

Ascorbic acid in the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*

By R. V. SHAH, P. K. HIRADHAR AND D. K. MAGON

*From the Division of Developmental Physiology, Department of Zoology,
M.S. University of Baroda, Baroda 2, India.*

SUMMARY

The concentration of ascorbic acid (AA) and the histochemical distribution of the vitamin in the normal and regenerating tail of the gekkonid lizard, *Hemidactylus flaviviridis*, have been investigated.

In the regenerating tail of the lizard the AA concentration almost doubles during wound healing and becomes fivefold during differentiation. However, it falls almost to the normal level during the blastema phase (i.e. period between wound healing and differentiation). Again, during the growth period (i.e. after differentiation) the AA concentration gradually becomes reduced, reaching the normal mark as the regenerate regains the full length of the original tail. Nevertheless, the vitamin level does not fall below the normal mark at any stage of regeneration. Increase of ascorbic acid during wound healing is thought to be mainly due to increased demand for the vitamin at the broken ends of the stump tissues, for their repair and formation of wound epithelium; the vitamin is known to help these processes. A fivefold increase of the vitamin during the differentiation period corresponds to an increased pace of laying down of the matrix material for the connective tissues, suggesting the role of ascorbic acid in the formation of collagen and mucopolysaccharides. Besides, the role of ascorbic acid in lipid and carbohydrate metabolism is also important during tail regeneration. Fluctuations in the vitamin level during different phases of tail regeneration are correlated with various states of metabolic activities of the corresponding phases.

INTRODUCTION

Recent studies on general morphological and histological aspects of the regenerating lacertilian tail include the works of Quattrini (1954) on *Lacerta sicula*; Hughes & New (1959) on *Sphaerodactylus* and Moffat & Bellairs (1964) on *Lacerta vivipara*. Only during the last two decades has work on biochemical and experimental aspects of reptilian regeneration been undertaken. Kamrin & Singer (1955) reported the influence of the nerve cord in the tail regeneration of *Anolis carolinensis*, while Simpson (1964) suggested that ependyma greatly influences the tail regeneration of *Lygosoma laterale*. Licht (1967) studied the effect of certain hormones on the growth and tail regeneration in the lizard *A. carolinensis*. Maderson & Licht (1968) reported the influence of temperature and nutrition on the regenerating tail in the same lizard. Shah & Chakko (1966, 1967a, b; 1968a, b; 1969) and Shah & Magon (1969) studied histochemical and some of the biochemical aspects of tail regeneration in the gekkonid lizard

Hemidactylus flaviviridis. Recently Cox (1969) with the help of autoradiographic study of the regenerating tail of *A. carolinensis* has tried to solve the much debated question of origin of the mesenchyme cells of the blastema in lizard tail regeneration.

The process of regeneration is an assemblage of various morphological changes which reflect the metabolic activities during the process. It is well established that restoration of the lost part is from the aggregated non-differentiated or differentiated mass of cells. The process demands a large amount of energy for the breakdown of metabolites and also for the ultimate building up of new material which obviously calls for very much increased metabolic activities in the tissues involved.

Tissues possessing high metabolic activities are known to contain AA in an appreciably high concentration. An active participation of AA in the metabolic activities had been suggested by Burns, Burch & King (1951) because of its higher concentration in tissues than in blood. A higher level of AA in the regenerating axolotl limbs was observed by Ryvkina (1940) as compared to that in the normal ones. Brachet (1950) suggested that AA promotes differentiation rather than growth. Gould (1963) stressed the role of AA in collagen synthesis and its importance in regeneration and repair.

Although scattered information regarding the role of AA during regeneration, as cited above, is available, the knowledge regarding its exact significance is yet to be gathered. So far, no reports regarding the study of AA in regenerating appendages of reptiles are known to the authors. Therefore, the present investigation was undertaken to determine quantitatively the levels of AA and to study its distribution histochemically in the normal and regenerating tail of the lizard, *Hemidactylus flaviviridis*.

