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Vitamin A-mediated homeotic transformation and histological changes in amputated tail tissues of tadpoles of *Rana tigerina*

P. Das* and P. Mohanty-Hejmadi[†]

Institute of Life Sciences, NALCO Square, Chandrasekharapur, Bhubaneswar 751 023, India

[†]Department of Zoology, Utkal University, Bhubaneswar 751 004, India

The most recent and dramatic effect of vitamin A in tadpoles is the homeotic transformation of tail to limbs, which is characterized by the development of ectopic limbs at the site of tail amputation. The morphological changes brought about by vitamin A after tail amputation are quite striking and amazing. Histological changes include thickening of the epidermis, enlargement of the notochord and the nerve cord, thickening of the sheath covering the notochord and the nerve cord, clumping of the muscles and, above all, development of ectopic limbs from the amputated

tail end. In this communication, the histological changes in the tail tissue after vitamin A treatment are discussed with reference to homeotic transformation.

EVER since the discovery of vitamin A in 1909 (ref. 1) and compounds of related structure and function (the 'retinoids'), numerous workers started probing the amazing nature of vitamin A. To head the list were Niazi and Saxena², who first discovered that vitamin A has an inhibitory and modifying influence on tail regeneration in the tadpoles of *Bufo andersonii*. Since then, there have been several subsequent reports on the effects of vitamin A on limb development and regeneration^{3–7}. It was in 1992 that the phenomenon of homeotic transformation was first reported by Mohanty-Hejmadi *et al.*⁸ in *Uperodon systoma*, later being confirmed in other species^{9–11}. This led us to explore the changes in the tail tissue following vitamin A treatment, in greater detail. Besides, the tail in anurans has received little attention probably because of its transitory nature. Although extensive work has been done on the histological effects of vitamin A on limb development and regeneration^{12,13}, the histological effects of vitamin A on tail regeneration have not been dealt with. Examining the histological changes will improve our understanding of vitamin A-induced homeotic transformation, where the tail tissues are converted to limb tissues. In *Rana tigerina*, the ectopic limbs were stunted and not well-developed unlike the ectopic limbs of *Polypedates maculatus*, where they resembled the normal limbs with well-differentiated digits. It was therefore mandatory to study the histological changes in the tail tissues and ectopic limbs of *R. tigerina* after vitamin A treatment.

Egg masses of the Indian bull frog, *R. tigerina* were collected from nature during the rainy season and raised in the laboratory up to the hindlimb bud stage. The tadpoles were fed with boiled egg and *Amaranthus ad libitum*. Prior to amputation, the tadpoles were anaesthetized in 1:400 solution of MS 222 (tricaine methanesulphonate, Sigma), amputated in the middle of their tails and reared in conditioned water containing vitamin A 10 IU/ml (Arovit, Roche). For morphology, the tadpoles were treated for 24, 48, 72, 96 and 120 h. After the treatment period, they were reared in aerated, conditioned water and fixed in formalin only after forelimb emergence or after their death (whichever was earlier). The controls were also reared in a similar way, but without vitamin A treatment. For histology, the tadpoles were treated for 72 h (3 days), since morphological studies showed that 72 h was the optimum time to induce maximum effect in the anurans. Following treatment, the tadpoles were removed, anaesthetized, their regenerated tails amputated and fixed in aqueous Bouin's fluid on days 1, 2, 3, 4, 5, 6, 10, 15 and 25 (i.e. both during and beyond the period of treatment). The controls were also fixed on

*For correspondence. (e-mail: pragdas@rediffmail.com)

the corresponding days. Four tadpoles from the experimental group and two from the control group were examined at each interval as specified, and then fixed. The fixed tissues were then embedded in molten paraffin (m.p. 58–60°C), serially sectioned either longitudinally or transversely (depending upon the orientation of the morphological abnormality) at 10 μ m thickness and stained with Mallory's triple stain for examination in the light microscope.

Vitamin A induced several morphological abnormalities in the tail tissue after treatment from 24 to 120 h post-amputation. These abnormalities include develop-

ment of a bulbular mass at the amputated tail end (Figure 1*a, b*), formation of a blunt tail tip (Figure 1*c*), development of an abnormal protrusion from the distal end of the tail (Figure 1*d*), downwardly curved axial tissue (Figure 1*e*), upwardly curved axial tissue (Figure 1*f*), and more importantly, development of homeotic limbs at ectopic sites (Figure 1*g, h*). All these anomalies were visible externally, 8–10 days post-amputation. With so many morphological changes brought about by vitamin A, it was essential to study the detailed histological changes brought about by vitamin A in the tail at the cellular level.

