Chapter 5

PROTEIN METABOLISM IN THE URODELE REGENERATING APPENDAGE

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THERE EXISTS TODAY considerable surgical excellence such that, if a man's hand is accidently severed, autoplastic replantation of that severed hand is possible with a good chance of success (Herbsman, Lafer, and Shaftan, 1966; and others). Furthermore, the conservative treatment of fingertip amputees in very young children has resulted in remarkable regenerative responses (Douglas, 1972; Illingworth, 1974). Where replantation is not possible or is unsuccessful, or surgical intervention is necessary to close a wound, a typical fibrous pad forms. Limb amputees in man or mouse (Schotteé and Smith, 1959) will typically repair through restricted regeneration and massive connective tissue synthesis forming a cicatrix at the wound surface of the amputation stump.

On the other hand, the repair and subsequent regeneration of an amputated limb of the adult newt, *Notophthalmus viridescens,* demonstrates a reconstitutive capacity far exceeding that observed among any mammals to date. Involved is the coordinated renewal of several tissues such as epidermis, glands, blood vessels, nerves, striated muscle, cartilage, bone, and connective tissue proper.

Observations on the progress of regeneration of a urodele limb amputee have led to staging of events based on progressive changes in the gross morphology (Fig. 5-1) and microscopy of the bud of tissue growing distal to the stump as it is shaped and undergoes differentiation to become a new and functioning appendage (see reviews by Goss, 1969; Schmidt, 1968). In brief, the cut surface of an amputee is viewed for the appearance of a clearly defined cover of wound epithelium (Fig. 5-2). In time this is followed by a rounded swelling of the distal end of the amputation stump heralding and subepithelial accumulation of a blastema of cells, forming a bulbous blastema (Figs. 5-1 and 5-3). Growth in size and a change in distal shape forming conical characteristics is identified as the cone blastema (Figs. 5-1 and 5-4), and its subsequent dorso-ventral flattening and blunting of its distal free end present a paddleform blastema (Figs. 5-1 and 5-5). Grooving of the paddleform blastema foretells the differentiation of digits, first two then three and four digits (the maximum number in the newt forelimb), followed with successive interdigital grooving and distal outgrowths that leads to a well-defined regenerated appendage (Figs. 5-1