MATERIAL AND METHODS

The lizards, *H. flaviviridis*, collected from the university campus, were maintained in the laboratory on a diet of insects. Autotomy was induced by pinching off the normal and regenerating tails. The cut surfaces of the autotomized tails were blotted free of blood and tissue fluids. The tails were then immediately fixed on the chuck of a microtome in a cryostat maintained at -20°C . Sections of $12\ \mu\text{m}$ thickness were cut. Histochemical demonstration of AA was carried out according to the method of Giroud & Leblond (1936) as modified by Chinoy (1969*a, b*). For control, some of the sections were devitaminized by keeping them for 3–4 h in 10% neutral formalin before staining.

For quantitative estimations of AA, the autotomized tails were quickly weighed and homogenized in 6% TCA in prechilled mortars. Aliquots of these extracts were utilized for the determination of AA levels in the normal and regenerating tails, employing the dinitrophenyl hydrazine method of Roe *et al.* (Roe, 1954).

RESULTS

Normal tail

In the normal tail a very high concentration of AA was noticed in the epidermal cells, epidermal basement membrane, dermis, epimysium, perimysium, endomysium and muscle fibres while the rest of the tissues, namely subcutaneous and submuscular adipose tissue, osteocytes, chondrocytes of the intervertebral cartilage, periosteum, perichondrium and nerve cord showed relatively less vitamin content (Figs. 1, 2).

Regenerating tail

After autotomy the wound-healing phase presented a picture where AA was noticed to be in much higher concentration in the wound epithelium and sub-apical cells than that which was observed in the epidermal cells of the normal tail skin (Fig. 3). It was noticed that tissues under repair at the cut end of the tail stump also showed a similarly high content of AA. During the blastema phase the AA level remained quite high in the stratified epithelium and the blastema cells close to the cut end of the stump, while its concentration in the mesenchyme cells adjacent to the cut end of vertebra, spinal cord and dermis was much higher (Fig. 4). As the differentiation in the regenerate started, the AA content in the differentiating tissues, namely mononuclear myoblasts, chondroblasts, cells of dermis and stratum germinativum showed almost a fivefold increase (Figs. 5, 6; Table 1). The undifferentiated regions (at the distal part of the regenerate), however, revealed relatively lower concentration of AA but its