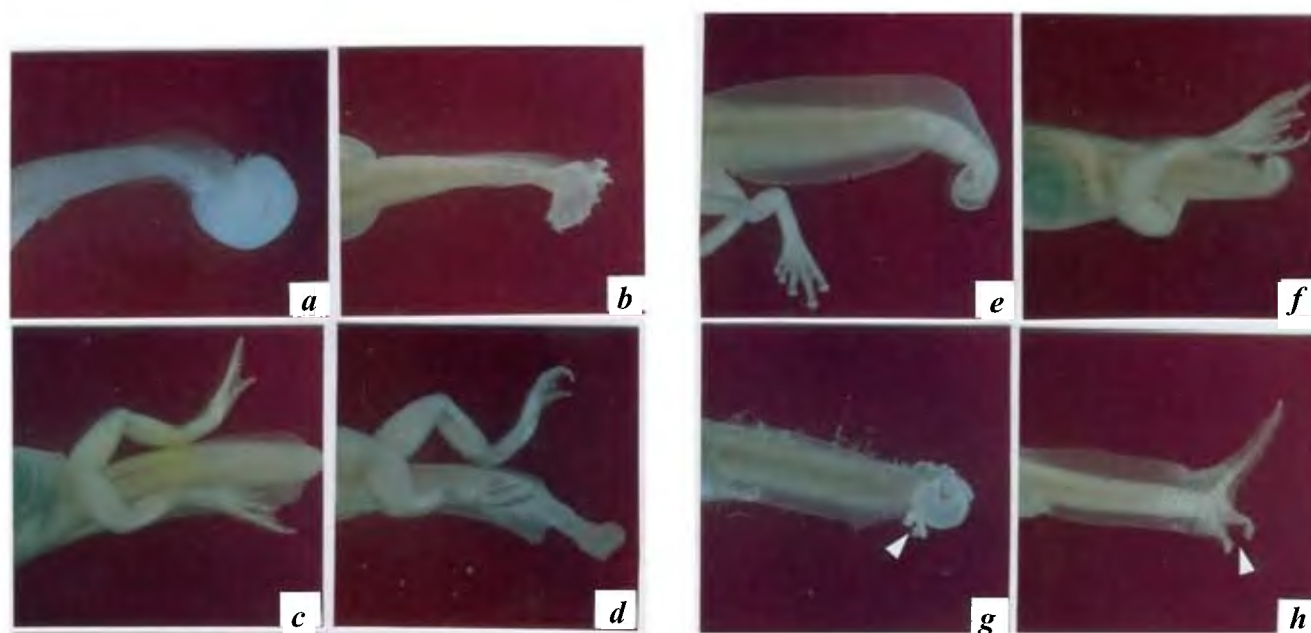


Figure 1. *a*, Large bulbular mass in tail of *R. tigerina* after vitamin A treatment; *b*, Small bulbular mass; *c*, Blunt tail tip; *d*, Abnormal protrusion at tail end; *e*, Downwardly curved axial tissue; *f*, Upwardly curved axial tissue; *g*, Pair of ectopic limbs (arrowhead) in initial stage of development seen arising from bulbular mass at distal end of the tail on day-14; and *h*, Ectopic limbs (arrowhead) at a slightly advanced stage of development on day-25. Tail fins are suppressed in *b, f* and *g*. *a-h*, magnification $\times 15$.

