Int. J. Dev. Biol. 36: 303-310 (1992)

Original Article

DNA synthesis decline involved in the developmental arrest of the limb buds in the embryos of the slow worm, *Anguis fragilis* (L.)

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ABSTRACT The present study was carried out to try and detect the biochemical mechanism involved in the developmental arrest of the limb bud in a serpentiform Reptile. Autoradiography, following tritiated thymidine incorporation, in embryos of the slow-worm (*Anguis fragilis*, L.) reveals a strong decrease in the rate of DNA synthesis in the mesodermal cells of the limb bud, after the degeneration of the apical ectodermal ridge (AER); the curve (a function of Gompertz) visualizing this decline shows that the drop in DNA synthesis becomes accentuated just after the degeneration of the AER. This decrease precedes the reduction of the mitotic index, the cell degeneration in the mesoderm and the other regressive changes occurring in the limb bud; it thus appears as the main causative factor of the developmental arrest of the limb bud. Furthermore, these results suggest that one of the functions of the AER would be to maintain a high level of DNA synthesis in the mesoderm underlying the AER in a normal limb bud.

KEY WORDS: *Anguis fragilis, limb morphogenesis, limb reduction, DNA synthesis*

Introduction

The slow-worm, *Anguis fragilis* (L.) (Fig. 1) is a serpentiform lizard, which in the adult is devoid of legs but with limb anlagen appearing temporarily in the young embryos. After a first protruding, their growth ceases, and they regress and disappear long before hatching (Born, 1883; Raynaud, 1962a, 1963). Our studies brought to light the key role, in this arrest of development, of deficiencies in the essential morphogenetic mechanisms responsible for the development of the limb: first, an initial somitic deficiency; and secondly, an incomplete differentiation (absence of gap junctions) and an early, premature degeneration of the apical ectodermal ridge (AER) that forms along the distal edge of the limb bud (Raynaud, 1962b, 1974, 1977, 1985; Raynaud *et al.*, 1979).

In order to further our knowledge of the biochemical mechanism at work in the developmental arrest of the limb bud, we undertook a study of the variations in the synthesis of nucleic acids in the anlage of the limb. The first part of this study related to the apical ridge and to its relationships with the mesoderm of the bud (Raynaud and Vasse, 1970, 1971, 1972). In order to accurately settle the variations in the rate of cellular proliferation in the mesoderm of the limb bud of *Anguis fragilis*, we investigated the variations in the rate of DNA synthesis in the mesodermal cells of the anlage of the bud at different stages of its development, and the variations in the mitotic index. This study was performed in the course of the three last years and a summary of its results was given in brief preliminary reports (Raynaud and Kan, 1989; Raynaud, 1990). We shall now proceed to the report of our whole study and of its results.

Results

Variations in the values of the labeling index (after incorporation of tritiated thymidine) for the subridge area of the mesoderm in the limb bud of Anguis fragilis, *at various stages of the development and regression of the bud*

The photographs in Fig. 2 are autoradiograms of the anterior limb buds of *Anguis fragilis* at stages defined by a length of the allantoic bud—1.5 mm (Fig. 2A), 3.2 mm (Fig. 2B) and 4.0 mm (Fig. 2C,D) (see Table 1).

The examination of these autoradiograms and of the values of the labeling index shows that the mesoderm of the limb bud of

Abbreviations used in this paper: AER, apical ectodermal ridge; Ara-C, Cytosine 1-ß-D-arabinofuranoside; S1-S13, post-otic somites 1 to 13; H-H stages, stages of development of the chick embryo defined by Hamburger and Hamilton (1951).

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0214-6282/92/$03.00

Printed in Spain

Fig. 1. Adult specimen of *Anguis fragilis* L.

Anguis fragilis is strongly labeled by the tritiated thymidine at the early stages of the development of the limb defined by a length of 0.2 to 1.5 mm of the allantoic bud. At this period of the development, 39% to 42% of the mesodermal cells are labeled, giving evidence of an active synthesis of DNA at these stages. Later on, in embryos whose allantoic bud length is 2.5 mm to 3 mm, the labeling index in the mesoderm is still 33% to 35%. Twelve to twenty-four h later, in embryos whose allantoic bud length ranges 3.2 mm and 3.9 mm, the value of the labeling index has dropped to 20.8% and 23.4%. Thirty h after the end of the degeneration of the AER (embryos whose allantoic bud length is between 4.0 mm and 4.5 mm), the value of the labeling index has strongly declined, ranging now between 19% and 12.5% (Table 2).