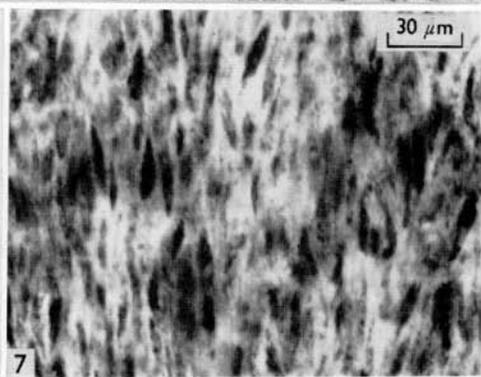
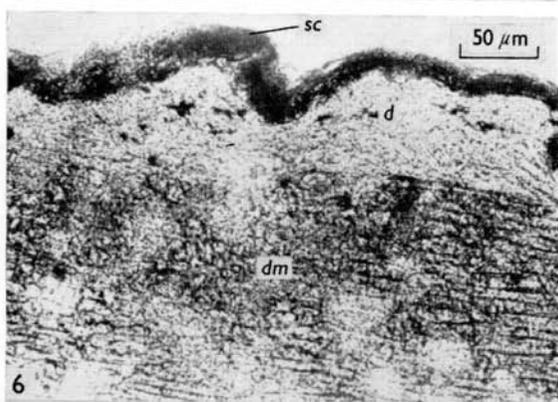
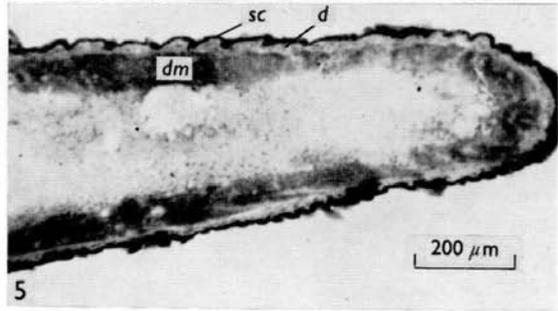
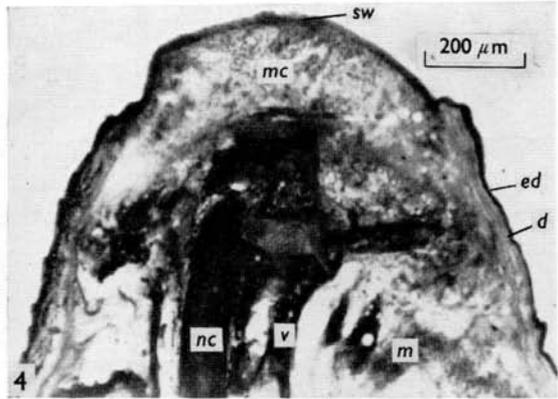
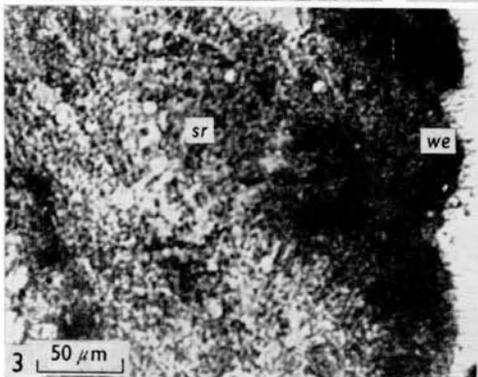
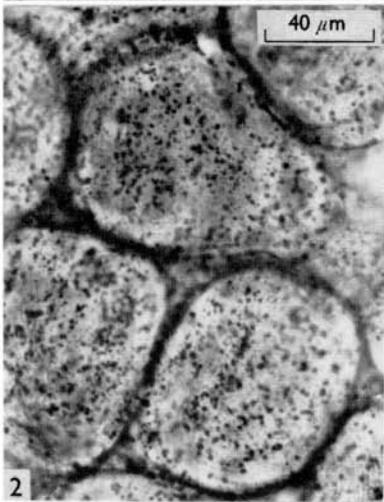
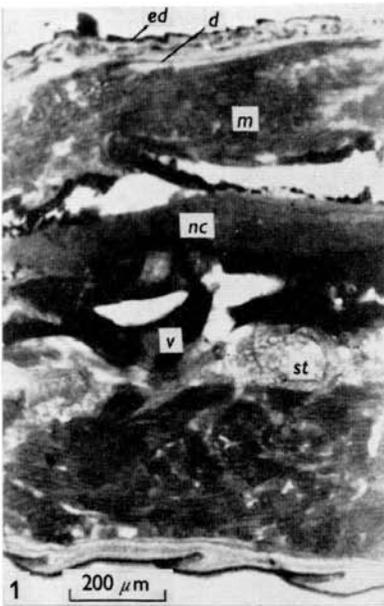
Table 1. *Levels of ascorbic acid in the normal and regenerating tail of the house lizard, Hemidactylus flaviviridis*

Normal tail and different phases of regenerating tail	Amount of AA (mg/100 g fresh tissue)	No. of animals used per experiment
Normal tail	4.881 ± 1.307 ‡	12
Regenerating tail:*		
Wound healing phase (4 days) †	8.196 ± 0.2682	12
Blastema phase (10 days) †	4.925 ± 1.742	10
Differentiation phase (18 days) †	25.36 ± 2.274	11
Growth phase (28 days) †	14.14 ± 1.11	10
Fully regenerated tail (70 days) †	5.312 ± 1.213	12

* The phases of regeneration are arbitrarily defined for the purpose of discussion, though the process of regeneration is a continuous one.

† Number of days after tail autotomy.

‡ Mean ± s.d.



level was as much as that which was observed in the blastema cells. AA concentration remained unchanged as myoblasts differentiated into myocytes and then in turn into myofibres. Nevertheless, at this stage AA was more evident in the interfibrillar regions than in the cells themselves. In the chondroblasts the AA content increased as they transformed into chondrocytes and further as the matrix was laid down (Fig. 7). Newly forming adipose tissue at the subcutaneous and submuscular regions showed higher localization of AA as compared to that seen in the same tissues of the normal tail.