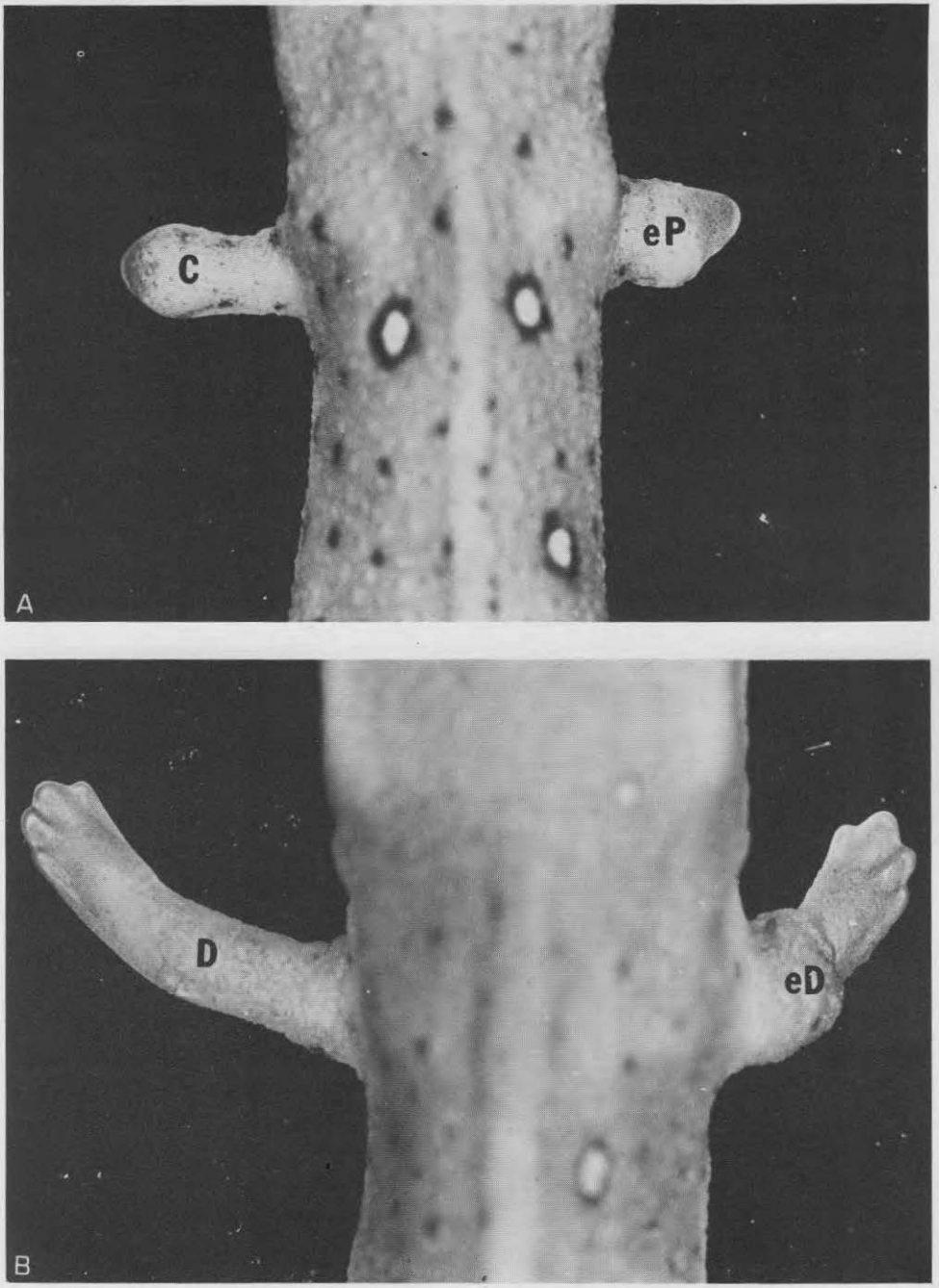


Figure 5-1. Regenerating forelimbs of the adult newt, *Notophthalmus v. A.* Twenty-five days after amputation, the left limb with humerus retained shows a small conic blastema (c); the right limb with humerus removed (eP) has flattened into an early paddleform regenerate. (×25). (From A. J. Schmidt, *Anat. Rec., 154:*417-418, 1966. Courtesy of the Wistar Institute, Philadelphia.) *B.* Forty-four days after amputation, both the humerus-retained left limb and the humerus enucleated (eD), right limb bear four digit buds (×25). (Reprinted from *Cellular Biology of Vertebrate Regeneration and Repair* by A. J. Schmidt by permission of the University of Chicago Press.)

