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Histochemical Study on the Appearance of Aminopeptidase in the Limb Regeneration of *Triturus pyrrhogaster*

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Studies on the chemical changes occurring in the successive phases of regeneration——dedifferentiation, blastema formation, and redifferentiation——must be fundamental for the full understanding of the phenomena. Indeed, there are some biochemical works on the regeneration of extremities and tail of Amphibians (OREKOVITSCH, BROMLEY and KUSMINA, 1935; OREKOVITSCH, 1936; WEBER, 1957; DEUCHAR, WEBER and LEHMANN, 1957). But so far, few effort has been made to correlate the chemical changes with the histological informations, except for the studies of GHIRETTI (1950), of KARCZMAR and BERG (1951), using histochemical method and of BODEMER and EVERETT (1959) applying the radioautographic technique. In the present work the distribution and activity of aminopeptidase were studied by means of the histochemical method. The informations gained from such study might contribute something to the understanding of the protein metabolism associated with the process of regeneration.

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Materials and Methods

Adult newts, *Triturus pyrrhogaster*, collected from the fields in the vicinity of Kyoto and reared at room temperatures (10-14°C) were used as materials. Both forelimbs of a newt were amputated through the distal part of the humerus, and histochemical observations were carried out at intervals of 10 days up to 160th day after amputation.

Histochemical procedures: Aminopeptidase determination was made according to the method of NACHLAS, CRAWFORD and SELIGMAN (1957) using DL-alanyl-β-naphtylamide as a substrate. The excised limbs carrying regenerating tissues

were embedded within fresh ox liver, frozen, and sectioned longitudinally at 20 micra in thickness in cryostat at −12.5°C (revised by SHIMIZU, KUBO and MORIKAWA 1956). Sections were mounted on the clean slides and dried in the air to ensure adhesion. After fixation in 10 per cent neutral formalin for 10 minutes, the slides were rinsed briefly in the distilled water, and incubated for 30 minutes at 37°C in the following mixture; 1 ml of DL-alanyl-β-naphthylamide (8 mg/ml), 10 ml of 0.1 M acetate buffer (pH 6.5), 8 ml of 0.85% sodium chloride and 1 ml of 2×10^{-2} M potassium cyanide. To this mixture 10 mg of Diazo Blue B (tetrazotized diorthoanisidine) was added just before using. After incubation, the slides were rinsed again in the distilled water for short time, then were treated with 0.1 M copper sulfate. Dehydration and mounting with balsam were done in usual manner.

Observations

Dedifferentiation phase of regeneration: Before describing the change of distribution of the enzyme in the course of regeneration, it should be mentioned here that the regenerating process of the Japanese newt *Triturus pyrrhogaster* proceeds very slowly in comparison with that of American and European species so far recorded. It takes more than 60 days after amputation at room temperatures (10–14°C) to reach the initial stage of accumulation of blastema cells.