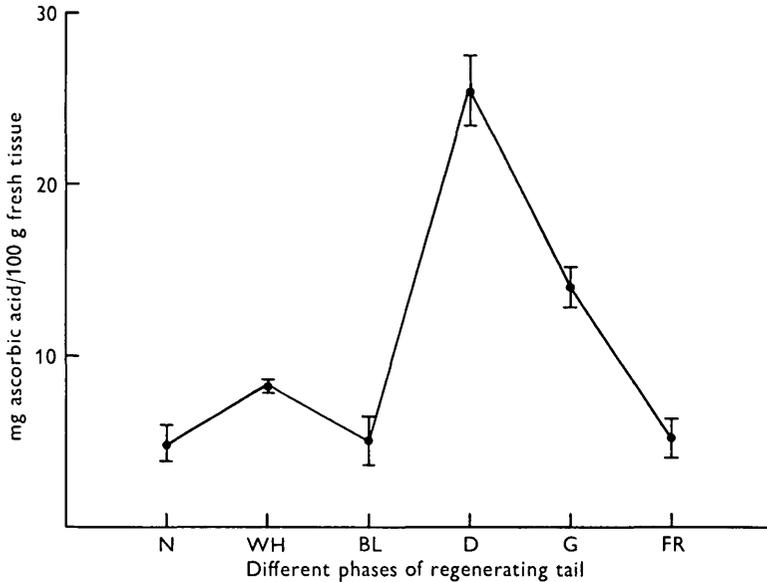


Fig. 8. Levels of AA in the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. N, Normal; WH, wound healing phase; BL, blastema phase; D, differentiation phase; G, growth phase; FR, fully regenerated tail.

Abbreviations on figures: *d*, dermis; *dm*, differentiating muscle; *ed*, epidermis; *m*, muscle; *mc*, mesenchyme cells; *nc*, nerve cord; *sc*, scale; *sr*, subapical region; *st*, submuscular adipose tissue; *sw*, stratified wound epithelium; *v*, vertebral column; *we*, wound epithelium.

Fig. 1. L.S. of normal tail showing ascorbic acid (AA) concentration in the epidermis, dermis, muscle, nerve cord and vertebral column.

Fig. 2. T.S. of caudal muscle of normal tail denoting high localization of AA.

Fig. 3. L.S. of regenerating tail (wound healing phase). Note a high level of AA in the wound epithelium and subapical region.

Fig. 4. L.S. of regenerating tail (blastema phase) showing AA localization.

Fig. 5. L.S. of regenerating tail (differentiation phase). Note a high concentration of AA in the epidermis, dermis and differentiating muscle.

Fig. 6. A higher magnification of Fig. 5, showing a high level of AA in the epidermis, dermis and differentiating muscle.

Fig. 7. Chondrocytes showing a high concentration of AA.

In the regenerate, differentiating parts of skin, namely dermis and different layers of epidermis, AA was more evident in fibroblasts, along the dermal fibres and in the cells of the stratum germinativum. Nevertheless, AA was localized in appreciable amounts in all the epidermal cells except the outer beta cells of the older epidermal layers of the skin (see Maderson, 1966). The ependyma and mesogial cells showed a high concentration of AA. When the regenerate was fully differentiated and later during the growth period, AA content in the growing tissues remained high, but was noticeably lower than that seen in the corresponding parts during the previous phase of the tail regeneration.

The quantitative biochemical data obtained from the study of AA in the normal and regenerating tail of *H. flaviviridis* are in conformity with the histochemical observations on AA in the tissues of the normal and regenerating tail of the lizard (Fig. 8; Table 1).

DISCUSSION

AA is known to be linked with lipid and carbohydrate metabolisms (Banerjee & Ghosh, 1947; Banerjee & Ganguli, 1962). It has been shown by Rusch & Kline (1941) that the presence of AA in a cell is correlated with breakdown of phospholipids or easy mobilization of lipids. From the study of the Krebs cycle enzymes and AA in scorbutic guinea-pigs, Banerjee, Biswas & Singh (1959) concluded that AA is essential for proper functioning of the Krebs cycle. Chinoy (1969*a*) histochemically demonstrated the presence of AA in the pectoralis muscle of some birds wherein the red fibres were greatly loaded with AA as compared with the white ones. She correlated the role of AA with higher metabolic activities of the red fibres which are known to be loaded with large amounts of lipids, mitochondria and oxidative enzymes (George & Berger, 1966). She further suggested that in the red fibres of the pectoralis muscle of birds, AA plays an important role in the energy transfer mechanisms by its transformation into its free radical – monodehydroascorbic acid – which is a more powerful electron donor than AA.