These results show that during and after the degeneration of the apical ectodermal ridge, the labeling index (after tritiated thymidine incorporation) declines in the mesoderm of the limb bud of *Anguis fragilis*. If the value of this index already decreases from the stage of an allantoic bud length of 3.2 mm, the decline becomes more pronounced at about thirty hours after the end of the degeneration of the ridge (that is to say around 80 h after the beginning of the degeneration of this AER): at these stages only 25% of the mesodermal cells are synthesizing DNA in the limb bud of *Anguis fragilis*.

From the values of the labeling index obtained by count of labeled cells in the autoradiograms, it was possible to draw a theoretical curve (Fig. 3) from estimated points corresponding to a function of Gompertz using the least squares method. This function fulfills the conditions of the following equation:

$$y = b_3\, e^{-e^{-\left(\frac{x - b_1}{b_2}\right)}}$$

where the constants b_1, b_2 and b_3 possess the following values: b_1: 4.2354 (inflexion point); b_2: 0.9654; b_3: 41.1556.

This curve (Fig. 3) shows the continuous decrease in the value of the labeling index in the mesoderm of the anterior limb bud during and after the degeneration of the AER; furthermore it shows that the decrease becomes strongly accentuated after the degeneration of the apical ridge (visualized by a solid line on the horizontal axis of the graph). This curve permits a comparison of the variations of the labeling index with the other modifications occurring at these stages in the limb bud (arrest of growth, variations in the values of the mitotic index, etc.); this comparison shows that the decrease in the value of the labeling index precedes the other regressive changes taking place in the limb bud, namely the reduction in mitotic proliferation and the occurrence of intensive cell degeneration.

Variations in the intensity of the labeling after incorporation of tritiated thymidine, in the mesodermal cells of the limb bud of* Anguis fragilis, *during its development and regression

Another phenomenon is brought to light by this labeling study: the decline in the intensity of the labeling in the mesodermal cells of the limb bud, soon after the end of the degeneration of the AER. This decrease is already apparent on the autoradiograms of Fig. 4. To obtain more precise data on this variation, we counted the number of silver grains above the nuclei of the labeled mesodermal cells, in the salient part of the limb bud, in ten embryos whose allantoic bud length ranged from 2.5 mm to 4.5 mm. We also counted the number of silver grains above the nuclei of mesoblastic cells located outside the limb bud, in the neighborhood of this bud (more precisely, in an area located between the basal part of the limb bud and the neural tube on transversal sections of the embryos). This made it possible to confirm the fact that the large variations in the intensity of labeling seen in the mesodermal cells of the limb bud are really peculiar to these cells and do not reflect general variations occurring in others tissues of the embryo.

The numbers show that from the 3.9 mm allantoic bud length stage—that is to say, 30 h after the end of the degeneration of the AER—a strong decrease occurs in the intensity of the labeling by the tritiated thymidine, in the mesodermal cells located in the salient part of the limb bud (Table 3).

TABLE 1

LENGTH OF SUCCESSIVE DEVELOPMENTAL STAGES FOR THE EMBRYOS OF THE SLOW-WORM (*ANGUIS FRAGILIS* L.) IN EGGS CULTURED *IN VITRO* AT A TEMPERATURE OF 25°C

Allantoic bud length (mm)	Duration of development (h)
0.5	
	48 h
1	
	24 h
1.5	
	55-65 h
3	
	27-30 h
4	
	24 h
4.5	
	15 h
5	

Note that the apical ridge on the anterior limb bud degenerates between the stages defined by an allantoic bud length of 1.5 mm to 3 mm.