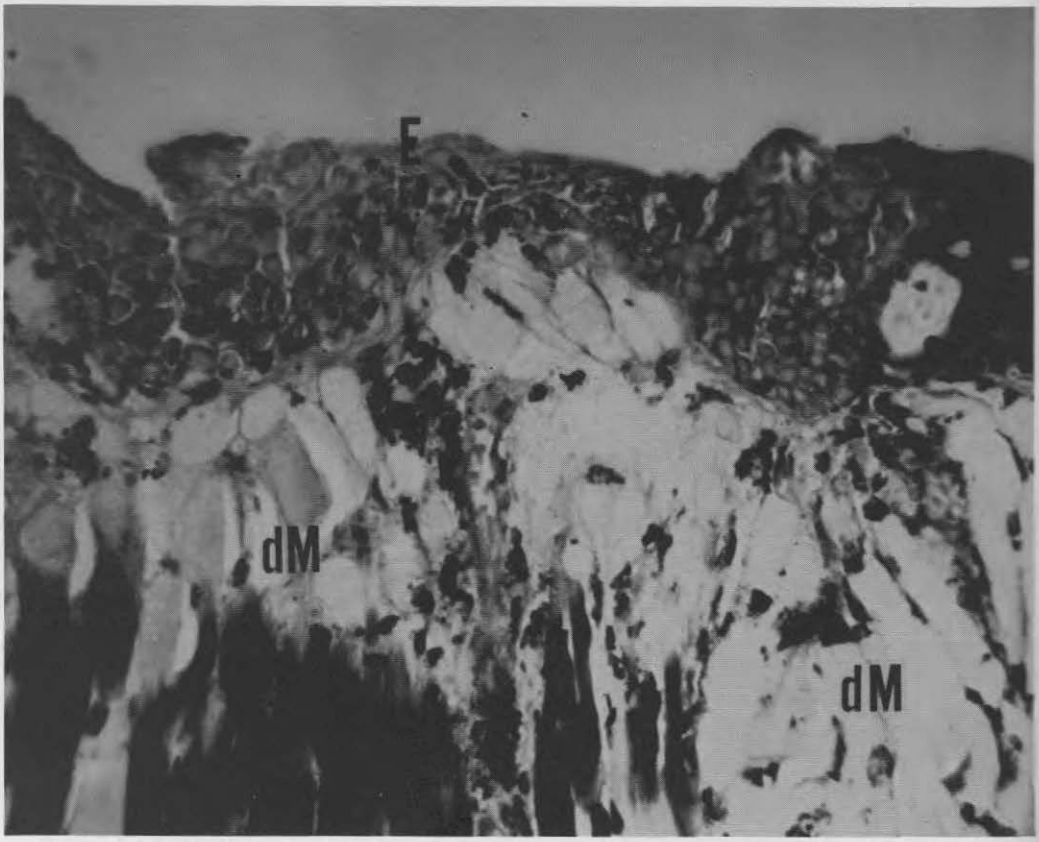


Figure 5-2. Adult newt forelimb twenty-four hours post-amputation. A thick epithelium (E) has formed over the wound. Glycogen is located within cells of this wound epithelium, within and between underlying muscle fibers except at their distal ends (dM) where they are depleted of glycogen content. (PAS, ×150). (From A. J. Schmidt, *J. Exp. Zool., 145:*43-48, 1960. Courtesy of the Wistar Institute, Philadelphia.)

and 5-6). It is in particular during the differentiative phases, when morphogenetic changes are clearly evidenced in the paddleform and digitform stages, that histogenesis of striated muscle, skeletal cartilage models with their ossification centers, peripheral nerves, microcirculation, and supporting connective tissues are all readily identified in histologic sections.

A conception of the phenomenon of regeneration has been proposed (Schmidt, 1968) where in the adult newt the individual limb tissues themselves regenerate, as is probably the case in all vertebrate species under appropriate conditions* (Fig. 5-7). The cellular population forming the

* It should be noted that various means have also been employed and reported to have stimulated regenerative events where they might otherwise not have occurred, for example, nerve augmentation in the frog (Singer, 1951, 1954), lizard (Simpson, 1961), opposum (Mizel and Isaacs, 1970); and electrical stimulus in the rat (Becker, 1972).

regeneration blastema of the newt limb appears to have much in common with the fibroblastic granulation tissue of repairing mammalian wounds. Mammalian repair and newt regeneration appear to differ in that the changing environment of the amputated newt limb permits tissues to manifest their regenerative capability, whereas in mammals, regeneration may be masked or prevented by a marked predisposition toward protein synthesis, particularly directed to forming a cicatricial dam of massive collagen fibers (Fig. 5-8).

Historically, the importance of proteins (as well as peptides and amino acids) in repair processes in mammals have been recognized for a number of years. For example, the body responds to trauma by entering into a negative nitrogen balance (Cuthbertson, 1960; Moore, 1959), and should this persist as it would in hypoproteinemic animals, wounds heal poorly if at all (Localio, Morton, and Hinton, 1948).

Proteins, peptides, and amino acids appear also to be appreciably important to regenerative processes in amphibians. For example, antibiotics such as chloramphenicol, puromycin, and actinomycin-D that interfere with protein synthesis have been shown to suppress limb and tail regeneration in urodeles (Burnet and Liversage, 1964; Liversage and Colley, 1965; Wolsky and Van Doi, 1965; Carlson, 1967).