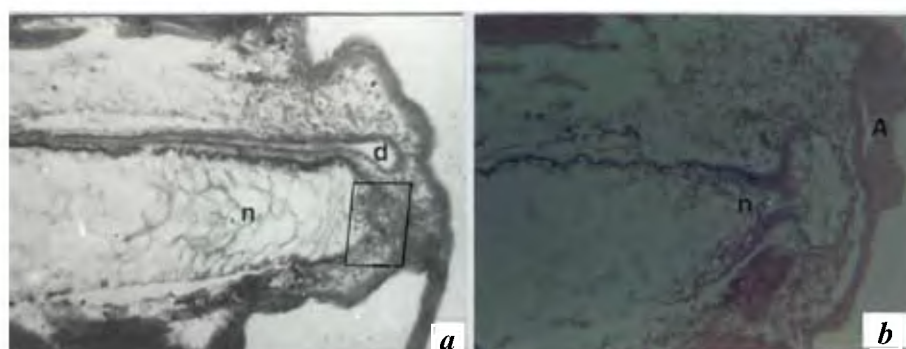


Figure 2. Longitudinal section (L.S.) of tail of *R. tigerina*: *a*, Control, one day post-amputation (p.a.). Notochord (n) is constricted, nerve cord (d) is vesicular, chorda cells are dedifferentiated (in box). AEC is not seen in this section, $\times 150$; *b*, Treated, one day p.a. AEC (A) is present, notochord (n) is constricted. Nerve cord is not seen in this section. There is not much difference between the control and treated tails; $\times 150$.

The histology of the tail tissues was studied from day-1 post-amputation up to the development of ectopic limbs. On day-1, in both the control (Figure 2a) and the treated tadpoles (Figure 2b), an apical epidermal cap (AEC) developed at the distal end of the amputated tail to close the wound surface. The epidermis was single-layered. The chorda cells of the notochord were dedifferentiated. The nerve cord was vesicular at the distal end. Muscles were absent in the regenerating tail tips. Thus, there was no difference between the control and the treated tails by day-1 post-amputation. On day-2, in the controls the AEC was no longer visible, as the wound had healed completely and tail regeneration was almost complete (Figure 3a). The epidermis was single-layered, the notochord was covered by a thin sheath. The nerve cord was small and also covered by a thin sheath. Muscles were beginning to redifferentiate and were arranged laterally in small bundles beneath the epidermis. In the treated tails (Figure 3b), the notochord was enlarged and covered by a thick notochordal sheath. The nerve cord was also enlarged and enveloped by a thick sheath. Muscles were in a dedifferentiated state. On day-3, in the controls regeneration was almost complete (Figure 4a); the epidermis was single-layered. The notochord, nerve-

cord and muscles had all redifferentiated and were therefore normal. The notochord and nerve cord were thin and slender as in normal tails because they had regenerated completely. Muscles were arranged in well-defined bundles and were present as V-shaped myotomes. In the treated animals, the epidermis was thick (Figure 4b). The notochord was enlarged and was somewhat irregular in shape, being covered by a thick sheath. The nerve cord was also covered by a thick sheath and muscles were dedifferentiated. On days 4–6 in the controls, all the tissues were normal and similar to those on day-3. The epidermis was multilayered. The notochord and the nerve cord were covered by thick sheaths. Muscles remained dedifferentiated up to day-4, and by day-6 though they had redifferentiated, they were not arranged in their characteristic bundles. Instead, they remained unorganized and clumped as dense masses and nearly covered the whole region (Figure 5a–c). On days 10–14, in the treated group, a large bulbular mass developed at the distal end of the amputated tail, as seen morphologically (see Figure 1a, b). The fins were suppressed. Histologically, all the features of the control and the treated groups were similar to those on earlier days, except that the bulbular mass, as seen in Figure 1a and b, consisted of a massive

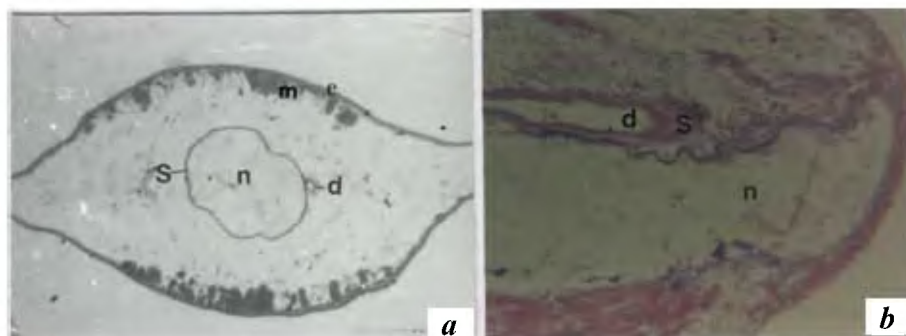


Figure 3. *a*, Transverse section (T.S.) of tail of *R. tigerina* (control) two days post-amputation. Epidermis (e) is single-layered, notochord (n) is covered by a thin sheath (S), nerve cord (d) is small and muscles (m) are arranged in bundles just beneath the epidermis, $\times 150$; *b*, L. S. of tail of *R. tigerina* (treated) two days post-amputation. Notochord (n) is irregular in shape, nerve cord (d) is vesicular and covered by a thick sheath (S); $\times 150$.