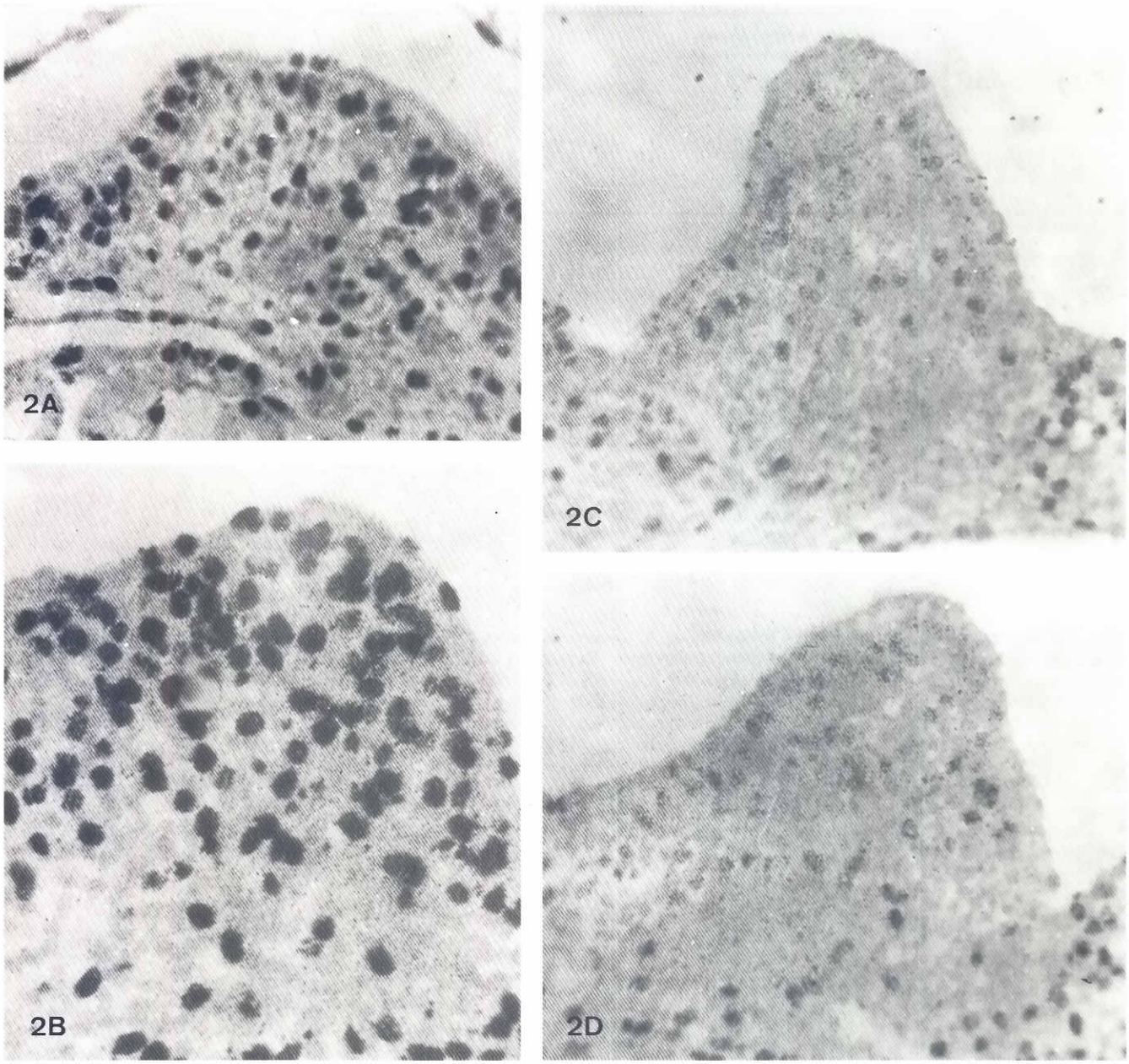

Fig. 2. Autoradiographs of sections of normal young limb buds of *Anguis fragilis*, after labeling with tritiated thymidine, during the period of the growth of the limb bud *(Mx186 for A and Mx242 for B) and at the stage of the beginning of the regression of the bud (C and D, Mx242).* **(A)** *Right anterior limb bud of an embryo at the 1.5 mm-long allantoic bud stage.* **(B)** *Right anterior limb bud of an embryo at the 3.2 mm-long allantoic bud stage.* **(C and D)** *Sections of the anterior limb buds of two embryos at the 4.0 mm-long allantoic bud stage. In these sections, the percentage of cells labeled with thymidine is only of 16.8% to 19% in the mesoderm of the limb bud. The autoradiographs show that at early stages of the limb bud, the DNA synthesis, which has stopped in the AER, is active in the mesoblast. Next a sharp decline in this synthesis occurs in the mesoderm after the degeneration of the AER.*

It should be observed that some decline in the intensity of the labeling appears in the cells located outside the limb bud, near its basal part, but it is of feeble amplitude in comparison with that which occurs in the mesodermal cells of the limb bud. It is probably in keeping with a general decline in the growth of this area of the thoracic wall of the embryo.

