Developmental mechanism involved in the embryonic reduction of limbs in reptiles

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ABSTRACT The purpose of the studies here reported was to explain the mechanisms responsible for the reduction of limbs in the serpentiform reptiles. Descriptive and experimental embryology, ultrastructural studies, chemical action (with Ara-C) on embryos and (3H) thymidine autoradiography of limb buds were used in this study; they provide evidence that defects in the morphogenetic mechanisms involved in the development of limbs (somitic deficiency, incomplete differentiation and premature degeneration of the apical ectodermal ridge) are responsible for the cessation of growth of the limb buds in these reptiles. At the biochemical level, a strong decline in the rate of DNA synthesis in the mesodermal cells of the limb bud (during and after the degeneration of the apical ridge) is the main causative factor of the evolutionary arrest of limb development in serpentiform reptiles.

KEY WORDS: *limb reduction, morphogenesis, Ara-C, DNA synthesis, reptile*

Introduction

An extensive literature has been devoted to the various conditions of reduction or loss of limbs in serpentiform reptiles. Numerous descriptive and anatomical studies on these reduced limbs in a wide range of living and fossil species were carried out; but until recent years, the mechanism involved in this evolutionary reduction remained largely unknown.

First, several hypotheses were formulated to explain limb reduction. In Lamarck's view (1809), the limbs regressed as a consequence of lack of use: in certain reptiles with fully developed legs, the trunk lengthened so as to be able to pass through narrow spaces and the animals began to crawl on their abdomens, and their limbs, which were less and less used, gradually atrophied. Darwin (1868) agreed with this concept of reduction of organs following continued disuse, but added control by natural selection of this effect of non-use.

Most authors now ascribe the origin of limb regression to mutations of genomic factors controlling limb development. These genetic defects give rise to variations in the degree of limb reduction under the control of natural selection. The effects of the selection are, for some authors, an all-important event able to direct the pattern of limb loss or reduction under pressure of mutation; by adding adaptation to the new conditions of life, the concept of "selection by adaptation" would be quite sufficient to explain the regressive evolution and the modeling of the structural pattern of the reduced limb in conformity with the new mode of life in the appropiate biotope.

Gans (1975) admits that an elongation of the body may occur as a consequence of some initial selective advantage (the facility to squeeze through narrow spaces) in certain reptiles with well-developed limbs; limb reduction then took place only as a secondary modification, also facilitated by natural selection. "Reduction by adaptation", as defined by Steiner and Anders (1946) would govern the pattern of reduction: applying this principle to the three-toed Skink, *Chalcides chalcides* (L.) these authors admit that it led to a peculiar capacity to dig, strengthening digits I, II and III and leading to the disappearance of digits IV and V. However, the frailty of this interpretation became apparent when an embryological study of the formation of the limb established that the digits present in the hands and feet of this Skink are digits II, III and IV (Sewertzoff, 1931; Raynaud *et al.*, 1987).

Quite different hypotheses suggest that the atrophy of an organ might be the consequence of the hypertrophy of some other part of the organism. These organic embryonic correlations may link the development of various parts of the embryo. Thus, L. Cuénot (1921) suggested: "Qui sait si ce n´est pas la multiplication des vertèbres, amenant corrélativement l´élongation du corps, qui a déterminé l´atrophie graduelle des pattes dans les séries parallèles et indépendantes de Lézards apodes (*Pseudopus, Anguis, Scincus, Seps, Chirotes, Amphisbena*) dont l´une a donné le phylum des Serpents?". Some years later, anatomical studies showed pre-

Abbreviations used in this paper:Ara-C: Cytosine 1-ß-D-arabinofuranoside; S_1-S_{13}: post-otic somites 1 to 13.

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cisely that in a given family of reptiles, the elongation of the body (the increase in the number of presacral vertebrae) is accompanied by a shortening of the limb (Sewertzoff, 1931; Stokely, 1947). An embryological interpretation of this observation (in keeping with it) suggests that in serpentiform reptiles, the increase in the number of somites of the trunk entails a reduction in the number of somites taking part in the first development of the limb; this somitic reduction would entail the reduction of the limb and, thus, the correlative modification makes it possible to understand how the elongation of the trunk may be accompanied by a reduction of the limbs.

In a recent publication, Gould and Levontin (1982) also suggest that the reduction of the anterior limbs in Dinosaurians might be an after-effect of an excessive growth of the posterior limbs and not an effect of adaptation.

These concepts are thus far from the Lamarckian hypothesis suggesting that a lack of limb use in adult life is the essential factor leading to the loss of limbs.

We will see how embryology has brought to light the existence of correlations between several morphogenetic mechanisms involved in the formation of limbs and other organs.

About thirty years ago, I broke with the hypothesis and put the problem of the reduction of limbs back on its true basis, an embryological basis: it thus became possible to detect, by way of comparative analysis, the defects of the developmental mechanisms leading to a partial or a total arrest of development of the limb bud. Two important factors were identified: a somitic deficiency, followed by incomplete differentiation and premature degeneration of the apical ectodermal ridge of the limb bud.

The next step forward came with the obtention of an experimental reduction of limbs in a tetrapod pentadactylous species, by a chemical effect on the embryo.

Finally, it was possible to detect, by means of autoradiography, the biochemical mechanism – a decline in the rate of DNA synthesis – responsible for the arrest of limb bud development in the evolutionary reduction.

The essential steps in this research will be now described.

An analysis of the developmental arrest of the limb buds in the embryos of serpentiform reptiles

This analysis was performed by means of a comparative study of the formation of the anlage of the limb bud in the embryos of some serpentiform reptiles and in the embryos of a tetrapod, pentadactylous species, the green lizard (*Lacerta viridis*, Laur.).

The serpentiform lizard, *Anguis fragilis* (L.) (the slow-worm) was extensively used in these studies; it is an ovoviviparous form, which in the adult is devoid of legs; however, anlagen of limbs appear during embryonic life (Fig. 1, a, b) but their existence is only temporary; their growth ceases and they regress and disappear in the young embryo, long before hatching.

Other species used were several Scincids, certain snakes and *Ophisaurus apodus* (Pallas) studied by Rahmani (1974)

The somitic deficiency in embryos of serpentiform species

In all reptiles with well-developed limbs, there are eight somites (S_6 to S_{13}, S_1 being the first post-otic somite) that send a ventral process into the somatopleural area of the future anterior limb. In the embryos of serpentiform species, the number of somites sending processes into the territory of the future limb is always less than eight: 4 (rarely 5) in *Anguis fragilis* (Raynaud and Vasse, 1968); 5 in *Ophisaurus apodus* (Rahmani, 1974); 5 in *Scelotes brevipes* (Hewitt) (Raynaud *et al.*, 1974); no somite forms ventral processes in embryos of snakes (*Natrix: Vipera*) (Raynaud, 1972a) (Fig. 1, c, d).