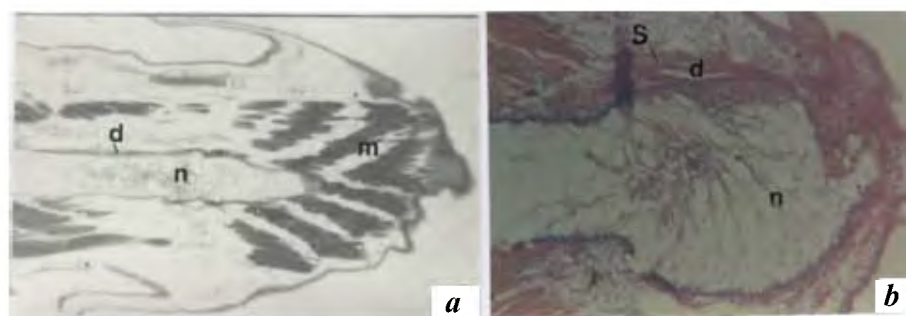


Figure 4. L.S. of tail of *R. tigerina*: *a*, Control, three days post-amputation. Notochord (n), nerve cord (d) and muscles (m) have all become normal. Muscles are arranged as V-shaped myotomes, $\times 150$; *b*, Treated, three days post-amputation. Notochord (n) is still constricted and enlarged considerably. Nerve cord (d) is vesicular and covered by a thick sheath (S); $\times 150$.

amount of thin-walled chorda cells. However, in the control, from day-14 onwards, post-amputation, the tail epidermis became thick and appeared multilayered (Figure 6a) in contrast to the epidermis from day-1 to day-13 post-amputation, where it was single-layered. From day-15 onwards, post-amputation, in the treated groups, a pair of ectopic limbs emerged from the tissue on the lateral sides of the tail (Figure 1g, h). These limbs internally consisted of mesenchyme only (Figure 1b) and exter-

nally resembled muscular outgrowths (Figure 7a). Histologically, all other characteristics being similar to those on earlier days, the ectopic limb consisted of muscle tissue only (Figure 7b). By day-25, in another tadpole with a pair of ectopic limbs, histology revealed a pair of cartilaginous structures, which represented the formation of pelvic girdle, from which the limbs arise (Figure 8). This indicated that the limbs were at a later stage of development. The pattern of limbs had probably been laid down from day-15 onwards.

The present study describes the detailed histological changes in both the control and vitamin A-treated tails of *R. tigerina*, with special reference to homeotic transformation. An AEC was found in both the control and