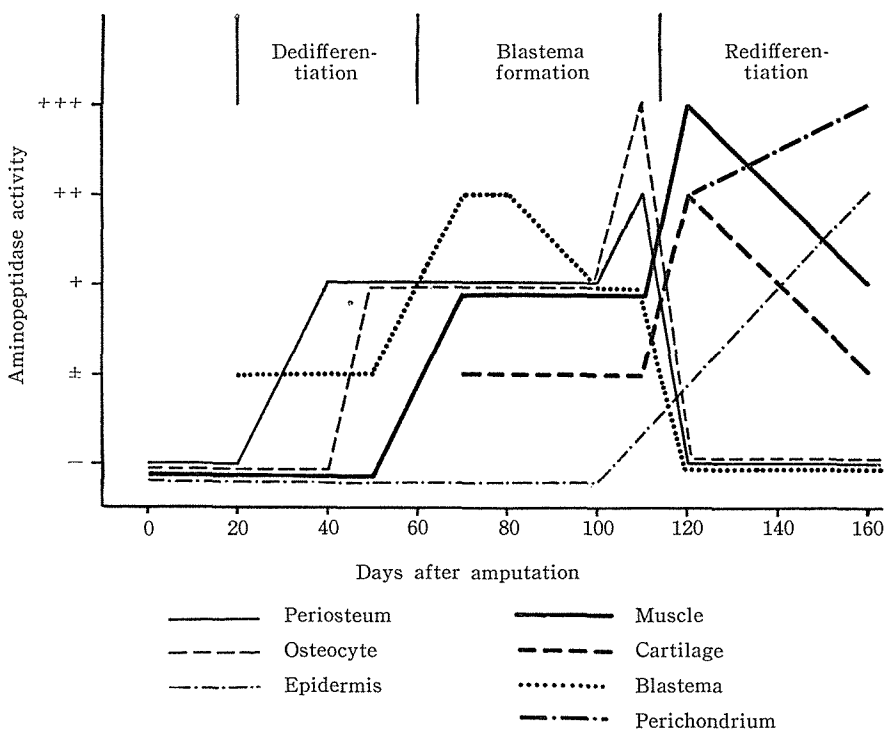
Within 20 days after amputation the aminopeptidase activity is still completely negative, even in the new epidermis over the cut surface. At 30 days a moderate activity of the enzyme begins to appear at the peripheral osteocytes and certain parts of the periosteum of the transected humerus, whereas the osteocytes located at the central portion of the humerus show no activity (Plate I, Fig. 1). The activity becomes very intense at all parts of the periosteum as well as the peripheral osteocytes at 40 days after amputation, when the degeneration of bone starts at the cut end. On the 80th day disintegration of the humerus has proceeded markedly, but is still under way. The lacunae of the bone in the peripheral part of the degenerating bone become open to the outside due to the dissolution of bony substance. Thus, the osteocytes are liberated towards the blastema which is now being established. These released cells show a marked activity (Plate I, Fig. 3). At this state the muscle indicates also a slight activity of the enzyme. Degeneration of the bone is almost completed about 110 days after amputation leaving only its debris surrounded by the periosteum and some freed osteocytes. At this state the highest activity of the enzyme is found in the periosteum and adjacent cells (Plate I, Fig. 3). The nerves and the wound epidermis do not yet show any detectable reaction.

Phase of blastema formation: Fibrocytes make their first appearance in the region between the new epidermis and the tip of the amputated bone during the 30th to 50th day after amputation. Later, some of the fibrocytes begin to aggregate into the procartilaginous assembly capping the degenerating bone.

The enzymatic activity is detected in this procartilage, with the increasing intensity as the time lapses. On the 80th day after amputation, the accumulated blastema cells display a strong aminopeptidase activity, especially near the distal end of the humerus (Plate I, Fig. 2). The thickened epidermis overlying the blastema, however, shows no activity at all.

Phase of redifferentiation: The distal part of the original muscle begins to regenerate by means of terminal formation of the sarcoplasmic buds at 70 days after amputation. A marked aminopeptidase activity is found to occur in these budding muscle fibers, showing the definite pattern of proximo-distally increasing gradient (Plate I, Fig. 4).

Cartilage is the first tissue to differentiate within the regeneration blastema, distinct cartilaginous tissue being observed at 120 days after amputation. The round chondrocytes found at the peripheral part of the new cartilage show strong activity, while the chondrocytes located at the central part of the cartilage indicate no activity at all (Plate II, Figs. 5, 6 and 8). The activity of chondrocytes, if any, is always restricted to the cytoplasm, and the intercellular



Text-fig. 1. Diagrammatic representation of the appearance and intensity of aminopeptidase activity in regenerating tissues.

matrix which has already been deposited around the chondrocytes never shows positive reaction (Plate II, Figs. 5, 6 and 8). The activity of perichondrium is intense throughout the course of the histogenesis of cartilage (Plate II, Figs. 6 and 8). The epidermis covering the blastema is always negative, while the intact epidermis located proximal to the amputation plane displays the strong activity. But, it is highly probable that this epidermal activity is due to the secreted substance, not to the epidermal cells, because the activity is sometimes especially intense at the part where the dermal gland opens, as well as in the mucous substance itself.

At 160 days after amputation, the strong aminopeptidase activity is recognized not only in cartilage and perichondrium but also in muscles, exhibiting a center of enzyme activity at the area proximal to the amputation plane, with the gradual decrease toward more distal part. The distal end of the new lower arm indicates no aminopeptidase activity.