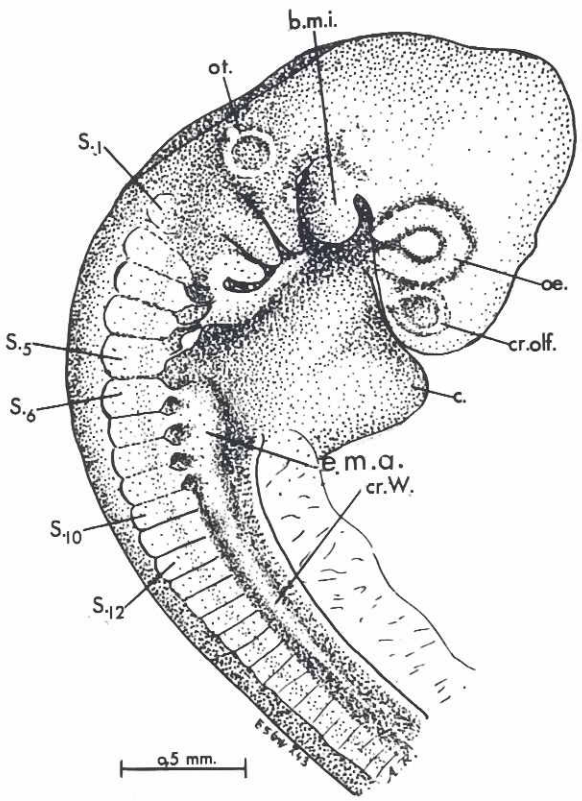
It is known, from studies and experiments carried out on amphibian larvae (Amano, 1960; Finnegan, 1963) and on avian embryos (Murillo-Ferrol, 1963, 1965; Pinot, 1970; Kieny, 1971) that the somites play an essential role in the initial development of the limb. Similar results were obtained in reptilian embryos (Raynaud, 1972b, 1977). The reduction in the number of somites participating in the formation of the limb bud in *Anguis fragilis* thus entails a first reduction of the limb anlage, which develops only opposite the somites forming ventral processes; the limb bud in this species is thus shortened and lacks a posterior part, that which develops opposite the other somites (S_{10} to S_{13}, for instance) in other tetrapod lizards. The same conclusion is valid for other serpentiform species (*Ophisaurus, Scelotes*) and similar observations were made on the anlagen of the posterior limbs.

The reduction in the number of somites participating in the development of the limb is accompanied, in *Anguis fragilis*, by other deficiencies: early degeneration of many cells in the somitic processes, segregation of their nucleolar components with a more or less complete separation of their granular and fibrillar structures.

Incomplete differentiation and early degeneration of the apical ectodermal ridge in the limb bud

In the embryos of *Anguis fragilis*, an incompletely differentiated apical ridge forms along the distal edge of the limb bud; this ridge never forms an apical fold with a notch as is the case in *Lacerta* embryos. An ultrastructural study showed that gap-junctions are absent in this ridge at all stages of development (Raynaud *et al.*, 1979); this absence, by preventing the coupling of the cells, very probably leads to a lesser activity of the ridge in *Anguis*. A similar absence of gap-junctions was later observed in the ridge of the "wingless" mutant in the chick embryo (Sawyer, 1982) and was interpreted as a "morphological indication of a waning inductive influence" of the ridge.

