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Local action of vitamin A on amphibian limb regeneration

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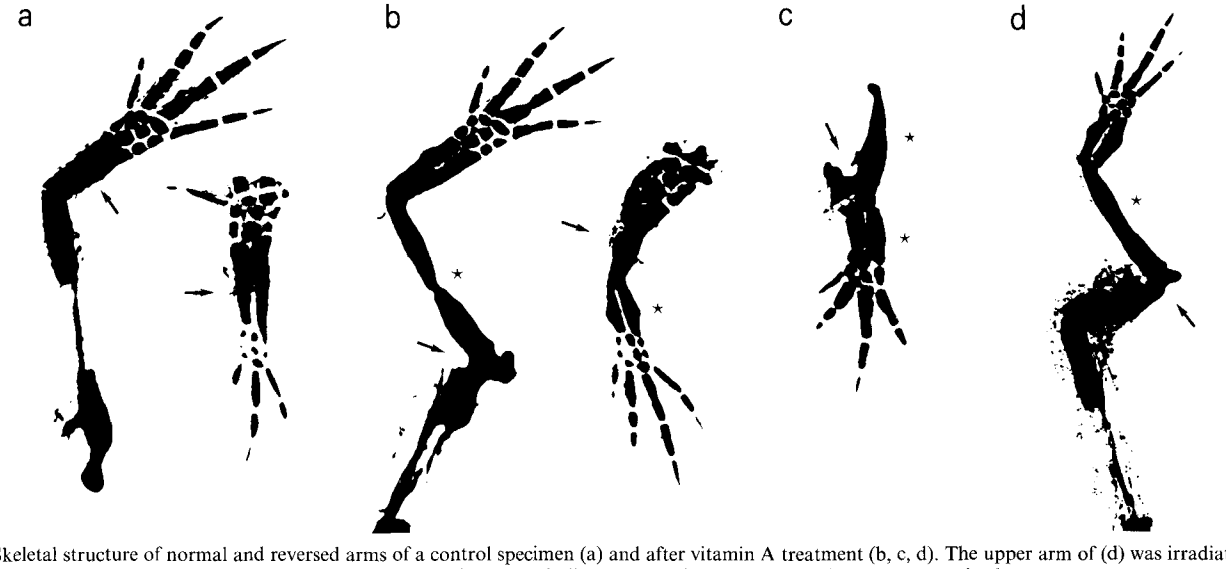
Summary. Axolotl forearms isolated from upper arm tissue either surgically or by local irradiation regenerate excessive structures after vitamin A treatment. This demonstration excludes the possibility of regeneration being altered by enhanced cellular migration and thus indicates that vitamin A respecifies cells close to the site of amputation.
Key words. Axolotl; *Ambystoma mexicanum;* limb regeneration; regeneration, limb; vitamin A.

In addition to impeding growth and metamorphosis of amphibian larvae, excess vitamin A inhibits the development and regeneration of their limbs. Niazi and Saxena[1] discovered 2 further effects of vitamin A on limb regeneration after immersing *Bufo* tadpoles in retinol palmitate. They obtained cases of triplicate regenerates and of excessively long regenerates containing a serial repetition of skeletal elements present in the limb stump. The latter effect has been confirmed in axolotls exposed to 15–150 IU/ml (international or USP units) of retinol palmitate and other retinoids for defined periods[2] and has excited interest as contravening the 'law of distal transformation'. This law[3] states that an amputated limb can only regenerate structures which are anatomically distal to the plane of section: so that a normal limb stump will be completed but a proximodistally reversed one will regenerate a mirror image of itself. The evidence for the law has been reviewed recently[4]. The excessive regeneration caused by vitamin A certainly violates the law in terms of gross morphogenesis, but it still needs to be demonstrated whether or not that implies a cellular respecification.

Limb regeneration occurs by the growth of a small population of dedifferentiated cells (the blastema) which normally arise from several tissues close to the end of the limb stump, as shown by the effects of local irradiation[4]. If that is still true after vitamin A treatment, then there must be an alteration of the cellular program for regeneration. Alternatively, vitamin A may preferentially inhibit those tissues exposed at the end of the limb stump and thus encourage other cells in more proximal regions to dedifferentiate and migrate distally, while retaining their normal program for regeneration of all parts distal to their former position. The most obvious ways of testing these alternatives are to repeat the classical experiments on reversed and locally irradiated limbs, to discover if their regeneration is influenced by vitamin A. We have applied both tests to the arms of 60–90 mm long axolotls *(Ambystoma mexicanum)*, with results which imply that seemingly determined blastemal cells can be respecified.

Arm reversal entailed severing the nerves in the brachial plexus, removing the first and last digits and inserting the rest of the skinned hand through a slit in the body wall. The arm then remains immobile while healing with a hand-in-pocket posture[5]. After 10 days healing and reinnervation from the shoulder and flank, the arm was amputated below the elbow to produce a normal and a reversed forearm stump. Experimental specimens were then immersed in 25 IU/ml vitamin A for 15 days (using Sigma water dispersible retinol palmitate, renewed at 3–4 day intervals), while control specimens were kept in water in the same 21 °C incubator. The controls regenerated only the distal forearm and complete hand. This is classed as typical regeneration in the table, as it completes the normal arm or forms a mirror image of the reversed forearm (fig. a). Vitamin A treatment usually either prevented regeneration or induced an excessive regeneration, as shown by the presence of a humerus or complete radius and ulna distal to the stump skeleton (fig. b), but some reversed arms formed typical mirror image regenerates after a delay of several weeks. Those classed as excessive (table) contained a complete radius and ulna partly fused to the reversed stump skeleton, indicating a mild proximal transformation of tissue well separated from the upper arm and shoulder. Figure c shows a more extreme example where the carpal region of an isolated hand has regenerated an almost complete arm, after treatment with 50 IU/ml vitamin A. Localized irradiation was achieved by 3 mm lead shields during exposure to 20 Gy (2 krad) of filtered Xrays, a dose which inhibits regeneration[6]. 2 shielding patterns were used: either both hands and forearms, or both upper arms and the inter-