The presence of AA in almost all the tissues of the normal tail where lipids and oxidative enzymes are also present (Chakko, 1967; Shah & Chakko, 1969; Shah & Magon, unpublished) appears to be correlated with their higher metabolic activities.

The role of AA in carbohydrate metabolism has been well explained by Banerjee & Ganguli (1962) in the scorbutic mammals. They suggested that AA influences hexokinase activity. In the lizard tail, Shah & Chakko (1967*b*) have reported a moderate amount of glycogen in the cells of the stratum germinativum in skin while peripherally situated muscle fibres showed a relatively higher content of the glycogen than the ones situated farther from the surface. In the muscle fibres of the tail where the glycogen content is relatively higher than that of the other tissues, a correspondingly high concentration of AA was also noticed. AA in these muscle fibres, by its influence on hexokinase activity,

could facilitate increased uptake of glucose by the cells from circulating blood, which might ultimately lead to synthesis of glycogen. This might also explain the coexistence of high concentrations of glycogen and AA in the peripherally situated muscle fibres.

It has been suggested that AA plays a very prominent role in the healing of wounds (Bourne, 1953). Lauber & Rosenfeld (1938) reported that when mammals with partial vitamin C deficiency were wounded, mobilization of AA from the other tissues and organs to the site of wound was noticed during the wound healing. Bartlett, Jones & Ryan (1942*a, b*) suggested that accumulation of fibroblasts and leucocytes at the wound site may be due to the high accumulation of AA there. Similarly high levels of AA in healing wounds have been reported by several other workers (Zamanskii & Lopushanskii, 1955; Schauble, Chen, Postlethwaite & Dillon, 1960; Crandon, Lennihan, Mikal & Reif, 1961). Accumulation of AA in a period shortly following incision was noted by Abt & Von Schuching (1961). Ksabyan (1956) noticed a threefold increase of AA content in the skin of albino mice in a couple of days after inflicting wounds. Similar observations in the present study support the contention that a higher concentration of AA greatly aids the process of wound healing. The requirement for AA occurs not only during the wound healing but also afterwards to provide tensile strength to the scar tissue, which in turn depends on the connective tissue formation at the site, so that it does not rupture (Bartlett, Jones & Ryan, 1942*a, b*). Abt, Von Schuching & Roe (1959) reported that the abdominal wounds in guinea-pigs allowed to heal for a long period could be ruptured on development of scurvy. Thus the formation and maintenance of connective tissue of the scar is to a certain extent dependent on AA. Since mucopolysaccharides and collagen are abundantly synthesized during the wound healing in the autotomized tail (Shah *et al.*, unpublished), one can safely say that AA could be well involved in the formation of connective tissue and in general wound healing. Nevertheless, the fact must be borne in mind that here the wound healing is not the end of this fascinating phenomenon of regeneration which is switched on as a result of autotomy, but it is just the beginning of the dynamic process of regeneration.

High AA concentration in the differentiating cartilage and muscle is quite logical, as in the former a large amount of matrix material is to be formed. As stated earlier, such matrix material can be easily formed when an adequate amount of AA is available. Thus, AA may well be involved in the formation of epimysium, perimysium and endomysium during the muscle regeneration where collagen fibre formation takes place. For the muscle fibres, AA may be helping in the metabolic activities of the cells, where lipid and glycogen are involved.

A fall in the level of AA soon after the differentiation phase (Table) strengthens Brachet's view (1950) that AA promotes differentiation rather than growth. Nevertheless, it could be said that AA does help growth, as it is evident from

the present observations that AA level is about 2–2.5 times more during the growth period than that found in the fully regenerated and normal tail.

Once the regenerate reaches its full-grown state, i.e. achieves the full length of the original tail (by about 70 days after autotomy), its AA concentration comes in line with that noticed in the normal tail. This perhaps marks the completion of the process of tail regeneration where the active morphologic and metabolic activities have more or less settled to the normal pace.