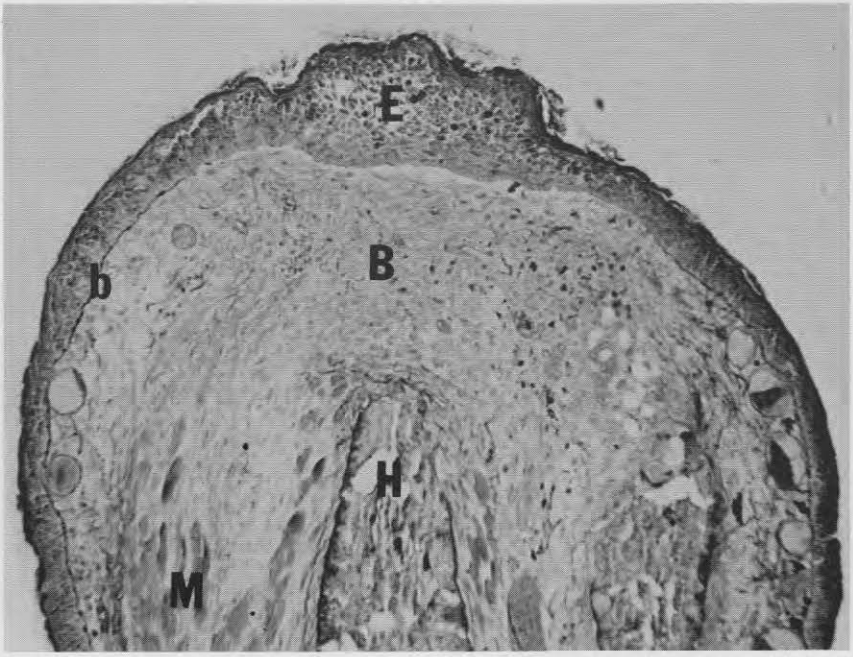


Figure 5-3. Adult newt forelimb regenerating for nineteen days. There is a thick wound epithelium (E), an underlying accumulation of cells forming a bulb blastema (B), a subjacent portion of the humerus (H), and nearby muscle fibers (M) containing glycogen. The basal membrane (b) underlies the epidermis at the limb perimeter, but none intervenes between the wound epithelium and blastema. (PAS, ×50).

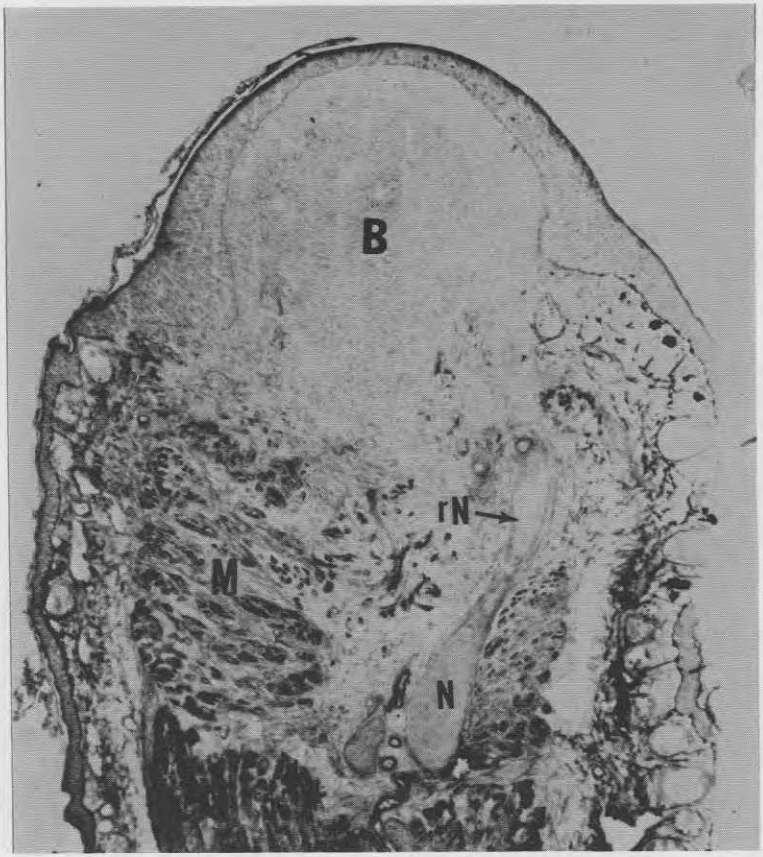


Figure 5-4. Adult newt forelimb regenerating for twenty-two days. An advanced conic blastema (B) illustrating cellular growth. Widely separated muscle fibers (M) that have recovered from the injury of amputation lie distal to darkly stained basal stump muscle. A brachial nerve (N) and its distal regenerating-myelinating (rN) portion are indicated. (Acetone-sudan black B, × 18.) (From A. J. Schmidt, *Anat. Rec., 155*:65-76, 1966. Courtesy of the Wistar Institute, Philadelphia.)