Mitotic index and labeling index for the mesodermal cells of the limb bud of* Anguis fragilis *at different stages of the development of the bud

The comparison of the variations in the values of the mitotic index with the values of the labeling index at different stages of limb bud development in *Anguis* embryos reveals (Fig. 3) that:

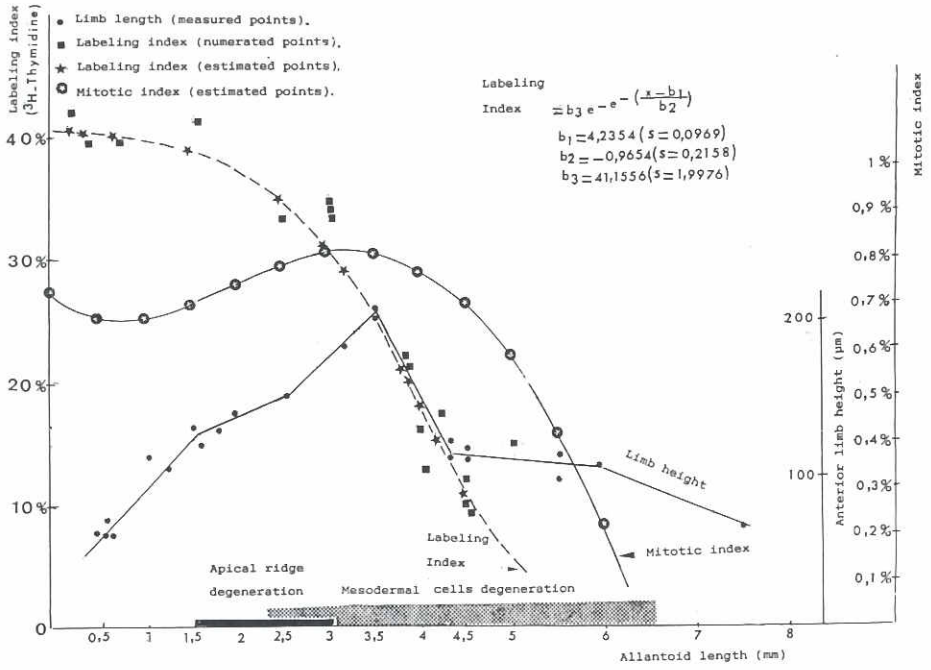

Fig. 3. Curves visualizing the variations in the mitotic index, in the labeling index and the growth and regression of the anterior limb buds of *Anguis fragilis*: *growth of the limb (right hand ordinate), variations of the mitotic index (right hand ordinate) and variations of the labeling index (after incorporation of tritiated thymidine) in the mesoderm of the limb bud (left hand ordinate). The curve showing the growth and the retrogression of the limb bud was drawn from measurements of the height of the limb above the thoracic body wall, on transversal histological sections of the embryos. The curve showing the variations of the mitotic index was drawn from data obtained by polynomial fitting of the values of the index obtained by count of mitotic figures per cent nuclei. The curve showing the variations of the labeling index (percentage of cells that had taken tritiated thymidine) before and after the degeneration of the apical ectodermal ridge, was drawn from estimated values given by a function of Gompertz (calculated by the least squares method, from the values obtained by count of labeled cells in sections of the mesoderm of the limb bud). The values of the parameters of this curve and of the corresponding standard deviations are given in this Figure. The successive stages of embryonic development are plotted on the horizontal axis as values of the allantoic bud length. The degeneration of the apical ectodermal ridge (indicated by a thick black bar) takes place between the stages of 1.5 mm and 3 mm of allantoic bud length. The degeneration of the mesodermal cells is indicated, along the horizontal axis, by a stippled strip.*

- when the mitotic index begins to decline, the labeling index has already distinctly decreased: its value is only about 27%;
- when the limb bud of *Anguis fragilis* ceases to grow, the values of the mitotic index for the mesodermal cells are still about 0.70% to 0.80%; thus no fall in this index occurs before the beginning of the regression of the limb bud. A decline in the rate of cellular proliferation takes place only some hours after the cessation of the limb outgrowth. The variations, always of feeble amplitude, in the values of the mitotic index do not appear to be involved in the initial phase of the regression of the limb bud of *Anguis fragilis* (Table 4). Another factor comes into play: cell death in the mesoderm.