REFERENCES

- ABT, A. F., SCHUCHING, S. VON and ROE, J. H. (1959). *Bull. Johns Hopkins Hosp.* **104**, 163. (Cited from G. C. Chatterjee (1967).)
- ABT, A. F. & SCHUCHING, S. VON (1961). *Ann. N.Y. Acad. Sci.* **92**, 148 (Cited from G. C. Chatterjee (1967).)
- BANERJEE, S. & GHOSH, N. C. (1947). Relation of scurvy to glucose tolerance test, liver glycogen and insulin content of pancreas in guinea pigs. *J. biol. Chem.* **168**, 207–211.
- BANERJEE, S., BISWAS, D. K. & SINGH, H. D. (1959). Dehydrogenase activity of the tissue in scurvy. *J. biol. Chem.* **234**, 405–408.
- BANERJEE, A. B. & GANGULI, N. C. (1962). Metabolic studies on scorbutic guinea pigs. 2. Hepatic glycogen synthesis *in vitro* and *in vivo*. *J. biol. Chem.* **237**, 14–18.
- BARTLETT, M. K., JONES, C. M. & RYAN, A. E. (1942*a*). Vitamin C and wound healing. I. Experimental wound in guinea pigs. *New Engl. J. Med.* **226**, 469–473.
- BARTLETT, M. K., JONES, C. M. & RYAN, A. E. (1942*b*). Vitamin C and wound healing. II. Ascorbic acid content and tensile strength of healing wounds in human beings. *New Engl. J. Med.* **226**, 474–481.
- BOURNE, G. H. (1953). *Biochemistry and Physiology of nutrition*, vol. 2, p. 86. New York: Academic Press. (Cited from G. C. Chatterjee (1967).)
- BRACHET, J. (1950). *Chemical Embryology*. New York. (Cited from A. E. Needham (1952).)
- BURNS, J. J., BURCH, H. B. & KING, C. G. (1951). The metabolism of I-C¹⁴ L-ascorbic acid in guinea pigs. *J. biol. Chem.* **189**, 203.
- CHAKKO, T. V. (1967). Ph.D. thesis entitled 'A histological and histochemical study of the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*', submitted to the M.S. University of Baroda 2, India.
- CHATERJEE, G. C. (1967). Effects of ascorbic acid deficiency in animals. In *The Vitamins*, vol. 1. (eds Sebrell & Harris). New York: Academic Press.
- CHINYOY, N. J. (1969*a*). Source of electron energy for animal metabolism. I. Role of ascorbic acid in metabolism of avian muscle. *Histochemie* **19**, 125–128.
- CHINYOY, N. J. (1969*b*). On the specificity of alcoholic acidic silver nitrate reagent for the histochemical localization of ascorbic acid. *Histochemie* **20**, 105–107.
- COX, P. G. (1969). Some aspects of tail regeneration in the lizard *Anolis carolinensis*. I. A description based on histology and autoradiography. *J. exp. Zool.* **171**, 127–150.
- CRANDON, J. H., LENNIHAN, R. Jr., MIKAL, S. & REIF, A. E. (1961). *Ann. N.Y. Acad. Sci.* **92**, 246. (Cited from B. S. Gould (1963).)
- GEORGE, J. C. & BERGER, A. J. (1966). *Avian Myology*. New York: Academic Press.
- GIROUD, A. & LEBLOND, C. P. (1936). *L'acido ascorbique dans les tissus et sa détection*. Paris: Herman Press.
- GOULD, B. S. (1963). Collagen formation and fibrogenesis with special reference to role of ascorbic acid. *Int. Rev. Cytol.* **15**, 301–361.
- HUGHES, A. & NEW, D. (1959). Tail regeneration in the gekkonid lizard, *Sphaerodactylus*. *J. Embryol. exp. Morph.* **7**, 281–302.
- KAMRIN, R. P. & SINGER, M. (1955). The influence of the spinal cord in regeneration of the tail of the lizard, *Anolis carolinensis*. *J. exp. Zool.* **128**, 611–627.
- KSABYAN, S. S. (1956). *Ark. Patol.* **18**, 91. (Cited from B. S. Gould (1963).)