Fig. 1. External view of the developing limb bud of the slow-worm (*Anguis fragilis*, L.). (a) *Connections between somites and anterior limb bud in an embryo at the stage when the allantoic bud is 0.3 mm long: somites S_6 to S_9 have sent ventral processes into the anlage of the anterior limb and the limb protrudes only in front of these four somites (c: heart; cr. olf.: olfactory oit; cr. W.: Wolffian crest; oe.: eye; ot.: otocyst; S_1 - S_{12}: post-otic somites S_1 to S_{12}). (x32). (From Raynaud and Vasse, 1969).* **(b)** *Scanning electron microscope view of the right anterior limb bud of an embryo at the stage when the allantoic bud is 3.5 mm long. (x128).* **(c, d)** *Graphic reconstructions showing up and comparing the somitic relationships of the limb bud in the embryo of* Lacerta viridis *(Fig. 1d) and in the embryo of* Anguis fragilis *(Fig. 1c). Note the reduction in the number of somites sending processes into the territory of the limb bud in the embryo of* Anguis fragilis *(ch.: notochord; c.m.b.: cells detached from the somitic extensions; e.e.p.: ectodermal thickenings in front of somitic extensions; e.m. ant.: anterior limb bud; ep.: ectoderm; 1.m.ant.: limit of the anterior limb area; S_1- S_{13}: postotic somites S_1-S_{13}. (from Raynaud and Vasse, 1968).*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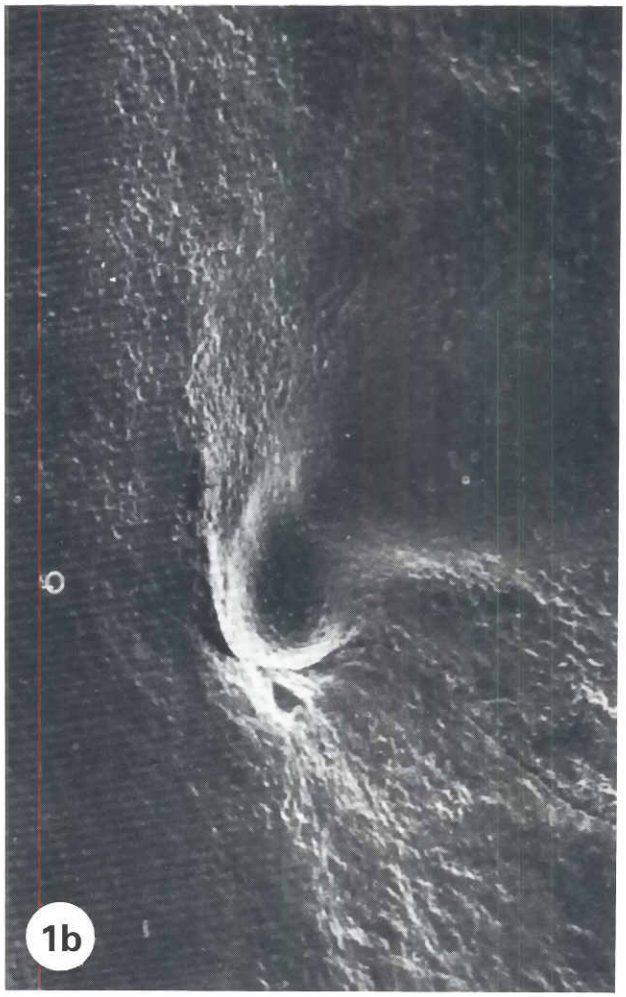
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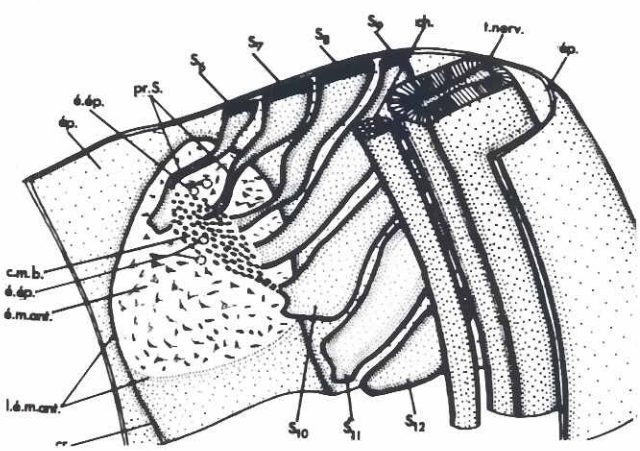
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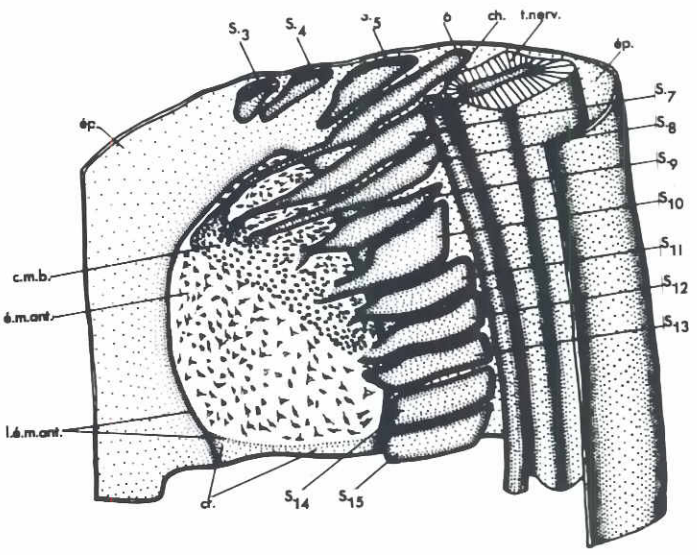
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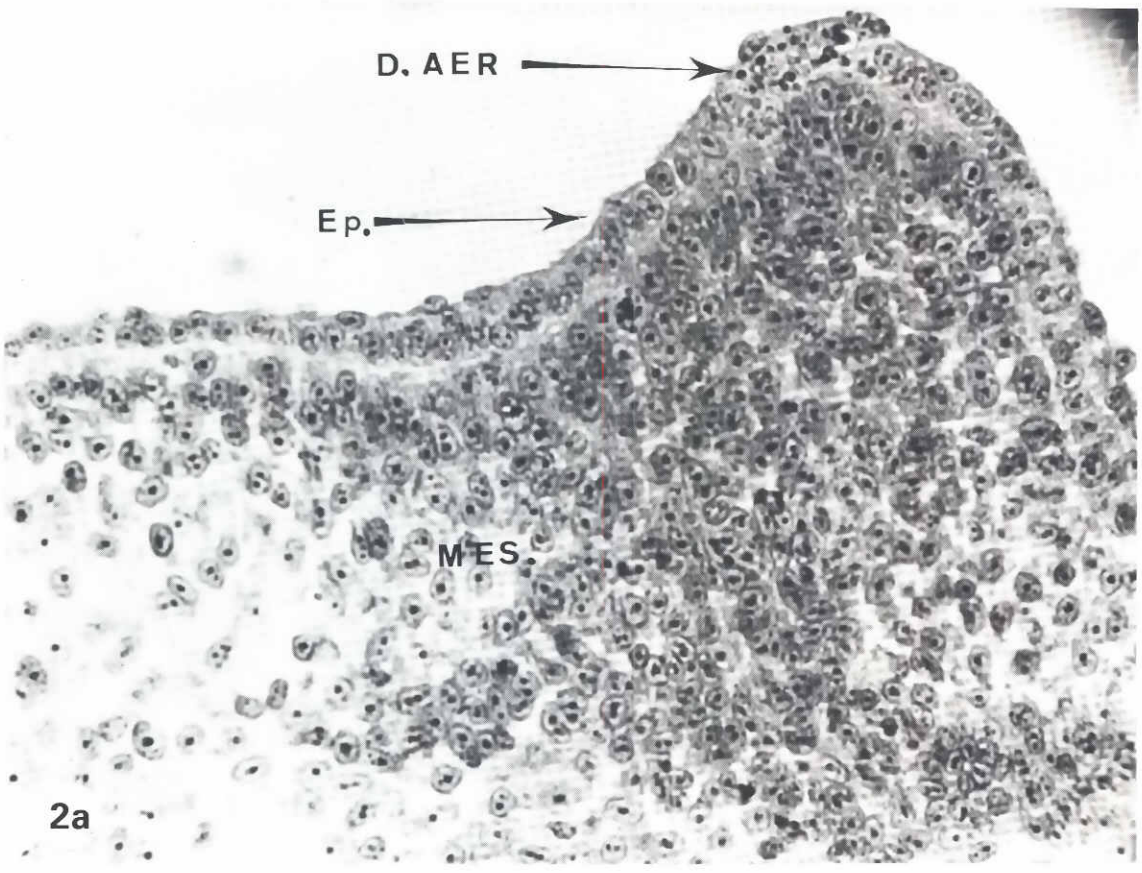