After completion of the histogenesis, the decrease of the aminopeptidase activity occurs in every tissue, and finally it attains to the negative level as in the normal limb exhibiting no aminopeptidase activity except for epidermis and dermal glands secreting mucous substance. The observations recorded above are diagrammatically indicated in the text-figure 1.

Discussion

Although the technique of histochemistry is well established for the detection of the various substances within the tissues, its application to regeneration processes has been rarely tried (KARCZMAR and BERG, 1951; BODEMER and EVERETT 1959). But, the present technique can be certainly a potent tool for studying the regeneration, in which such drastic changes as wound healing, dedifferentiation and redifferentiation take place in a number of tissues. From the present histochemical observations it is apparent that aminopeptidase is active in both phases of dedifferentiation and redifferentiation, but the appearance of its activity is markedly different between the tissues involved. That is, in the periosteum and osteocytes the present enzyme appears associated with the event of dedifferentiation, whereas in the muscle and cartilage, it appears rather in the phase of redifferentiation.

It seems to be worthy of mention that there is no appreciable enzyme activity in the wound epidermis, since this fact seems to be in contradiction with the data of BODEMER and EVERETT (1959), who have suggested a high protein synthesis in the wound epidermis, by means of measuring the incorporation of the radioactive amino acid. The present finding that degenerating muscle does not show any activity, is also against BODEMER and EVERETT's view (1959) of the high amino acid incorporation in degenerating muscle fragments.

On the other hand, the aminopeptidase activity arises during the differentiation phase of the periosteum and osteocytes, especially at the peripheral part

of the bone. The possible role of aminopeptidase in association with the tissue dedifferentiation may be to disintegrate protein. According to WEBER (1957) cathepsin also acts to dissolve the proteins in the course of dedifferentiation of the tail of *Xenopus* larvae. In the dedifferentiation phase of bone, the high incorporation of methionine-S^{35} has been recorded to occur in the osteocytes and periosteum (BODEMER and EVERETT, 1959). This increasing methionine-S^{35} incorporation must be for the synthesis of such enzymes as aminopeptidase and cathepsin which digest the bone matrix.

Aminopeptidase activity is also recognized at the phase of redifferentiation. It becomes higher at the time of blastema formation and attains to maximum coincident with the histogenesis of muscle, cartilage and perichondrium in the regenerate. The observation on this paper will be comparable with the quantitative study of dipeptidase content of the regenerating tail of Amphibians, which shows the highest activity of this enzyme during histogenesis rather than dedifferentiation phase (OREKOVITSCH, 1936). According to BODEMER and EVERETT (1959), the incorporation of methionine-S^{35} also reaches to its maximum at the time of histogenesis. On the other hand, immunological evidence has been presented to show the appearance of structural proteins such as actomyosin and myosin in this redifferentiating phase of regeneration (DEHAAN, 1956; OGAWA, KAWAHARA and MIURA, 1958; LAUFER, 1959). Consequently, the increase of the aminopeptidase activity in the redifferentiating phase can be interpreted as relating to the synthesis of tissue specific proteins or to that of some enzymes necessary for synthesizing them.

Summary

1) Using the adult *Triturus pyrrhogaster*, changes of the aminopeptidase activity during limb regeneration were histochemically studied by means of NACHALAS, CRAWFORD and SELIGMAN's method.

2) Aminopeptidase activity arises in the dedifferentiating phase of the periosteum and osteocytes, and in the redifferentiating phase of the muscle, cartilage and perichondrium.

3) The blastema cells indicate the intense aminopeptidase activity.

4) Wound epidermis hardly displays the activity of the present enzyme throughout the course of regeneration.

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Explanation of Plates I-II

Abbreviations. B: Accumulated blastema. C: Cartilage. D: Dermal Gland. E: Epidermis. H: Humerus. M: Muscle. N: Nerve. Os: Freed osteocytes. P: Perichondrium. P.o.: Periosteum. S.b.: Sarcoplasmic buds.

Plate I

Fig. 1. A longitudinal section through a transected humerus at 30 days after amputation. A part of the periosteum shows a weak aminopeptidase activity, no activity being detectable at the proximal portion of the transected forelimb muscles and the wound epidermis.