- LAUBER, H. J. & ROSENFELD, W. (1938). *Klin. Wochschr.* **17**, 1587. (Cited from B. S. Gould (1963).)
- LICHT, P. (1967). Interaction of prolactin and gonadotropins on appetite, growth and tail regeneration in the lizard *Anolis carolinensis*. *Gen. comp. Endocr.* **9**, 49-63.
- MADERSON, P. F. A. (1966). Histological changes in the epidermis of the Tokay (*Gecko gecko*) during the sloughing cycle. *J. Morph.* **119**, 39-50.
- MADERSON, P. F. A. & LICHT, P. (1968). Factors influencing rates of tail regeneration in lizard, *Anolis carolinensis*. *Experientia* **24**, 1083.
- MOFFAT, L. A. & BELLAIRS, A. d'A. (1964). The regenerative capacity of the tail in embryonic and post natal lizard (*Lacerta vivipara* Jacquin). *J. Embryol. exp. Morph.* **12**, 769-786.
- NEEDHAM, A. E. (1952). *Regeneration and Wound Healing*. New York: John Wiley.
- QUATTRINI, D. (1954). *Arch. ital. Anat. Embriol.* **54**, 226. Cited from S. B. Simpson, Jr., Regeneration of the Lizard Tail. In: *Regeneration of Animals and Related Problems*. Eds V. Kiortsis and H. A. L. Trampusch (1965). Amsterdam: North Holland Publishing Company.
- ROE, J. H. (1954). Chemical determination of ascorbic and dehydroascorbic and diketogulonic acid. In *Methods of Biochemical Analysis*, vol. 1 (ed. D. Glick.) New York: Interscience Publishers.
- RUSCH, H. P. & KLINE, B. E. (1941). *Cancer Res.* **1**, 465 (Cited from Sebrell & Harris (1967).) In *The Vitamins*, vol. 1. New York: Academic Press.
- RYVKINA, D. E. (1940). *C.r. Acad. Sci. URSS* **27**, 380. (Cited from A. E. Needham (1952).)
- SCHAUBLE, J. F., CHEN, R., POSTLETHWAITE, R. W. & DILLON, M. L. (1960). *Surg. Gynecol. Obstet.* **110**, 314. (Cited from B. S. Gould (1963).)
- SHAH, R. V. & CHAKKO, T. V. (1966). Histochemical localization of acid phosphatase in the adult normal and regenerating tail of house lizard, *Hemidactylus flaviviridis*. *J. anim. Morph. Physiol.* **13**, 169-188.
- SHAH, R. V. & CHAKKO, T. V. (1967a). Histochemical localization of alkaline phosphatase in the adult normal and regenerating tail of house lizard, *Hemidactylus flaviviridis*. *J. anim. Morph. Physiol.* **141**, 69-88.
- SHAH, R. V. & CHAKKO, T. V. (1967b). Histochemical localization of glycogen and phosphorylase in the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. *J. anim. Morph. Physiol.* **14**, 257-264.
- SHAH, R. V. & CHAKKO, T. V. (1968a). Histological observations on the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. *J. anim. Morph. Physiol.* **15**, 29-39.
- SHAH, R. V. & CHAKKO, T. V. (1968b). Preliminary observations on the changes in the thyroid activity in the house lizard, *Hemidactylus flaviviridis*, during the different phases of regeneration. *J. biol. Sci.* **11**, 41-44.
- SHAH, R. V. & CHAKKO, T. V. (1969). Histochemical localization of succinate dehydrogenase in the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. *J. anim. Morph. Physiol.* **16**, 88-96.
- SHAH, R. V. & MAGON, D. K. (1969). Histochemical demonstration of α -glycerophosphate dehydrogenase in the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. *J. anim. Morph. Physiol.* **16**, 97-105.
- SIMPSON, S. B. (1964). Analysis of tail regeneration in the lizard, *Lygosoma laterale*. I. Initiation of regeneration and cartilage differentiation: the role of ependyma. *J. Morph.* **114**, 425-436.
- ZAMANSKII, L. N. & LOPUSHANKII, A. I. (1955). *Ukrain. Biokhim. Zbur.* **27**, 25. (Cited from B.S. Gould (1963).)

(Manuscript received 24 November 1970, revised 18 January 1971)