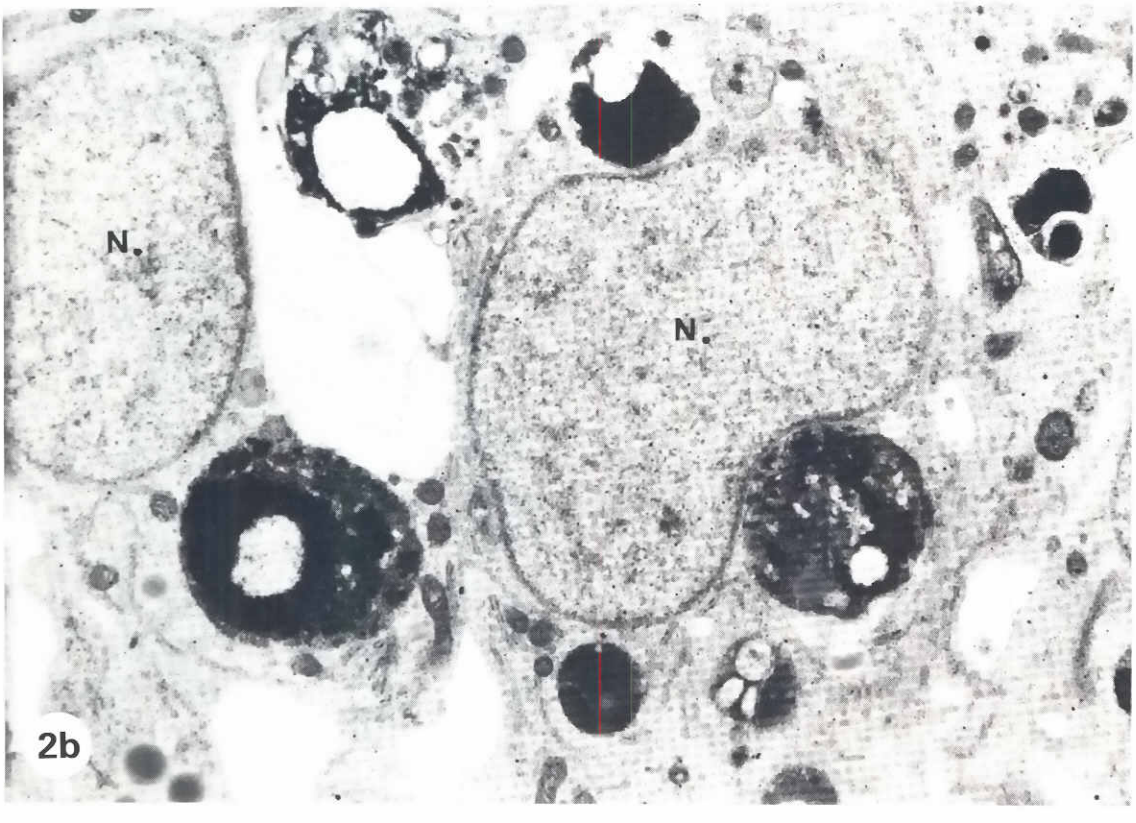


Fig. 2. Degeneration of the apical ectodermal ridge in the limb bud of the young embryo of the slow-worm (*Anguis fragilis*, L.). **(a)** *Histological section through the limb bud of an embryo at the stage when the allantoic bud is 1.5mm long. At the top of the limb bud, the apical ectodermal ridge (D. AER) is degenerating. Note that cell degeneration is sharply localized. (Ep.: epiblast; Mes.: mesoderm). (x530).* **(b)** *Ultrathin section of the apical ridge showing the mode of degeneration of a cell: numerous large, intracytoplasmic autolytic centers of degeneration, in which organelles are destroyed, wrinkle the membranes of nuclei that have remained intact (embryo at the stage when the allantoic bud is 2.0mm long) (x18,200).*

Furthermore, I discovered that the apical ridge degenerates spontaneously at an early stage (defined by an allantoic bud length of 1.2 to 1.5 mm) of the embryonic development of *Anguis fragilis* (Raynaud, 1962); this degeneration is strictly localized at the site of the ridge (Fig. 2a) and still proceeds for two to three days until the stage of an allantoic bud length of 3 to 3.5mm is reached; at this stage, almost all the cells of the ridge have died.

After degeneration of the apical ridge, the limb bud continues to grow for 48 hours; then, growth ceases and regression of the anlage begins.

The degeneration of the apical ridge in the limb bud of *Anguis fragilis* takes place at a stage preceding by eight days the stage at which the apical ridge of the limb bud in *Lacerta viridis* begins to reduce and retrogress as formation of the digits begins.

Ultrastructural study shows (Fig. 2b) that degeneration of the ridge cells is a consequence of the formation of broad intracytoplasmic centers of autolysis in which the organelles of the cell (mitochondria, endoplasmic reticulum, etc.) are destroyed by lysosomal enzymes; the cell nuclei are not, at first, affected, but their membranes soon become wrinkled under the pressure of the surrounding cytolysosomes and autophagic vacuoles (Fig. 2b). The majority of organelles are soon destroyed and the cells die. The same process of degeneration affects the apical ridge of the posterior limbs some time later, 24 to 36 hours after degeneration of the anterior ridge (Raynaud, 1963).

The observations relevant to the embryos of *Anguis fragilis* can be generalized to other serpentiform reptiles. In *Ophisaurus apodus*, the apical ridge of the limb bud also degenerates prematurely (Rhamani, 1974). A similar degeneration was observed in the embryos of *Scelotes inornatus* and *Scelotes brevipes* (Raynaud *et al.*, 1975; Vasse *et al.*, 1974).

In all these species, growth and differentiation of the limb bud ceases a few hours after degeneration of the apical ridge; this arrest of development is probably the consequence of the mesoderm's being deprived of some stimulating substances normally elaborated by the cells of the apical ridge, and will be further discussed. This phenomenon is probably similar to the arrest of limb bud development as experimentally obtained after surgical excision of the apical ridge, in the chick embryo (Saunders, 1948; Summerbell, 1974).

Effects of cytosine-arabinofuranoside on the morphogenesis of the limbs, in reptiles

Cytosine 1-ß-D-arabinofuranoside (Ara-C), a potent inhibitor of DNA synthesis was known as an acute teratogen to the chick embryo (Karnofsky and Lacon, 1966) and to mammalian embryos (rat and mouse) (Chaube *et al.*, 1968; Ritter *et al.*, 1971, 1973; Scott *et al.*, 1977; Kochhar *et al.*, 1978). Among the numerous developmental abnormalities that this compound provoked, a wide range of limb malformations was observed.

These observations led me to undertake a study of the effects of Ara-C on the limb buds of reptilian embryos.

For these experiments, Ara-C in physiological saline was injected into the yolk sac of green lizard (*Lacerta viridis*, Laur.) eggs at different developmental stages of the embryo, between the sixth and the twelth day of incubation (at a constant temperature of 25ºC). Different Ara-C doses of 17 µg, 20 µg, 40 µg or 60 µg were injected into the yolk (single injection). A total of 135 embryos were treated by Ara-C; 94 embryos treated only by physiological saline were used as controls.

The Ara-C doses between 17 and 60 µg were compatible with the survival of numerous embryos: 126 among the 135 treated were kept alive after the administration of the drug; these embryos were killed at an age of 7 to 60 days to study the modifications induced by Ara-C in body morphogenesis and, in particular, to study the structural changes induced in the limb buds by this drug (Raynaud, 1989b).

When Ara-C was administered to young embryos (aged 6-8 days) it brought about a general, sometimes strong, reduction in the size and weight of the embryo, accompanied by more or less severe malformations of the body, namely modifications of head shape, skeletal defects in skull, vertebrae, ribs, shortening of the lower jaw, labial clefts, microphtalmia and developmental defects in limbs and tail. The limb defects consisted essentially of a developmental arrest of the limb bud, leading to ectromelia, phocomelia, ectropodia or different degrees of micromelia and ectrodactyly (Fig. 3). Low doses usually reduced the size of the stylopod and of the zeugopod. These modifications were accompanied by changes in the girdles and in the external conformation of the cloacal region.

Treatment by Ara-C at a somewhat advanced stage (between 9 and 12 days of incubation) with doses of 20 µg to 30 µg/egg resulted mainly in ectrodactyly, and the incidence of the digital reductions among the treated embryos was rather high: for instance, 21.5% of the treated embryos possessed at least one tetradactylous leg. Furthermore, the same pattern of reduction frequently occurred in the four limbs of a given embryo. For instance, with treatments applied between 6 and 10 days of incubation, 21.7% of the embryos showed four digits on the four legs; for treatments between 7 and 11 days, 10.8% of the embryos had only one digit on the four lefs; for treatments between 6 and 11 days of incubation, 20.7% of the embryos displayed ectropodia on the four limbs.

Several other patterns of reduction have been obtained: embryos with four digits on the anterior legs and five digits on the posterior limbs; embryos with four digits on anterior legs and three digits on the posterior limbs; embryos with three digits on the anterior legs and four digits on the posterior limbs.