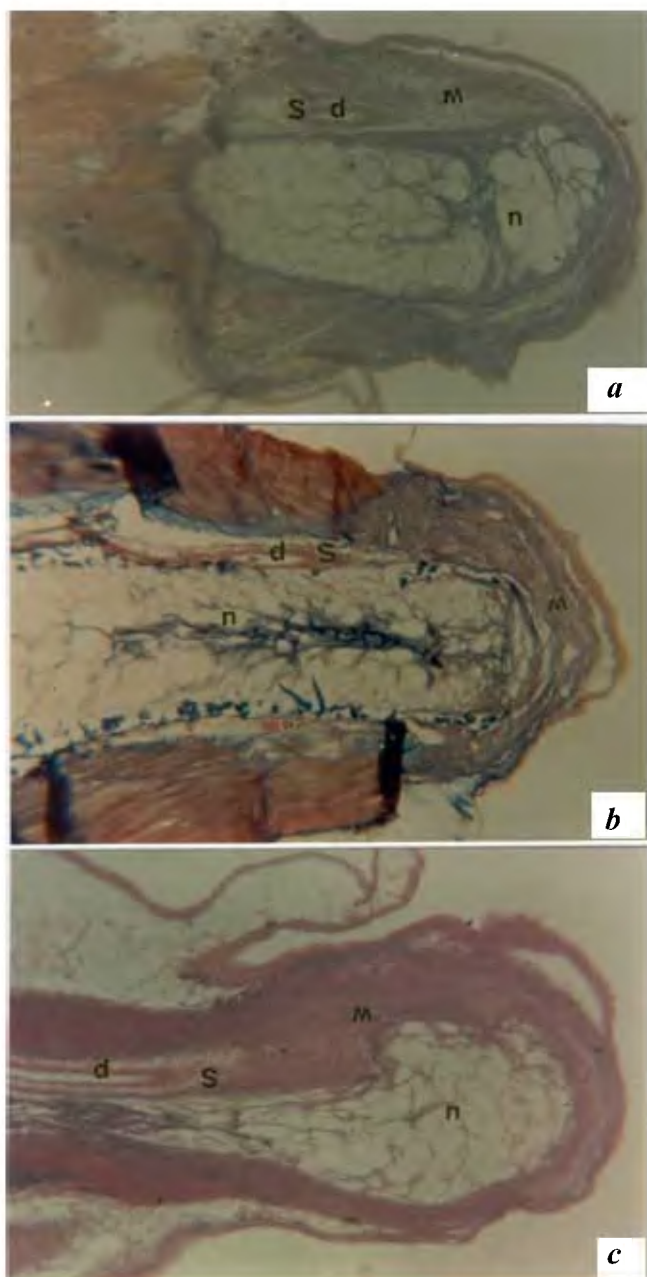


Figure 5 a-c. L.S. of tail of *R. tigerina* (treated) on days 4, 5 and 6 respectively, post-amputation. Notochord (n) is enlarged, nerve cord (d) is vesicular and covered by a thick sheath (S) and muscles (m) are dedifferentiated even up to day-6 in contrast to the controls where dedifferentiation is completed within 2 days post-amputation; $\times 150$.

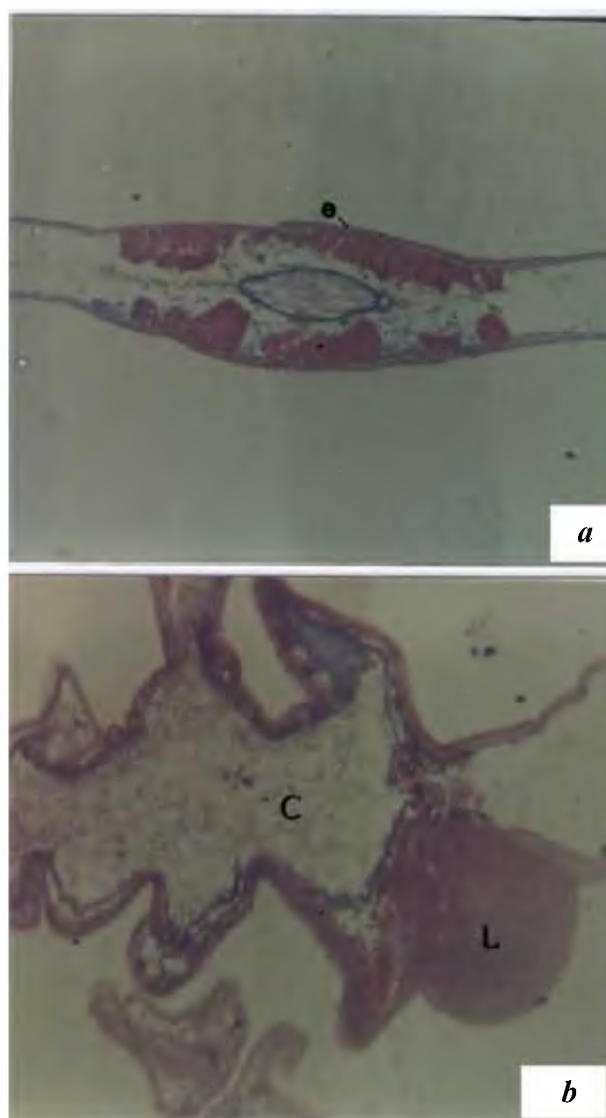


Figure 6. L.S. of tail: a, Control on day-14 post-amputation. Epidermis (e) has become thick, while all the other structures are normal, $\times 100$; b, Treated on day-14 post-amputation as seen in Figure 1g. Bulbar mass from which ectopic limbs (L) have arisen consists of chorda cells (C) only. Ectopic limb, which is in the initial stage of development, consists of mesenchyme; $\times 150$.

treated tadpoles, which persisted for just one day. The AEC which covers the wound surface after amputation plays an important role in amphibian regeneration. The conspicuous thickness of the epidermis of the AEC is involved in the cellular processes involved in the regeneration of the stump. It is perhaps related to the removal of cellular debris from the damaged surface of the wound by either providing adequate proteolytic enzymes or perhaps involved in some phagocytic activities¹⁴.