Cell degeneration in the mesoderm of the limb bud, in Anguis fragilis

Histological studies have shown (Raynaud, 1972a,b, 1974) that numerous cell degenerations occur in the mesoderm of the limb bud of the slow-worm. Cell death first occurs at the relatively early 1.8 mm-to-2 mm allantoic bud length stage; that is to say, soon after the beginning of the degeneration of the apical ectodermal ridge. At these stages cell death is chiefly located in the basal part of the limb bud.

After the degeneration of the AER, the proportion of dead cells increases. At the stage when the allantoic bud is 4 mm long, numerous degenerating cells are present in the somitic tissue—in the axial part of the limb bud and among the mesodermal cells of the sub-ridge area. At this stage, the limb bud has begun to sink and the layers of the mesodermal cells under the epiblast have lost their basophilia and the size of their cells drops steeply. The span of the degenerating cells aggregates being unknown, it is difficult to estimate quantitatively the proportion and the incidence of cell death at any stage. However, a relatively much higher number of dead cells than of dividing cells seems to be present at these stages and cell death appears at this period to be the most likely essential factor involved in limb bud reduction.

Discussion

The curves of Fig. 3 and other observations show that the decline in the rate of DNA synthesis for the mesodermal cells represent the first change occurring in the mesoderm of the limb bud. The other regressive changes—reduction in cell size (Raynaud, 1972b, 1974), decline in the rate of RNA synthesis 38 to 48 h after the beginning of AER degeneration (Raynaud and Vasse, 1972; Raynaud, 1985), ultrastructural changes (Raynaud *et al.*, 1973; Raynaud, 1974), cell death, decline of the mitotic index, etc.—occur later in the meso-

TABLE 2

VARIATION IN THE VALUE OF THE LABELING INDEX, AFTER TRITIATED THYMIDINE INCORPORATION, IN THE MESODERM OF THE RIGHT ANTERIOR LIMB BUD OF *ANGUIS FRAGILIS*

Allantoic bud length (mm)	Observed values of the labeling index (% of labeled cells)	Standard deviation (s)	Variance coefficient (v)
0.2	42.0	2.71	6.45
0.4	43.9	5.79	13.18
0.4	39.7	2.79	7.02
0.7	39.6	6.58	16.61
1.5	41.5	4.13	9.95
2.5	33.0	1.71	5.19
3.0	35.0	2.26	6.45
3.0	34.9	4.10	11.76
3.0	34.0	3.26	9.58
3.2	23.4	1.80	7.67
3.8	23.1	4.29	18.56
3.9	20.8	1.98	9.55
4.0	19.0	3.69	19.43
4.0	16.8	3.53	21.02
4.0	12.8	4.00	31.23
4.2	17.7	1.88	10.59
4.5	12.2	2.34	26.45
5	15.1	4.25	21.22

derm and very probably are subordinated to the reduction in the rate of DNA synthesis.

The drop in the rate of DNA synthesis thus appears, besides the degeneration of the AER, as the main causative factor of the developmental arrest of the limb bud in *Anguis fragilis* embryos. This conclusion comes up with the one proceeding from the results of a comparative study of limb reduction in the embryos of *Lacerta viridis* treated by Ara-C (Raynaud and Kan, 1988) and in the embryos of the serpentiform Reptiles (Raynaud, 1986, 1989, 1991). Thus the evolutionary reduction of the limbs in nature would result from a decrease or temporary arrest of DNA synthesis in the mesoderm of the limb bud at definite stages of embryonic life.