Skeletal structure of normal and reversed arms of a control specimen (a) and after vitamin A treatment (b, c, d). The upper arm of (d) was irradiated prior to amputation. Arrows mark the site of amputation; stars indicate a complete radius and ulna or humerus in the regenerate.

vening shoulder region, were irradiated. Specimens were then amputated through both forearms and kept in 25 IU/ml vitamin A for 15 days at room temperature. Irradiated forearm stumps did not regenerate even after vitamin treatment (table), thus providing no evidence of cellular migration from the shielded upper arm. Shielded forearm stumps regenerated a virtually complete arm, containing a partial or entire repeat of the stump humerus (fig. d). Since all proximal tissue had been irradiated and thus rendered incapable of regeneration, this result demonstrates that local forearm cells have been respecified to attain the status of upper arm cells.

We used retinol palmitate because it is available in a relatively stabilized form which can be accumulated by storage in the liver and thus metabolized less rapidly than retinoic acid[7]. Our treatment caused a temporary reduction of appetite and growth, and the regenerates contained a variable amount of structural repetition. Higher concentrations of retinol palmitate produce more extreme responses[2], but also increase the incidence of limbs whose regeneration is greatly delayed or inhibited. The delay of regeneration during treatment is not responsible for the serial repetition. Even greater delays resulting from higher doses tend to result in typical regenerates, as does the delay caused by repeated denervation[8]. Reamputated limbs also regenerate typically, indicating that excess retinoids do not persist very long in the body and do not have a permanent effect on mature tissues. We conclude that vitamin A affects cells close to the site of amputation, probably after they have dedifferentiated to form blastemal mesenchyme, by respecifying them to adopt a more proximal character.

Types of regenerate scored 6 weeks after forearm amputation

Pretreatment	Vitamin A IU/ml	Regenerated structure None	Typical	Excessive
Normal arms	none	0	12	0
Reversed arms	none	0	12	0
Normal arms	25	6	0	6
Reversed arms	25	2	4	6
Forearms X-rayed	25	12	0	0
Upper arms X-rayed	25	0	0	12

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Occurrence of polyploidy and multinuclearity in the differentiating liver of chick embryo

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Summary. Increase in nuclear size in liver has been used as an index of polyploidy. It has long been considered that the occurrence of polyploidy and multinuclearity are characteristics of mammalian liver. The present study shows the occurrence of these phenomena in the liver of birds, so these features are not confined to mammals. 3 classes of nuclear size groups have been identified. The simultaneous occurrence of polyploidy and binuclearity indicates some sort of interrelationship between them.
Key words. Chick embryos; liver, chick; chick, liver; nuclear size; polyploidy; multinuclearity.

Differences of nuclear size in the hepatic parenchyma of mammals have been observed by several investigators[1,2]. Nuclear size has frequently been used as an index of ploidy by investigators working with hepatic tissue. Several authors[3-12] have shown that doubling of the DNA content in liver nuclei is indeed reflected by roughly a doubling of the volume. Jacoby[1] concludes that the higher nuclear class or polyploid classes are characteristic of mammalian liver, and are absent in any other groups of vertebrates.

In addition to the polyploid nuclei, liver cells frequently contain more than 1 nucleus, binuclearity being the most common type of multinucleated condition. Munzer[13] reported that there are wide species differences in the frequency of binucleated cells, and also noted that they are few in the new born and appear with greater frequency in adults.

Most work on liver polyploidy and binuclearity has been carried out in mammals, but practically no work have been done in this regard on avian liver. Medda and Shamsuddin[14] observed that up to the 12th day of incubation, the increase and decline of DNA content per unit weight of liver tissue run parallel with the increase and decline of mitotic index, but after the 12th day there is a decrease in mitotic index and an increase in the DNA content. The high DNA content, in spite of the lower rate of cell proliferation, after the 12th day of incubation, led them to speculate about the development of polyploidy and binuclearity in chick liver cells. However, only direct microscopical observation could confirm the above speculation. For this reason, in the present work it was decided to study the percentage of multinucleated cells and the average volume of the nuclei, which may be correlated with the degree of ploidy, at different developmental stages of liver in chick.

Materials and methods. The materials were the eggs and embryos of white Leghorn chicks obtained from Govt. Poultry Farm, Burdwan, W. Bengal. The eggs were incubated at 38 °C, with 75% relative humidity.

Since EDTA has been extensively used as a cell-separating detergent[15-17] we used EDTA solution for separting cells. By trial and error methods we devised the following procedure for separating liver cells. The livers of the embryos were dissected out quickly, cut into small pieces and then placed in a dissociating mixture (equal volumes of 0.015 M EDTA solution and 0.22 M NaCl solutions) in the proportion of 2 ml per 100 mg of tissue, along with some glass beads, and shaken for half an hour. The entire mass was then passed through a fine-meshed silk to remove the glass beads and tissue clumps. The dissociated cells in solution were fixed by 0.01 ml formalin/ml of dissociating mixture, and shaken for 5 min. After fixation samples were centrifuged and the supernatant discarded. The fixed