Fig. 2. A section through the regenerating limb at 70 days after amputation, indicating a strong activity in the accumulated blastema. The activity of fibrocytes near the blastema is moderate.

Fig. 3. A section through the distal end of the transected humerus at 110 days after amputation, indicating an intense activity in the periosteum and in the freed osteocytes: Digestion of bone matrix is under way.

Fig. 4. A section through the regenerating limb at 80 days after amputation, indicating a marked activity in the sarcoplasmic buds with definite proximodistal gradient.

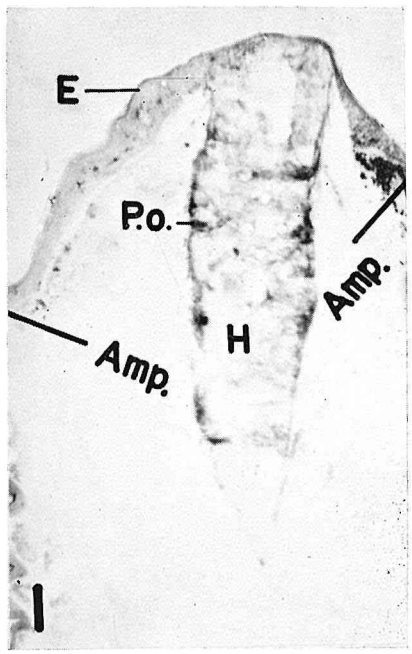
Plate II

Fig. 5. A section through the regenerating limb at 120 days after amputation, indicating a moderate activity in the sarcoplasmic buds and chondrocytes: Activity of perichondrium is intense, but is little in the cartilage matrix.

Fig. 6. A section through the regenerating limb at 120 days after amputation, indicating moderate activity at the proximal part, but none at the distal part.

Fig. 7. Details of the proximal part to the amputation plane of the same regenerate as given in Fig. 6: The enzyme activity is recognized in the muscle, nerve, dermal gland and epidermis.