The curves of Fig. 3 show that the decrease in the rate of DNA synthesis in the mesoderm of the limb bud of *Anguis fragilis* occurs shortly after the beginning of the AER degeneration. They show, furthermore, that this decline becomes accentuated from a stage defined by an allantoic bud 3.5 mm long, that is to say just after the total regression of the apical ridge. The decrease in the rate of DNA synthesis in the mesoderm is thus very probably the consequence of the apical ridge regression. This conclusion is apparently in contradiction with the observations made on the chick embryo: after surgical excision of the AER, at H-H stage 19, Janners and Searls (1971) note that this ablation has but little effect on the labeling index (after tritiated thymidine incorporation) for the mesodermal cells of the wing bud. The study of these authors concerns chick embryos sacrificed 4 to 36 h after the excision of the AER. Now, the curve of Fig. 3 relating to the labeling index for the mesoderm of the limb bud of *Anguis fragilis* shows that the decline of this index becomes substantial (inferior to 27%) only some hours (10 h to 15 h) after the end of the degeneration of the apical ridge (stage of an allantoic bud of 3.5 mm in length). This degeneration continues 55 to 65 h, with the accentuated decrease in DNA synthesis occurring around 80 h after the beginning of the involution of the AER. Taking into account the differences in the rate of embryonic development between *Anguis* embryos (developing at 25-26°C), and the chick embryo (developing at 38°C), we reach the provisional hypothesis that a decline in DNA synthesis might be predicted to occur only 40 h after the excision of the apical ridge in the chick embryo. Now, since in the work of Janners and Searls (1971), the time elapsing between the excision of the AER and the evaluation of the rate of DNA synthesis does not extend beyond 36 to 40 h, it may be suggested that a prolongation of this study beyond this period would appear necessary.

The observations relating to this problem are somewhat difficult to interpret, due to the fact that in the first stages of the chick wing bud, a feeble decline in the labeling index (after thymidine incorporation) occurs in the mesoderm (Hornbruch and Wolpert, 1970; Janners and Searls, 1970; Searls and Janners, 1971; Lewis, 1975; Summerbell, 1977). Furthermore, a feeble retardation in the rate of

TABLE 3

INTENSITY OF THE LABELING, AFTER TRITIATED THYMIDINE INCORPORATION, IN THE MESODERMAL CELLS OF THE RIGHT ANTERIOR LIMB BUD OF *ANGUIS FRAGILIS* AND IN MESODERMAL CELLS OUTSIDE THE LIMB

Allantoic bud length (mm)	% of nuclei, surmounted by 20-40 grains	40-60 grains	60-80 grains	80-100 grains	>100 grains	Background (*)
In the mesoderm of the salient part of the limb bud						
2.5	1	7	8	14	70	12
3.0	4	7	6	2	81	12
3.0	1	2	5	2	90	22
3.0	4	7	4	13	72	15
3.2	2	9	2	11	76	12
3.8	1	7	6	12	74	11
3.9	7	16	6	41	30	11
4.0	44	42	7	2	5	12
4.0	40	45	6	1	8	9
4.2	24	17	20	14	25	14
In the mesoderm outside the limb bud, near its basal part						
2.5	6	2	2	6	84	
3.0	1	3	6	2	88	
3.0	0	3	8	4	85	
3.0	2	4	2	12	80	
3.2	0	12	6	2	72	
3.8	0	8	3	15	74	
3.9	3	6	4	18	69	
4.0	13	15	14	6	52	
4.0	9	21	10	6	54	
4.2	0	9	3	24	64	

(*) The intensity of the background is here defined by the number of silver grains (average of 10 counting), on a surface outside the section of the embryo equal to that of a nucleus. This value is not given in the lower part of the Table, the sections used for the determination of the intensity of the labeling being the same as those used in the upper part of this Table.