The epidermis became multilayered in the treated tadpoles from day-3 onwards post-amputation, whereas in the controls they remained single-layered for about 15 days, and became thick and multilayered (adult-type) only when the tadpoles approached metamorphosis (a characteristic feature in anuran metamorphosis)¹⁵. This shows that vitamin A probably induces adult-type epidermis in the embryonic tail by increasing the number of basal cells in the tail epidermis, which appear during metamorphic climax. The basal cells are present in the body epidermis and show up only when there is neogenesis of connective tissue in the body region. But since neither normal tails nor regenerating tails of *R. tigerina* contain a skeleton, the most striking result is the appearance of multilayered epidermis in the tail region. It is

therefore possible that vitamin A probably induces neogenesis of connective tissue in the tail region, thereby giving rise to homeotic limbs at ectopic sites. As described earlier, due to neogenesis of connective tissue in the tail, basal cells are proliferated in the tail epidermis thereby causing thickening of the epidermis and making it multilayered, as seen in all the treated tadpoles from day-3 onwards. The whole process is probably regulated by a positive feedback mechanism.

After amputation, the notochord dedifferentiated. The chorda cells remained in a dedifferentiated state up to one day post-amputation in the control and for two days in the treated, and by day-3 they had regenerated completely in both the groups. But in the treated group, the notochord remained enlarged and was covered by a thick sheath throughout. The chorda cells solely occupied the bulbular mass as seen at the distal end of the amputated tail, but the reason for such accumulation is not yet known.

The nerve cord also became vesicular at the level of amputation in both the control and treated tails. The vesicular nerve cord persisted in both the control and treated tails up to day-2, and by day-3 it became thin and slender, as in normal ones. But in the treated tadpoles it became vesicular and remained as such, being enlarged and covered by a thick sheath. Hunter *et al.*¹⁶ have reported that retinoids are involved in the development of the nervous system by stimulating the outgrowth of neuritis from axolotl spinal cord explants. In the present study, it is therefore quite possible that the thickening of the nerve cord may actually be due to the presence of extra neuritis, which vitamin A might have induced from the axons of the nerve cord.

Muscles also dedifferentiated after amputation in both the groups, but again redifferentiated completely within three days of initial amputation. In the control tails, they arranged themselves in their usual bundles and were or-

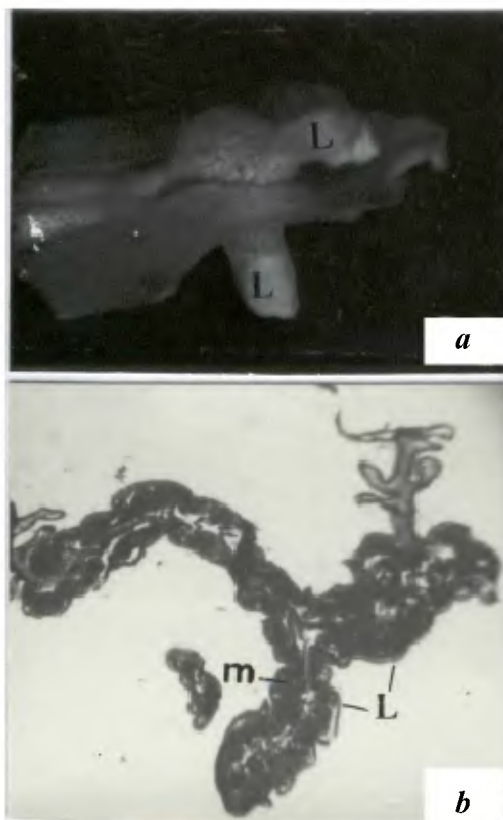


Figure 7. *a*, Morphology of tail (treated) 20 days post-amputation. A pair of ectopic limbs (L) arising from either lateral side of the tail resemble muscular outgrowths only; $\times 15$; *b*, L.S. of tail (treated) as seen in (*a*). Muscular ectopic limbs (L) internally consist of muscle tissue (m) only; $\times 150$.

