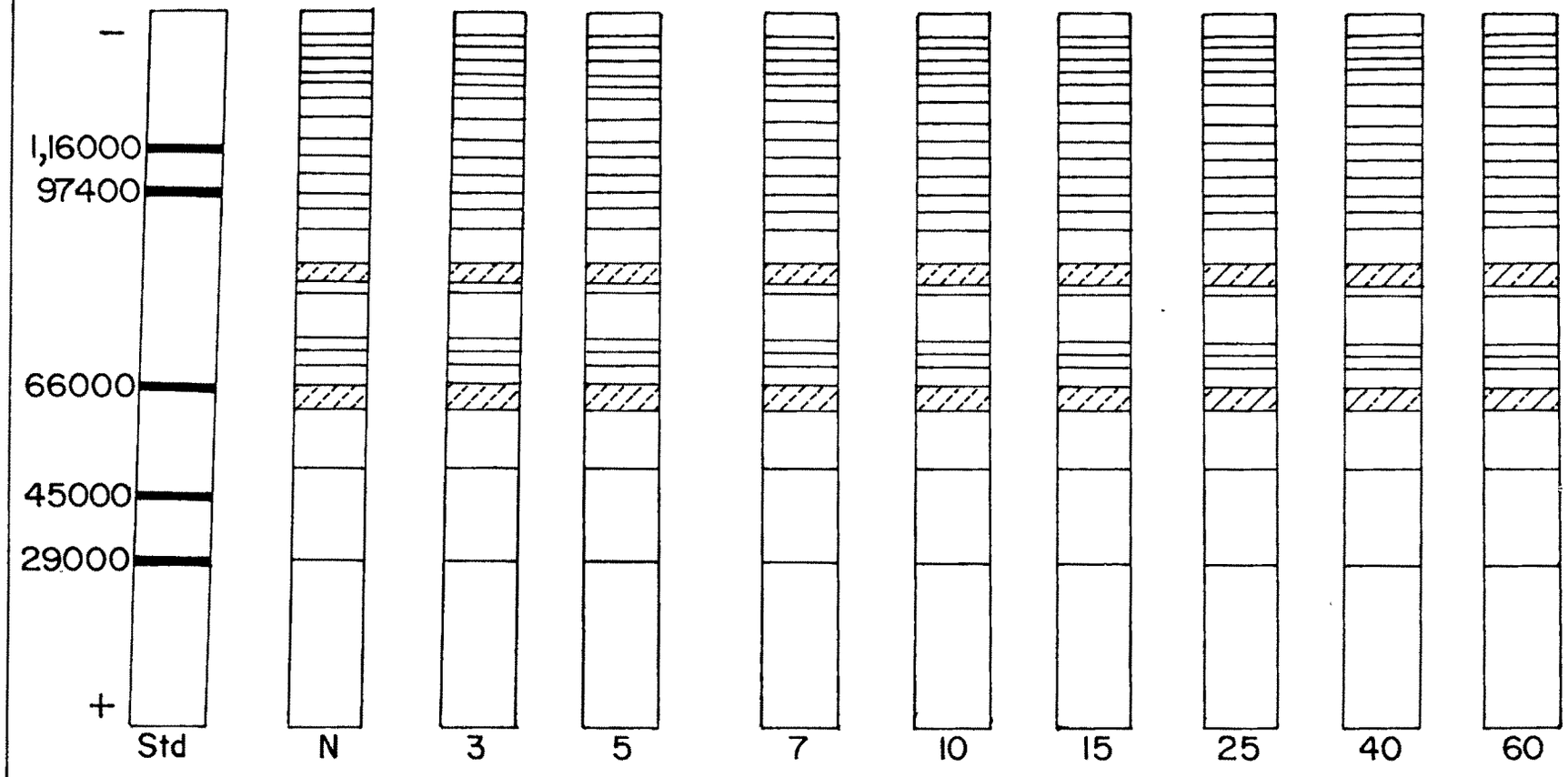

Fig. 6. Schematic electrophoretogram depicting hepatic protein profile of hypothyroidic H. flaviviridis during tail regeneration.

Lanes 2–10 represent various periods of regeneration in days.