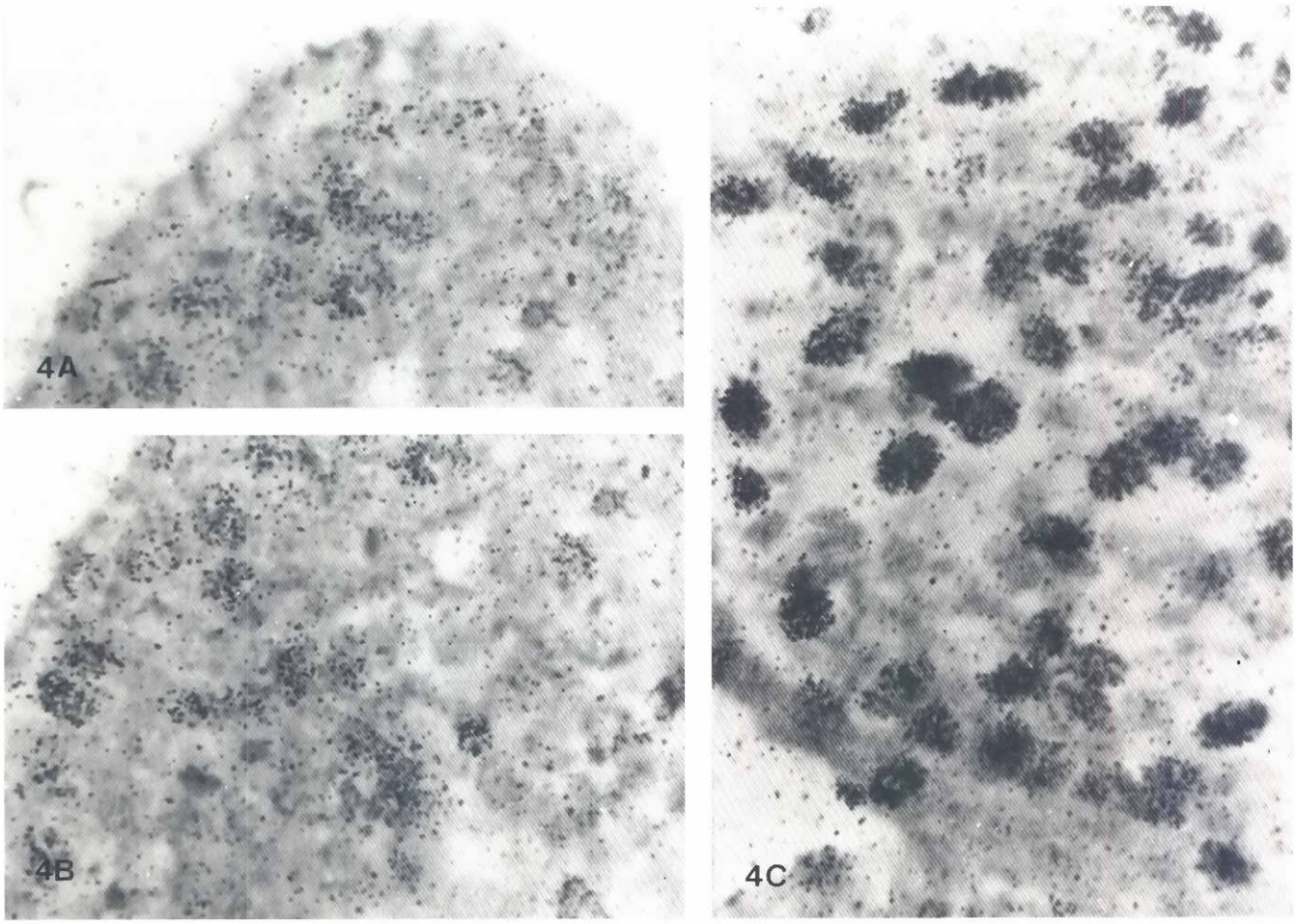

Fig. 4. Variations in the intensity of DNA synthesis in the mesoderm of the limb bud: *decline in the intensity of the labeling after incorporation of tritiated thymidine, for the mesodermal cells in the salient part of the limb bud (A,B) of the embryo of the slow-worm* (Anguis fragilis *L.) after the retrogression of the apical ectodermal ridge (Mx537 for all the photographs).* **(A and B)** *Distal (A) and central (B) part of the limb bud, on the same section of the anterior right limb bud of an embryo at the 4.0 mm-long allantoic bud stage. Over the nuclei of the majority of the labeled cells, the number of silver grains is reduced (grain count: between 20 and 50).* **(C)** *On the same section of the limb bud, the mesoderm located near the lower part of the limb bud but outside of the limb is highly labeled: most of the nuclei are densely labeled (grain count: between 80 and 100).*

growth of the wing bud was observed after excision of the AER (Summerbell, 1977) at H-H stage 19. It would thus be useful to study, in the chick embryo, the effects of an earlier excision of the apical ridge of the wing bud at a stage equivalent to the one at which the apical ridge of the limb bud of *Anguis fragilis* begins to degenerate.

Conclusion

Autoradiography after tritiated thymidine incorporation clearly shows a decrease in the rate of DNA synthesis in the mesodermal cells of the limb bud of the slow-worm (*Anguis fragilis*, L.) from the stage at which the apical ectodermal ridge (AER) begins to degenerate. After the total degeneration of the AER, the decline in DNA synthesis strongly accentuates; moreover, a high percentage of labeled cells in the salient part of the limb bud shows a greatly reduced number of silver grains over their nuclei.

The decrease in the rate of DNA synthesis in the mesodermal cells precedes the other regressive changes occurring in the mesoderm of the limb bud. The study of the mitotic index and the occurrence of cell death in the mesoderm suggest that the cell degeneration in the mesoderm, subsequent to the decline in DNA synthesis, plays a key role in the arrest of growth of the limb bud. The subsequent retrogression of this bud would result from the decrease in the proliferative activity of the mesoderm and from the associated cell death, all together induced by the accentuated decline, at these stages, in the rate of DNA synthesis.