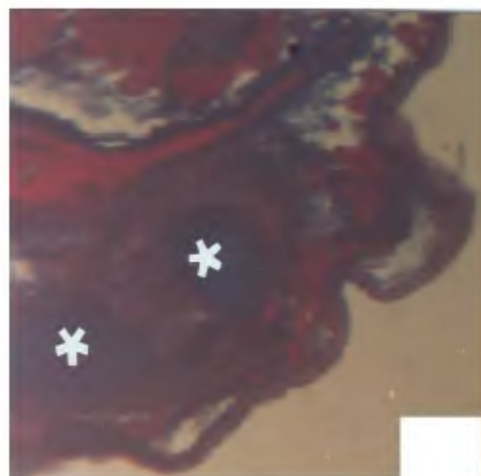


Figure 8. T.S. of tail (treated) 25 days post-amputation, as seen in Figure 1 *h*. Base of the limbs shows a pair of condensed cartilage (asterisks) which forms the base of a pair of pelvic girdle from which the limbs would arise. Rest of the limbs consisted of muscle tissue only; $\times 250$.

ganized on either lateral side of the tail beneath the epidermis. But in the treated group they remained in a dedifferentiated state up to day-4 of initial amputation, and thereafter remained clumped and unorganized instead of being arranged in their usual bundles. Iten and Bryant¹⁷ had reported such type of muscle disorganization in the amputated tails of the adult newt, *N. viridescens* and found that the failure to form bundles is due to disorganization of striations in individual muscle fibres. In the treated tails of *R. tigerina*, the clumped muscles in the regenerating tail are therefore the result of the effect of vitamin A on the muscle fibres.

The ectopic limbs were visible from day-15 onwards and were underdeveloped, unlike the ectopic limbs of *U. systoma*⁸, *R. temporaria*⁹, *P. maculatus*¹⁰ or *Microhyla ornata*¹⁸. *R. tigerina* was perhaps the only anuran tadpole that did not give rise to well-developed ectopic limbs as in other anuran tadpoles, after treatment with vitamin A. On day-15, these ectopic limbs looked like mere muscular outgrowths and by day-25, they appeared slightly developed but were still small in size and not differentiated into the usual parts like those found in normal limbs. The digits were absent. Supernumerary ectopic limbs never arose in contrast to other species, as described earlier. The ectopic limbs always arose in pairs, perhaps because their origin was associated with the development of a pelvic girdle⁹. This was also evident in the present study. Histology revealed that the ectopic limbs consisted of muscle tissue only and a pair of condensed cartilage-like structures (which would give rise to a pelvic girdle) at the base of the limbs. Other tissues were never found in the limb region. The underdeveloped ectopic limbs of *R. tigerina*, in contrast to the well-developed ectopic limbs of *U. systoma*, *P. maculatus*, *R. temporaria* or *M. ornata*, might be due to the species-specific effect of vitamin A. There are reports³ that the action of vitamin A on animal systems is paradoxical in nature. It suppresses cell division; yet once released from this inhibition, it regenerates extra pattern. This probably holds true for the anuran tadpoles because vitamin A has definitely interfered in the cell cycle of *R. tigerina* causing inhibition in creation of extra pattern, whereas in *P. maculatus*, *U. systoma*, *M. ornata* or *R. temporaria*, it is able to overcome the inhibition thereby bringing about well-defined homeotic limbs.

So far, it is not clear how limbs are formed in the tail region. The transformation of one organ into another at a fairly different far-off site, associated with a transitory organ, can be interpreted within the current concepts relating to retinoids, *Hox* type homeobox genes and developmental patterning in vertebrates^{19,20}. *Hox* genes determine the segmental identity along the body axis²¹⁻²³, and retinoids are able to modify the expression of these genes and the resulting morphological identities²¹. However, once the *Hox* genes of a specific axial level have been activated in normal development, cells must continue the

expression cascade from that activation site, and can only regenerate more caudal structures²⁴. The limbs which are usually present in the trunk region are respecified in the tail region from the amputation plane due to the action of vitamin A which has the ability to anteriorize (proximalize) *Hox* gene activation in the dedifferentiated cells of a regeneration blastema²⁵. This is supported by *in vitro* studies demonstrating that retinoids have a differential effect on *Hox* gene activation, essentially upregulating genes from the 3' half and downregulating genes from the 5' half of the *Hox* clusters. The respecification of anterior body pattern in anuran tail regeneration provides strong empirical evidence that retinoids have similar effect on *Hox* gene activation *in vivo*.

This study reveals characteristic cellular changes effected by vitamin A on regenerating amphibian tail tissues. Many questions remain unanswered – What causes the enlargement of the notochord and the nerve cord? Why are only chorda cells accumulated in the bulbular swelling? Why do thick sheaths envelope around the notochord and nerve cord? And many more.

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