Lane 1 is of standard proteins. Figures left of lane 1 represent molecular weights. of standard proteins.

of new protein bands. One new band of molecular weight 1,18000-1,20000 (between 8 and 9) appeared on the 3rd day and three more with molecular weights 1,00000 (between 15 and 16), 1,25000 (between 6 and 7) and 1,35000 (between 2 and 3) appeared on the 5th day of which the 1,25000 band disappeared on the 7th day. On day 15, protein band of molecular weight 1,35000 disappeared, while the 1,00000 molecular weight band was lost by the 40th day. However, by the 60th day two more protein bands appeared, one between 3 and 4 and the other between 12 and 13.

DISCUSSION

The present study on electrophoretic separation of proteins of tail, liver and skeletal muscle (femoral) both prior to and after caudal autotomy has revealed distinct alterations (both qualitative and quantitative), albeit differential, in the 3 tissues during the course of tail regeneration in H. flaviviridis. In general, the present study and the results obtained have relevance in two different contexts. The study itself is relevant in the context that it is the only one of its kind on lizard tail regeneration, though there are many such studies on amphibian limb regeneration, thus permitting a comparison of the regenerative mechanics between two classes. Such comparisons could reveal the underlying

unifying principles and/or dissimilarities if any that control the process of regenerative development.
In another context, the results obtained in the present study have given further credence to the theme that has been developed over the years from this laboratory of a definite supportive systemic participation for meeting the exigencies of vertebrate epimorphic regeneration.
The in loco changes in the protein profile obtained from the tail regenerate during the course of regeneration when compared with the anautotomised tail reveals the appearance of 4 new protein bands and loss of 6 old ones. It is quite logical to presume the new proteins appearing subsequent to autotomy to be regeneration specific, though their exact functions cannot be visualised. All these proteins are induced within the first 5 days post-autotomy and remain for varying periods of time. The differential longevity of the new proteins when equated with the time scale of regeneration seems to suggest their having involvement in the various developmental stages of regenerative ontogeny. Viewed in this perspective, the very short-living protein (3rd - 7th day) of approximate molecular weight 1,00000 could be associated with the regressive phase of regeneration characterised by wound closure, dedifferentiation and establishment of a blastema. The two transitory proteins making their appearance on

3rd and 5th day (mol. wts. 40000 and 42000) and remaining till the 25th day of regeneration could be accredited with the progressive phase of regeneration marked by proliferation and differentiation. Finally, the protein which was induced on the 3rd day and was constant for all post-autotomy periods (mol. wt. 70000-72000) could be considered as the morphogenetic guiding principle. Interestingly, lizard tail regeneration is also marked by loss of as many as 6 protein bands in a wide molecular weight range of 30000-130000 relative to the unautotomised tail. This loss of original tail proteins in the regenerate is understandable as the regenerated lizard tail is atypical in terms of reduced number of epidermal scales, loss of segmentation, the replacement of spinal cord by an ependyma and replacement of caudal vertebrae by a cartilaginous neural canal due to non-ossification. Apart from these qualitative differences, the general reduction in staining intensity of the protein bands during the first 10 days (except for the 5th day) and an increased intensity in the period between 15 and 25 days post-autotomy are indicative of the quantitative alterations in the synthesis of various tail proteins. Obviously, the increased staining intensity visible after the 10th day is reflective of the increased protein synthesis occurring concurrent to peak histodifferentiation.

The present findings when compared with the relatively more number of studies of this nature conducted on amphibians, reveals a commonness with respect to the appearance of new proteins during the regenerative process. However, the recorded number of new protein bands appearing post-amputation seems to show quite a bit of variation in the various studies. Whereas Garling and Tassava (1984) had observed the appearance of 4 new bands as in the present study, Schmidt (1966a) showed the appearance of as many as 10 new bands, 7 of them at the stage of transition from morphological indifference to initial histogenesis, and Dearlove and Stocum (1974) noted 5 new proteins. Variations with respect to the number of proteins disappearing subsequent to amputation as well as the number recovered at the end of regeneration are also evident. In this respect Garling and Tassava (1984) showed loss of only one protein permanently, while Schmidt (1966a) observed loss of as many as 12 proteins of which 9 were regained and Dearlove and Stocum (1974) of 5 bands all of which were however regained at the end. Apart from the differences in the number of proteins induced as well as lost during regeneration, the above studies on amphibians have also portrayed marked temporal difference with respect to appearance and disappearance of bands as well as their longevity.