All these patterns of digital reduction experimentally obtained by the action of Ara-C are similar to almost all of the patterns of reduction normally occurring in serpentiform reptiles (Raynaud, 1986, 1989, 1990). Furthermore, the same rules govern the loss of certain digits in the experimental (by Ara-C) and in the evolutionary reduction: thus, in both cases, in tetradactylous legs, digit I is lacking in 80% of the cases and digit V is missing in 20% of the cases. In the tridactylous legs experimentally obtained or naturally occurring, the missing digits are digits I and V in 80% of the embryos and digits I and II in 20% of the embryos. In cases of bidactyly, the remaining digits are always digits III and IV and in cases of monodactyly, the remaining digit is always digit IV (Raynaud and Clergue-Gazeau, 1986: Raynaud *et al.*, 1989).

Moreover, histological studies showed that the embryonic stages of limb reduction are very similar in the case of experimental reduction by Ara-C and in the case of evolutionary reduction (Raynaud, 1986; Raynaud, 1989).

All these similarities in the modalities of the reduction process suggest that a similar mechanism might be at work in the experimental reduction by Ara-C and in evolutionary reduction. As it is established that Ara-C acts at the cellular level in inducing a decline

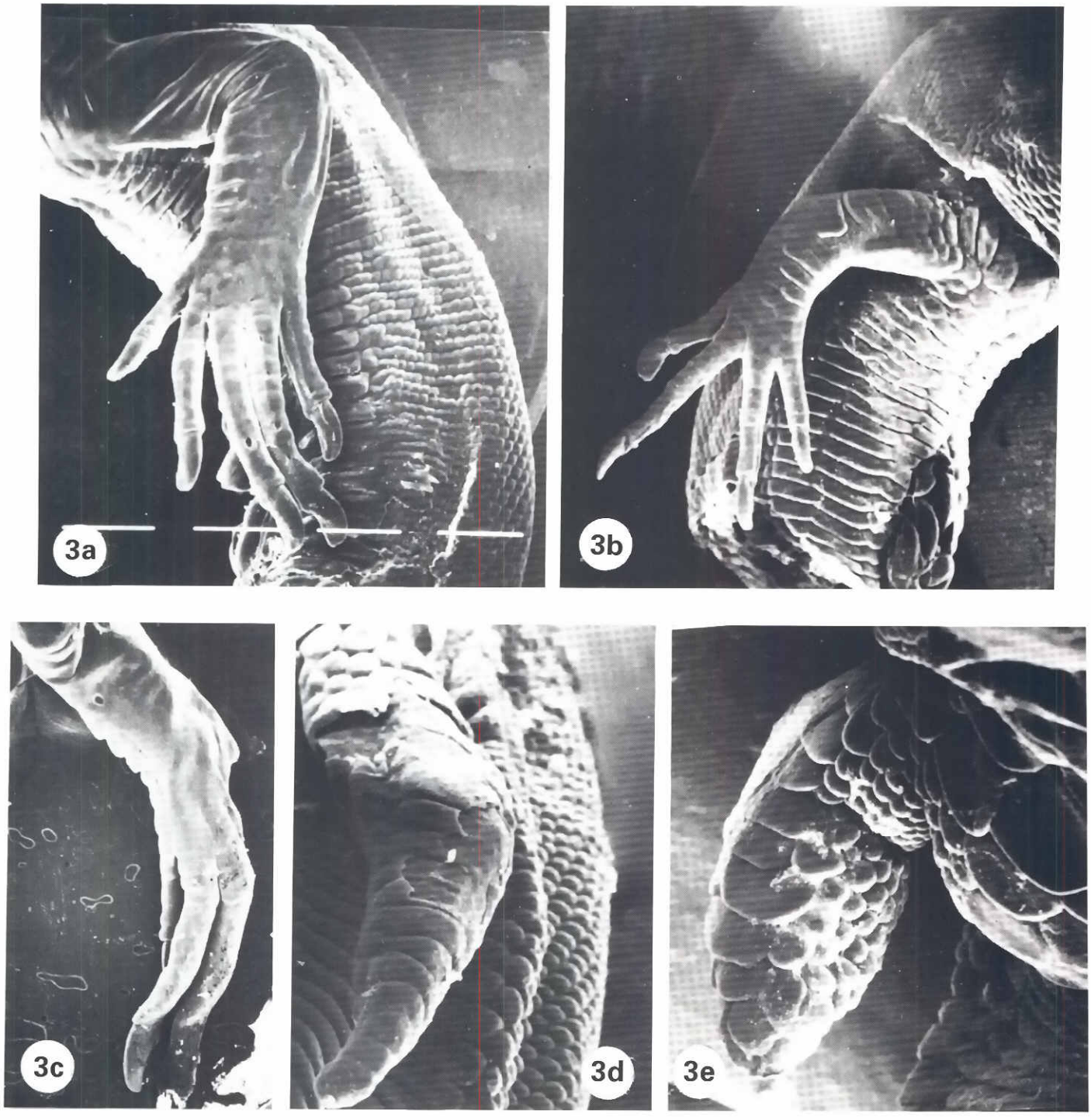


Fig. 3. Structural modifications induced by cytosine-arabinofuranoside (Ara-C) in *Lacerta viridis* embryo limb buds. (a) *Normal, pentadactylous anterior leg of a control embryo treated by physiological saline at the age of 8 days 20 h., sacrificed at the age of 35 days (weight: 298.4 mg). (x30).* **(b)** *Tetradactylous anterior leg obtained from administration of 20 μg of Ara-C into the yolk sac of an embryo aged 8 days 20 h; the embryo was sacrificed at the age of 34 days 20h (weight: 219.5 mg). (x30).* **(c)** *Tridactylous posterior leg obtained by the action of 20 μg of Ara-C injected into the yolk of an embryo aged 7 days 11 h; the embryo was killed at the age of 33 days (weight: 214.0 mg) (x39).* **(d)** *Monodactylous leg obtained under the effect of 20 μg of Ara-C administered into the yolk sac of an embryo at the age of 7 days 11 h; the embryo was killed at the age of 34 days (w eight: 193.3 mg) (x57).* **(e)** *Ectropod leg of an embryo treated at the age of 8 days 16h, with 40 μg of Ara-C and sacrificed at the age of 37 days 16 h (weight: 222.8 mg) (x55).*

in the rate of DNA synthesis, our results suggest that the evolutionary reduction of the limbs in nature could be the result of a reduction of DNA synthesis in the limb bud (Raynaud, 1985, 1986, 1989).

Mode of action of cytosine-arabinofuranoside on the limb bud

It is known that Ara-C acts on proliferating cells through an inhibition of DNA synthesis as a consequence of an inhibition of DNA-polymerase. To have a thorough knowledge of the range of Ara-C action on the limb anlage, an autoradiographic study was undertaken after incorporation of tritiated thymidine on the limb buds of *Lacerta viridis* embryos treated with Ara-C (Raynaud and Kan, 1988); this enabled us to investigate the changes induced by the drug in DNA synthesis in the mesoblastic cells of the limb buds.