Denervation (or irradiation) of an appendage prior to the formation of a blastema will normally prevent regeneration from ensuing. Polezhaev et al. (1961) have successfully restored regenerative capability in irradiation-suppressed axolotl limbs by administering crude acid extracts of proteins. In adult newt limbs denervated prior to blastema formation, protein levels (as well as DNA and RNA) declined to between 40 to 60 percent of control quantities (Lebowitz and Singer, 1970; Singer and Caston, 1972; Morzlock and Stocum, 1972). This is due to an actual reduction in the rate of protein synthesis (Singer and Ilan, 1977; Bast et al., 1979). Dresden (1969) on the other hand found an average decline of only 14 percent protein synthesis following denervation of paddleform limb regenerates. However, this

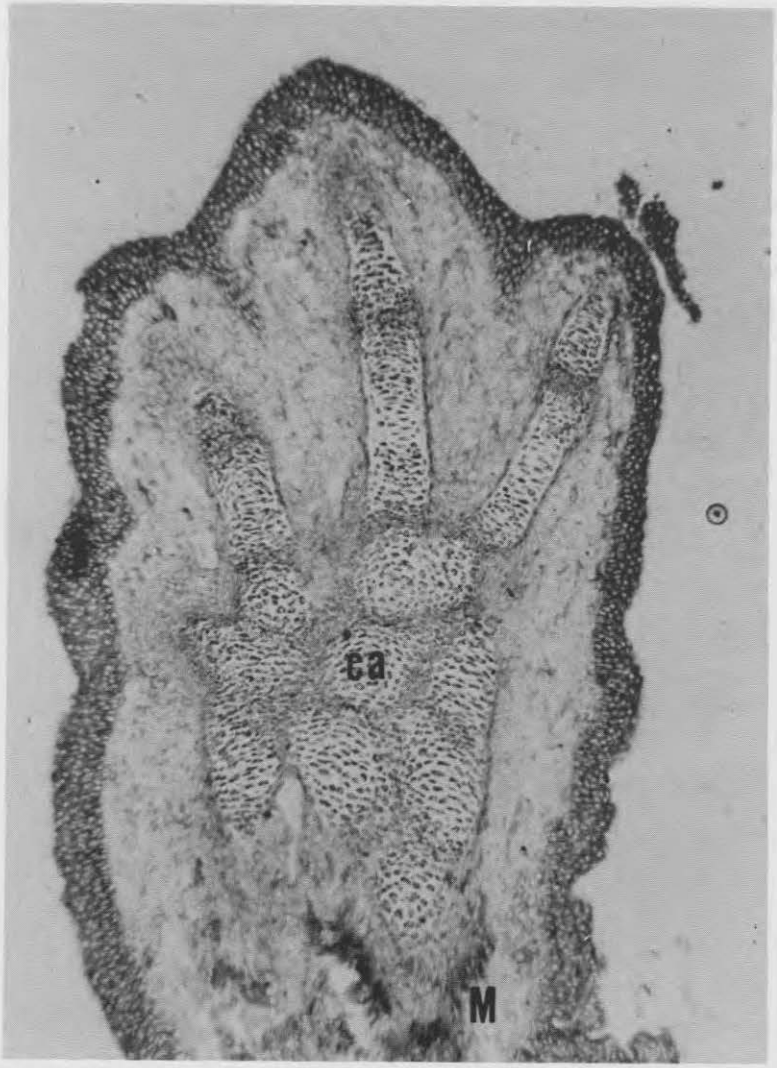


Figure 5-6. Adult newt forelimb regenerating for 34 days. A digitform regenerate with cartilaginous carpalia (ca) articulating with four digits distally, three of the phalanges here illustrated. Portions of ulna and radius lie proximal to the carpalia, and regenerating muscle (M) are evident. (Nitro BT/Lactate dehydrogenase, ×65). (From A. J. Schmidt and T. Weidman, *J. Exp. Zool., 155:*303-316, 1964. Courtesy of the Wistar Institute, Philadelphia.)

protein profiles within these biological systems. Other early investigations have revealed that labeled amino acids (e.g., leucine-^{3}H or methionine-^{35}S) are avidly incorporated within hours after amputation of a urodele limb (Fig. 5-9), with apparent synthetic activity generally increasing during subsequent regenerative events (Anton, 1961, 1965, 1968; Bodemer and Everett, 1959).