Fig. 8. A section through the regenerate with three digits at 160 days after amputation, indicating the enzyme activity at the proximal part to the amputation plane. The perichondrium and the chondrocytes located at the peripheral part of the cartilage show a strong activity, no reaction occurring in the chondrocytes located at the central part of the cartilage.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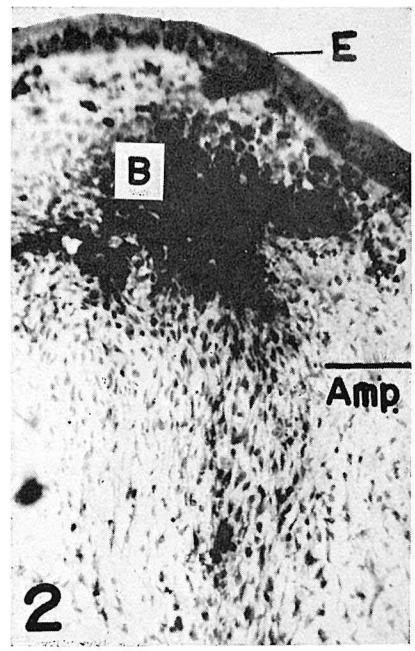
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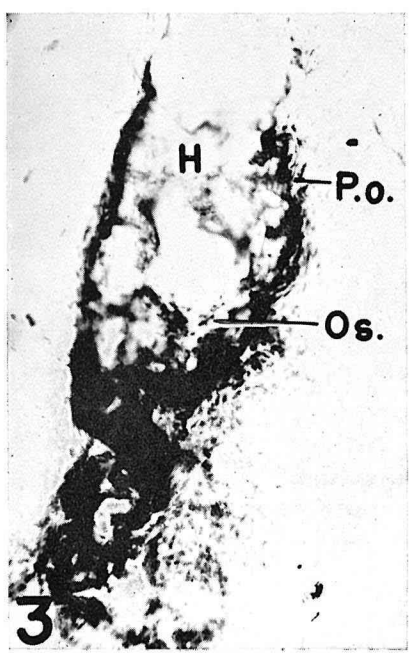
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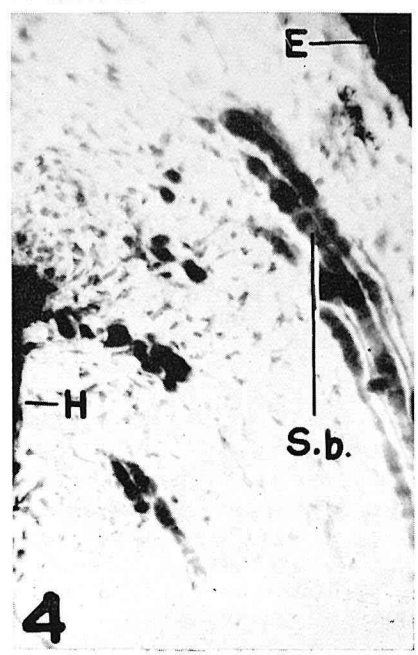
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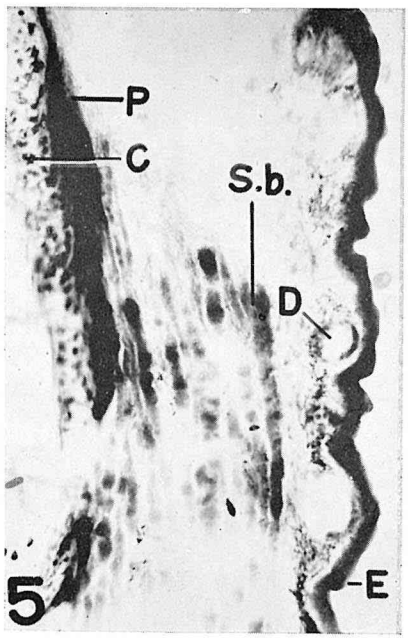
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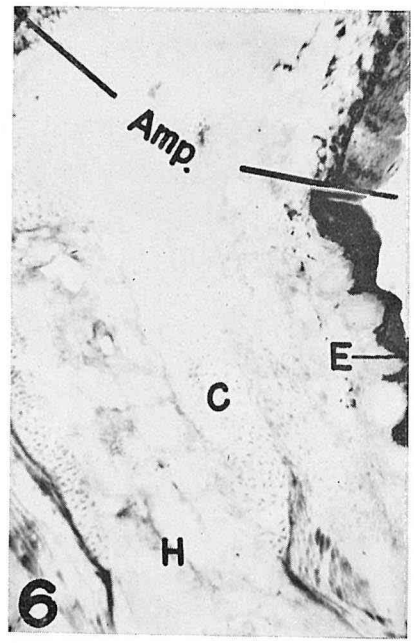
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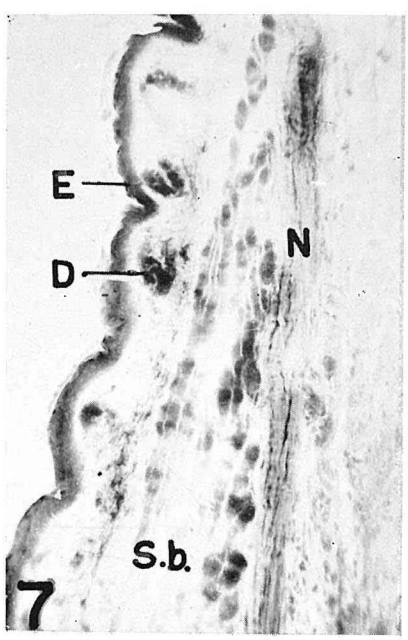
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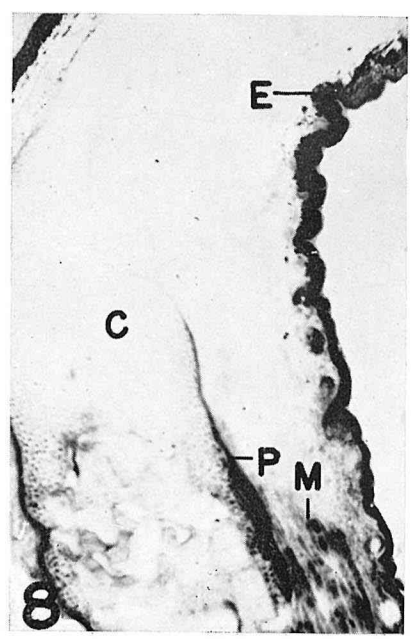
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