For this study, eggs of *Lacerta viridis*, at 11 days of incubation (at 26°C) received an injection of 40 µg of Ara-C into the yolk sac; next, on the 16th day of incubation, every egg received an injection of tritiated thymidine (10 µCi); the control eggs received first, at 11 days of incubation, an injection of physiological saline (10 µl) into the yolk; next, at 16 days of incubation, an injection of tritiated thymidine (10 µCi). Treated and control embryos were sacrificed 5 hours after the injection of thymidine, fixed in Bouin´s solution, then embedded in paraffin and serially sectioned. In treated embryos, the limbs were reduced and several of them displayed conditions of tetradactyly or of tridactyly, with a shortening of the remaining digital rays. Autoradiography of histological sections through these reduced autopods showed a strong reduction of the rate of DNA synthesis in the mesodermal cells of the digital rays: in these rays the labeling index, which reached 40% to 50% in controls declined to values of between 25% and 17% in the embryos treated with Ara-C. Moreover, the number of silver grains above the nuclei was higher than 100 in controls and less than 38 in embryos treated with Ara-C (Fig. 4a, b).

These autoradiographic studies establish that Ara-C induces a strong decrease in DNA synthesis in the mesodermal cells of the autopod in the limb bud of *Lacerta viridis* embryos. This decline in DNA synthesis results in a reduction of cell proliferation and in the death of numerous mesodermal cells. These changes lead to an arrest of development or a shortening of the digital rays. Thus, the digital reduction originates from a decrease in the rate of DNA synthesis in the mesodermal cells of the limb bud (Raynaud and Kan, 1988).

Decline in the rate of DNA synthesis in the mesodermal cells of the limb bud in *Anguis fragilis*, during and after the degeneration of the apical ectodermal ridge

The experimentally-induced limb reduction in *Lacerta viridis* embryos under the effect of Ara-C, a potent inhibitor of DNA synthesis, sheds light on the similarities between experimental and evolutionary limb reduction. These similarities suggested that the same mechanism might be involved in both types of reduction: a temporary decline or arrest in DNA synthesis in the mesodermal cells of the limb buds. This suggestion caused us to try and detect a decrease in the rate of DNA synthesis at a developmental stage preceding the reduction or the arrest of limb development in species of serpentiform reptiles normally displaying reduced limbs.

In collaboration with Mrs. P. Kan, I undertook a study of the DNA synthesis rate in the mesodermal cells of the limb bud of the slow worm *Anguis fragilis*. As mentioned above, this lizard is serpentiform and the adult, limbless; however, anlagen of limbs do form briefly in the young embryos, but only to quickly regress and disappear long before birth. Several observations suggest that developmental arrest of the limb bud, in *Anguis fragilis* is a consequence of the apical ectodermal ridge degeneration that occurs in the young embryo. The direct way of approaching this problem was then to compare the rate of DNA synthesis in the mesoderm of the limb bud before, during and after degeneration of the apical ridge. Autoradiography was used, after (^{3}H)-thymidine incorporation. Normal eggs of *Anguis fragilis* received, at various stages of their development, a single injection of tritiated thymidine (10 µCi/egg) in the yolk. The 22 treated embryos were at this time either at development stages previous to degeneration of the apical ridge, at the very stage of this degeneration, or else at stages posterior to the degeneration of the ridge. They were killed 5 hours after injection of the radioactive precursor and fixed in Bouin´s solution. After several days they were embedded in paraffin and serially sectioned: the sections were dipped in Ilford nuclear emulsion. After 13 days, the emulsion was developed and the sections stained with hemalun.

In the mesoderm of the salient part of at least six sections through the anterior limb bud, the total number of labeled and unlabeled cells was counted; the percentage of mesodermal cells that had incorporated thymidine (labeling index) was determined in this way. The values of this index at different stages of development (Table I) enabled us to monitor the variations in DNA synthesis before, during and after the degeneration of the apical ridge.

Before degeneration of the apical ridge, a high labeling index (38% to 45%) was found in the mesoderm of the limb bud (Table I); during the phase of regression of the apical ridge, the labeling index showed only a slight decrease (its value is 33% to 35%) (Fig. 4c). After degeneration of the apical ridge, the decline of the labeling index was strongly accentuated: thirty hours, for instance, after degeneration of the ridge, the labeling index had decreased by between 12.85% and 19% (Fig. 4d); 24 hours later, the index was 10% to 14% (Raynaud and Kan, 1989).

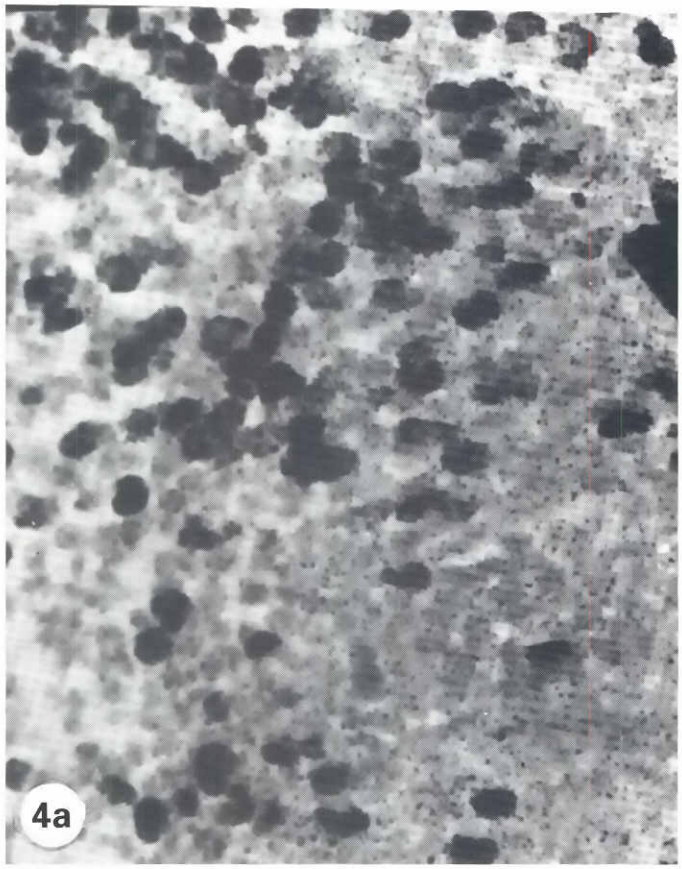
This decline in the rate of DNA synthesis precedes the other regressive changes occurring in the mesoderm of the limb bud (decline of the mitotic index, in the rate of RNA synthesis, etc.); it leads to a reduction in cell proliferation and to associated cell

TABLE 1

VARIATIONS OF THE LABELING INDEX, AFTER TRITIATED THYMIDINE INCORPORATION, IN THE MESODERM OF THE LIMB BUD OF EMBRYOS OF *ANGUIS FRAGILIS*, AT DIFFERENT STAGES OF THEIR DEVELOPMENT