Quantitative changes in soluble protein extracted in water or dilute

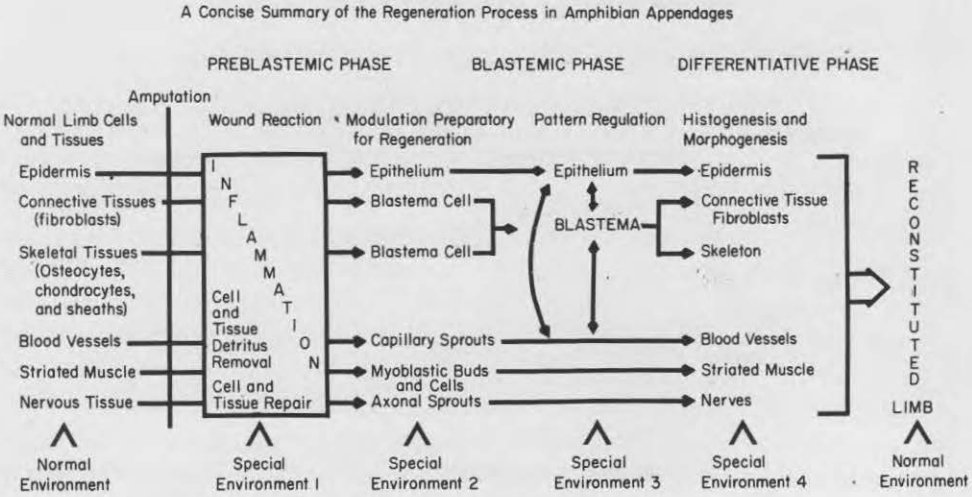


Figure 5-7. The diagram summarizes our concept of the regeneration process in the amphibian amputated appendage. With the exception of the final reconstitution of a regenerated limb, this diagram could also serve as a summary of repair processes in all vertebrates. (Reprinted from *Cellular Biology of Vertebrate Regeneration and Repair* by A. J. Schmidt by permission of The University of Chicago Press.)

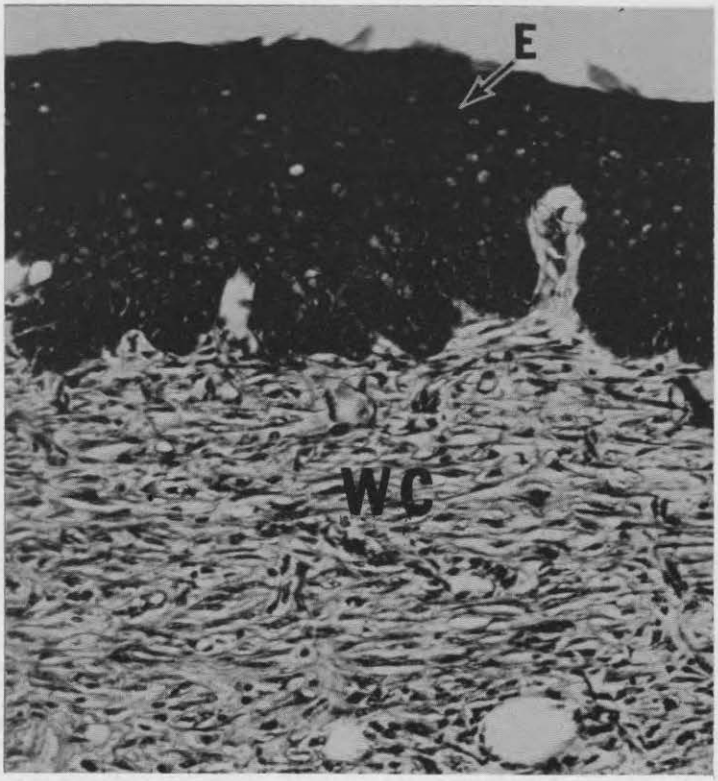


Figure 5-8. Wound repair eleven days after a localized burn of the dorsal skin of a sixty day old mouse. There is a thick wound epithelium (E) and a developing subepithelial fibrocellular wound cicatrix (WC). (McFarlane's trichrome, ×256)