The protein profile of liver and femoral muscle studied essentially due to the by now well recognised involvement of liver and muscle as part of the supportive systemic response, depicted noticeable changes providing further irrefutable evidence in favour of the significant systemic participation highlighted from this laboratory. The differential response of liver and muscle is indicated by the appearance of new protein bands in liver and loss in muscle post-caudal autotomy. Whereas 4 proteins were lost from the femoral muscle as early as 3rd day post-autotomy, an equal number appeared as new proteins in the liver by day 5. Except for the permanent loss of the most anodic protein (no. 30) all the other proteins were represented in the muscle at the end of regeneration though the disappearance and reappearance of a few proteins occurred in between. In contrast, the liver had 3 more protein bands on the 60th day of regeneration relative to the unautotomised state and the periods in between were marked by loss of the new bands acquired by the 5th day. These qualitative differences coupled with the quantitative alteration as marked by the decreased staining intensity of the major protein bands of liver and muscle denote elaboration of some specific proteins in liver and generalized protein catabolism from both liver and muscle as has been previously recorded and inferred (Chapter 2).

The loss of protein bands from liver and muscle might also suggest presence of labile pools of protein for meeting emergency exigencies.

Hypothyroidism *per se* reduced the number of stainable protein bands substantially in all the three tissues in the unautotomised condition. As many as 14 bands were lost from both tail and liver while 8 were lost from the muscle. None of these proteins reappeared at any stage post-caudal autotomy. Post-autotomy, the tail did show the appearance of the 2 long-living regeneration specific proteins exactly in the same temporal sequence relative to the euthyroidic lizards. The fact that the permanent regeneration specific protein was not detectable at all and the short living one only very feebly indicates the induction of these regeneration specific proteins at concentrations so low, that they are beyond the level of resolution of the present technique. The poor quality of the regenerate as well as the retardation in regeneration observed in hypothyroidic lizards previously (Chapter 4 and Ramachandran *et al.*, 1984) may find relevance in the present context of quantitatively reduced content of regeneration specific proteins. Apart from this, the tail protein pattern did not depict any difference relative to the unautotomised state all throughout. Similarly, the liver pattern too remained unchanged at all stages post-autotomy.

The only qualitative change recorded was in the muscle involving loss of 3 bands by the 3rd day post-autotomy of which two were regained by the 5th day itself and the remaining one by the 40th day. However, differences in staining intensity of the protein bands could be discerned in all the three tissues. Apparently, hypothyroidism *per se* does not seem to have any qualitative influence on the *in loco* mechanisms underlying regeneration though quantitative effects are evident. In this perspective the retardation in regeneration as well as the poor quality of the regenerate might indicate an indirect effect of hypothyroidism. This indirect effect of hypothyroidism could be visualised in terms of loss of systemic store of labile proteins as well as its suppressive influence on regeneration specific adaptive systemic protein metabolism as can be inferred from the present observations on the protein profile of liver and muscle.

SUMMARY

In loco (tail) and systemic (liver and muscle) alterations in protein profile during tail regeneration in euthyroidic and hypothyroidic *H. flaviviridis* have been analysed electrophoretically. Hypothyroidism

significantly reduced the total number of stainable protein bands in all the three tissues prior to autotomy. Post-caudal autotomy during the course of tail regeneration, qualitative and quantitative alterations were discernible in the protein profile of all the three tissues in euthyroidic lizards. Appearance of 4 'new' proteins and loss of 6 were the characteristic feature in the regenerate. Differential response of the liver and muscle was marked by appearance of 'new' protein bands in the liver and loss in the muscle during the course of regeneration. Hypothyroidic lizards showed appearance of two regeneration specific proteins in the tail. Relative to the unautotomised state where as the muscle showed some minor alterations, the liver did not depict any alterations in the hypothyroidic condition. These observations suggest that hypothyroidism does affect the qualitative and quantitative protein profile of liver and muscle while it is ineffective in preventing the appearance of some of the regeneration specific proteins in the tail.