Length of the allantoic bud of the embryo (mm)	Minimal and maximal values of the labeling index (percent of labeled cells) in the mesoderm.
0.3 - 1.5	38% - 45%
2.5 - 3.0	33.0% - 35.0%
3.9 - 4.5	12.0% - 20. 8%
5.0 - 8.0	10.5% - 15.2%

The values of the labeling index show a strong decrease after the degeneration of the apical ridge (occurring between the stages defined by an allantoic bud length of 1.5 to 3.0 mm).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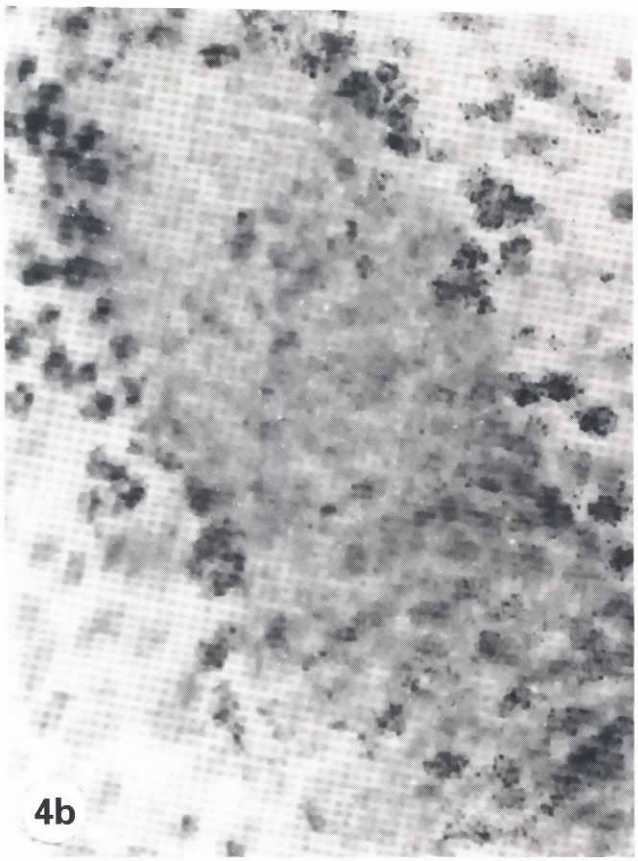
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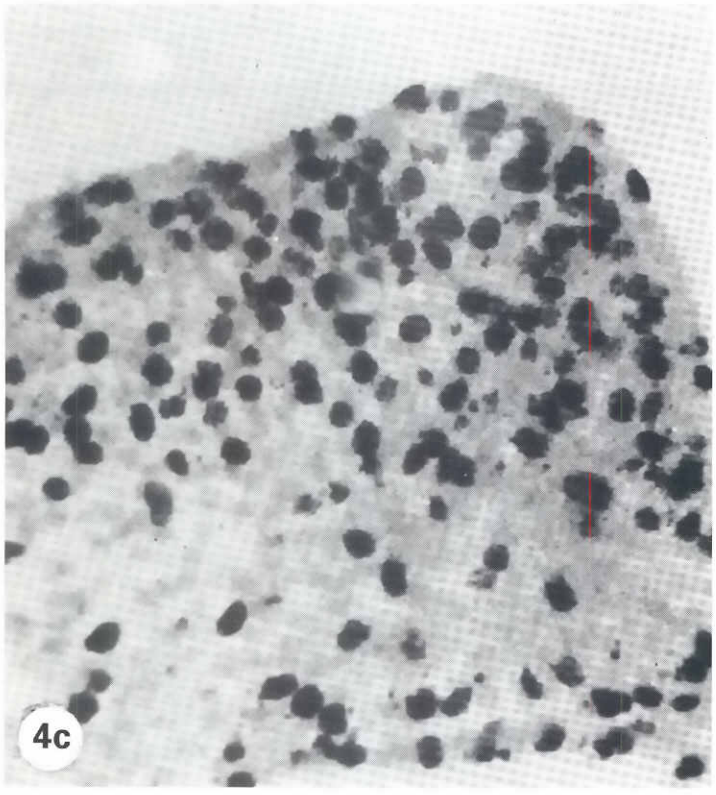
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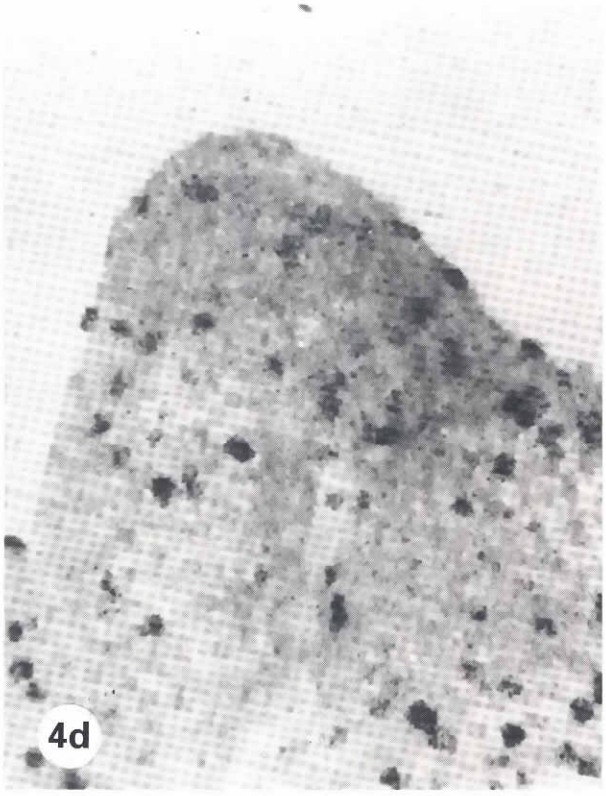
4d

death; it thus appears, besides degeneration of the apical ridge, as the main causative factor of the developmental arrest of the limb bud, in embryos of *Anguis fragilis* (Raynaud and Kan, 1989).

Generalizing, we may suggest that the terminal mechanism at the level of the anlage of the limb inducing the evolutionary reduction of limbs during embryonic life in serpentiform reptiles, would involve a decrease in the rate of DNA synthesis in the mesoderm of the limb buds.

Discussion

Our studies have shed light on the mechanisms involved in the reduction of limbs in serpentiform reptiles. We now are clearly on the way to identifying the various perturbations of the morphogenetic mechanisms leading to the partial or complete arrest of limb bud development, resulting in amelia, micromelia, ectropodia, hypodactylia, etc.

A somitic deficiency is at the origin of a first limb reduction. If no ventral processes are formed by the somites opposite the future territory of the limb bud, no limb develops; such is the case for most Ophidians. If there is only a partial somitic deficiency, that is to say, if only a reduced number of somites form processes penetrating into the future somatopleural territory of the limb bud, a reduced limb bud develops in which the posterior part is lacking (opposite the missing somites); in this limb bud anlage, the apical ectodermal ridge is always incompletely differentiated and it degenerates early (this regressive condition being very probably secondary to a failure in morphogenetic somitic stimulation).