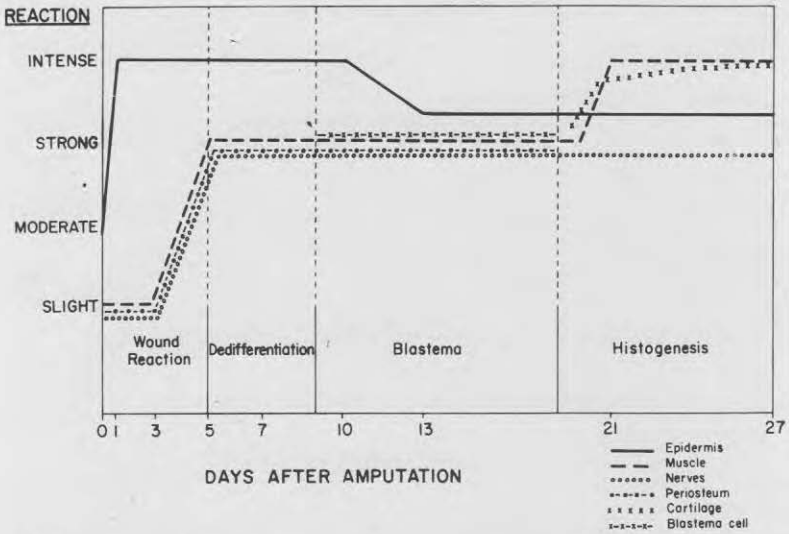


Figure 5-9. Summary of autoradiograms produced by methionine-^{35}S incorporated into tissues of regenerating forelimbs of the adult newt over the course of twenty-seven days after amputation. Regenerating limbs were sampled six hours after injection of isotope. (From C. W. Bodemer and N. B. Everett, *Develop. Biol., 1*:327-342, 1959. Reprinted by permission.)

saline (0.14M NaCl) solution during the course of regeneration (Fig. 5-10) have been reported (Schmidt and Woerthwein, 1966; Schmidt, 1968). Soluble protein initially increases in amount to the fifth day of regeneration, and then declines. This early postamputational rise may be attributed to a number of factors such as blood flow, injury detritus, and attendant protease activity. The lowest level reached is at ten days when the formative blastema first begins to appear; subsequently, the quantity of soluble protein increases gradually then sharply to its highest level with the growth of the blastema into the paddleform and digitform stages. High levels of soluble protein correlating with blastemic and subsequent differentiative events have recently been verified by Mailman and Dresden (1976).

The soluble protein extracted from urodele limb tissues likely consists of cellular enzymes, substrates, and products of both catabolic and synthetic metabolism. Not only are there quantitative differences among extracted proteins but there exist indicators of protein populations being qualitatively unique to regenerating and normal tissue (Schmidt, 1968). In preliminary attempts to establish the existence of these differences, we (Schmidt, 1966, 1968) initiated a study on electrophoretic patterns of extractable native protein on polyacrylamide gels. In comparing the separations obtained from normal unamputated limb tissue to the progressive events in regeneration (Fig. 5-11), we observed thirty bands during the late cone

blastema and paddleform stages and what appeared to be seven new protein or polypeptide species. Similar investigations by Dearlove and Stocum (1974) and Donaldson et al. (1974) with the denaturing agent sodium dodecyl sulfate (SDS) added to the polyacrylamide gels or buffer resulted in improved resolution of polypeptide bands and the identification of several so-called regeneration proteins. However, in recent experiments in our laboratory (Guy, 1979a, b) we employed tissue extracts treated with mercaptoethanol and SDS before electrophoretic application according to the procedures of Cohen et al. (1977). Employing a slab separating gel in which there is an acrylamide gradient varying linearly from 5 to 15 percent, and containing 0.1% SDS, some sixty protein bands with molecular weights ranging between 11,000 and 250,000 daltons were resolved in each group investigated. Comparisons were made of the polypeptide profiles between intact regenerates, separated epithelial and mesenchymal tissue of regenerates, and nonregenerating limb tissue. All polypeptide bands that appeared in the regenerating arrays had comigrat-