Occurring during the phase of the retrogression of the AER of the limb bud, the decline in DNA synthesis in the mesodermal cells is likely the consequence of the involution of this ridge.

Retrogression of the apical ectodermal ridge and decline in the rate of DNA synthesis in the mesoderm of the limb bud thus appears as the main causative factors of the evolutionary regression of the limb in the embryos of *Anguis fragilis*.

TABLE 4

VALUES OF THE MITOTIC INDEX, OBTAINED BY COUNTING AND ESTIMATED VALUES (AFTER POLYNOMIAL FITTING) FOR THE MESODERMAL CELLS OF THE RIGHT ANTERIOR LIMB BUD OF *ANGUIS FRAGILIS*

Allantoic bud length (mm)	Mitotic index: Values obtained by counting	Mitotic index: Estimated values
0.5	0.61	0.675
0.6	0.79	0.671
0.7	0.61	0.669
1	0.70	0.673
1.5	0.65	0.701
1.6	0.66	0.709
1.8	0.74	0.726
1.8	0.81	0.726
2.0	0.82	0.743
2.2	0.68	0.760
3.0	0.77	0.800
3.0	0.84	0.809
3.2	0.66	0.813
3.5	0.84	0.809
3.5	0.96	0.809
4.5	0.51	0.703
4.5	0.69	0.703
4.5	0.74	0.703
4.5	0.85	0.703
5.0	0.58	0.588
6.0	0.28	0.227
6.0	0.18	0.227

Materials and Methods

For the study of the rate of DNA synthesis in the limb bud, 27 embryos of *Anguis fragilis* were used. Their allantoic buds ranged between 0.2 mm and 6 mm in length. Of the embryos, 4 were at a development stage prior to degeneration of the AER, 5 were at the very stage of this degeneration (the length of their allantoic bud was between 1.5 mm and 3 mm). Nineteen embryos were at stages posterior to the degeneration of the ridge (allantoic buds ranging between 3.2 mm and 6 mm in length). Each embryo received in the yolk sac a unique injection of 10 µCi of tritiated thymidine (specific activity: 25 Ci/mM). The allantoic bud length was measured just after the injection. The laboratory temperature was kept at 23°C during the experiment. The treated embryos were killed 5 h after the injection of the precursor and fixed in Bouin solution. Some days later, the embryos were transferred to a 70% ethanol solution and next examined under the binocular microscope for staging (by morphologic criteria). Then they were embedded in paraffin and serially sectioned transversally (sections 7.5 µm thick). After removing paraffin with toluene, the sections were washed first in running water and then in distilled water, and dipped, still wet, in Ilford nuclear emulsion (type K5, diluted by half). After 13 days of exposition, at a temperature of 17°C, the emulsion was developed and the sections were stained with Mayer's hemalun.

The labeling index, i.e., the percentage of cells that had taken up tritiated thymidine, was obtained by count of labeled and non-labeled nuclei in the mesoderm of the salient part of the bud, in at least six sections through a given limb bud; the value of the labeling index is the average value for these six sections.

From the values obtained by counting, «estimated values» were determined for several stages of the embryos defined by allantoic bud length, from a function of Gompertz (see above), making it possible to draw a theoretical curve (Fig. 3) visualizing the continuous variation of the labeling index before, during and after the degeneration of the AER.

The intensity of the labeling by tritiated thymidine was determined by counting the number of silver grains above the nuclei of labeled mesodermal cells, using the method mentioned above. The mitotic index was also determined in the mesoderm for different stages of development of the limb bud of *Anguis fragilis* and for that of *Lacerta viridis*.

Acknowledgments

We would like to thank Professor E. Angelier (head of the laboratory of Zoology), Dr. A.M. Duprat and Prof. J. Cl. Beetschen (Center of Biology of Development), University Paul-Sabatier, and Prof. Et. Wolff for their useful comments and encouragement. A part of this work was performed at the Center of Biology of Development. Professor L. Bonnet gave us his invaluable help for the statistical evaluation of the results and plotting of the curves of Fig. 3. This work was supported by CNRS and Singer-Polignac Foundation.

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Accepted for publication: February 1992