The premature degeneration of the apical ectodermal ridge has been observed in the limb bud of many species of serpentiform lizards (*Anguis*, *Ophisaurus*, several *Scelotes*) and in *Python*. If the degeneration is early and complete, the limb anlage stops growing and regresses; in species whose apical ridge degenerates belatedly or incompletely, the mesodermal component of the limb bud can survive and form a reduced limb, more or less structurally simplified; moreover, an incomplete differentiation (absence of gap-junctions) must weaken the activity of the ridge and, thus, also contribute to various dificiencies of the limb bud.

Thus, the spontaneous and premature degeneration of the apical ridge in the limb buds of serpentiform reptiles, partially reproducing the experimental excision of the ridge in the chick embryo, may explain the developmental arrest of the limbs in reptiles: deprived of the stimulating substances normally built up by the apical ridge, the limb bud mesoderm can no longer grow normally, but either stops growing completely or forms only a rudimentary limb.

Moreover, the experimental reduction of the limbs in embryos of *Lancerta viridis* obtained by action of Ara-C shows that the young limb bud is a plastic structure: it can be modeled into a normal, pentadactylous limb as well as into a variety of reduced limbs, with all stages of loss of digits. We thus obtained several patterns of reduction (amelia, micromelia, ectropodia, hypodactylia) by a direct change in the morphogenesis of the young limb bud. For instance, from a normal young bud, it is possible to obtain limbs with four digits or with only two digits, without passing through a series of gradual regressive changes. Our studies also make it possible to establish the rules governing experimental digital reduction and to show that they are similar to those governing evolutionary digital reduction (same lost digits and same loss sequences). Since it is unlikely that these rules changed in the past, they must be taken into account by paleontologists in interpreting the reduction occurring in the limbs of fossil reptiles and identifying the missing digits in the fossil limbs.

Furthermore, the similarities existing in the modes of experimental (with Ara-C) and evolutionary reduction suggested that the same mechanism – a decline in the DNA synthesis rate in the anlage of the limb – might be at work in both types of reduction.

Finally, direct evidence was obtained by autoradiography for such a decrease in the rate of DNA synthesis in the mesodermal component of the limb bud of *Anguis fragilis*, during and after degeneration of the apical ridge. This makes it possible to characterize, at the cellular level, the biochemical mechanism inducing the evolutionary reduction of the limbs.

Our studies thus provide insight into most of the mechanisms involved in the evolutionary reduction of limbs: these mechanisms appear as defects in the morphogenetic mechanisms underlying the development of a harmonious limb.

The defects of these morphogenetic mechanisms probably result from genetic changes (as is the case with the conditions of "winglessness" or "limblessness" in bird embryos, or the hereditary hypodactyly observed in several mammals including Man). The pathways from gene activity to developmental limb abnormalities still remains partly unknown; but, in the broadest sense of the word, these genetic changes are mutations encoding aberrant versions of the mechanism of limb development. These gene disorders are expressed in the course of development, at different stages of limb bud formation, impairing the somitic action, the function of the apical ridge, etc.

It is difficult to link these perturbations of morphogenetic mechanisms to a lack of limb use in adult life, as is still held by followers of the Lamarckian theory of non-use and of the adaptative reduction theory (see Gans, 1975). It is an embryonic mechanism which determines limb reduction and we are led to consider that, at some time in the remote past, mutations developed in the genome, inducing alterations of somitogenesis, with all their consequences. Here it is worth mentioning that some link may exist between an increase in the number of somites in the trunk of serpentiform reptiles and a decrease in the number of somites (and in their functional capacities) involved in the initial development of the limb bud. This presumed morphogenetic correlation would make it possible to explain how the elongation of the body is associated with a reduction of the limbs in serpentiform reptiles.

In conclusion, our studies show that the mechanism of limb reduction is essentially an embryonic process determining an arrest

Fig. 4 Autoradiographs, after incorporation of tritiated thymidine, of histological sections through the limb buds of reptiles. **(a, b)** *Sections through the distal part of the digital ray IV of young embryos of* Lacerta viridis *at 16 days of incubation (at 26° C) (x654 for the two photographs).* **(a)** *Control embryo; the anlage of digit IV is highly labeled.* **(b)** *Embryo treated on day 11 with 40 µg of Ara-C and killed on day 16: the low labeling shows that Ara-C has provoked a strong decline in the rate of DNA synthesis in the cells of the digital ray and in the surrounding mesenchyme.* **(c, d)** *Sections through the anlage of the limb in two young normal embryos of the slow-worm* (Anguis fragilis). *x316. In the younger embryo, at the stage when the allantoic bud is 3.0 mm long, the limb bud is strongly labeled. In the older embryo at the 4mm-long allantoic bud stage, the faint labeling indicates a strong decline in the rate of DNA synthesis in the mesodermal cells of the limb bud.*

of development of the limb anlage. Besides the insight provided into the alterations of the morphogenetic mechanisms responsible for this arrest of growth, we extended at the cellular and molecular levels our knowledge of the processes of reduction, and I believe that these studies afford new insight into the general mechanisms of regressive evolution.

Summary

Until thirty years ago, our understanding of the causality of regressive evolution of the limbs in serpentiform reptiles was most unsatisfactory and researchers could only formulate hypotheses.

Around 1960, the problem of limb reduction was put back into the field of ontogeny. In serpentiform reptiles (*Anguis, Scelote, Ophisaurus*, etc.), an embryological analysis makes it possible to detect the defects of the morphogenetic mechanisms involved in the embryonic reduction of limbs: first, a somitic deficiency; next, an incomplete differentiation and a premature degeneration of the apical ectodermal ridge.

By a chemical action (effect of cytosine-arabinofuranoside), it became possible to induce a reduction of limbs in the embryos of the lizard, *Lacerta viridis*, a tetrapod pentadactylous species. This experimental reduction provides evidence that the limb bud is a malleable structure that can be fashioned into a normal limb as well as into various types of reduced limbs: from the young anlage of the limb, it is possible to directly obtain several patterns of reduction and, in particular, several patterns of digital reduction without passing through a gradual series of regressive changes. This study afforded the opportunity of bringing to light the rules governing experimental and evolutionary digital reductions. Moreover, the similarities between the modalities and patterns of this experimental reduction and those of evolutionary reduction suggested that an identical cellular mechanism – a decrease in the rate of DNA synthesis – might be at work in both types of reduction.

Autoradiography makes it possible to obtain a direct demonstration of this interpretation: a strong decline in the rate of DNA synthesis was observed in the mesodermal cells of the limb bud of the slow-worm (*Anguilus fragilis*) during and after degeneration of the apical ectodermal ridge. This decrease, associated with a reduction in cell proliferation and with cell death, appears at the cellular level as the main causative factor of the developmental arrest of the limb.

The reduction of limbs is thus the result of an embryonic arrest of development of their anlagen and this arrest is a consequence of defects in the morphogenetic mechanisms normally building a harmonious limb. These defects are very probably the consequence of genetic abnormalities which occurred in the past, in the genomic factors controlling embryonic development of the limb.

This embryological explanation of the developmental arrest of the limb buds as a consequence of defective morphogenetic mechanisms should replace the old Lamarckian concept of the loss of limbs through non-use in adult life.

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