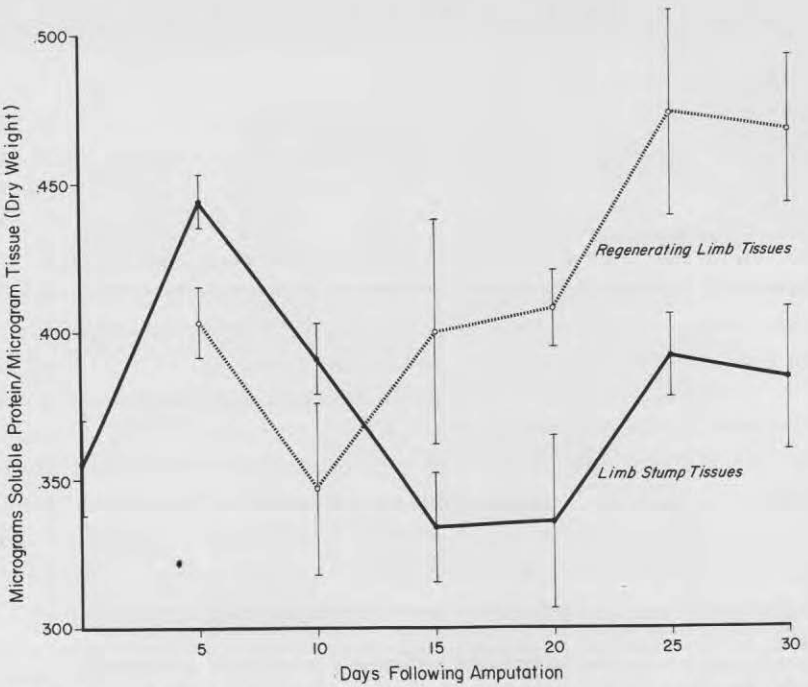
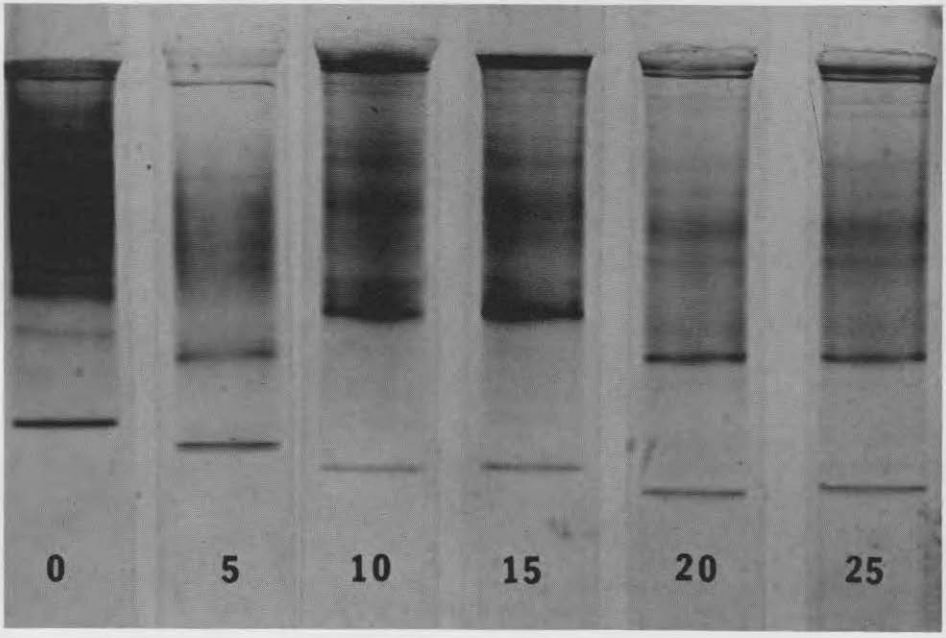


Figure 5-10. Quantitative estimation of water-soluble protein extracted from freeze-dried sections of regenerating forelimbs and adjacent limb stump tissue on specified days after amputation. Freeze-dried sections were weighed, and protein was determined by the Lowry Folin-phenol procedure.Each point is the mean ± S.E. of three replicate determinations from samples of three to six limbs/day. (From A. J. Schmidt, *Anat. Rec., 154*:417-418, 1966. Courtesy of the Wistar Institute, Philadelphia, 1966.)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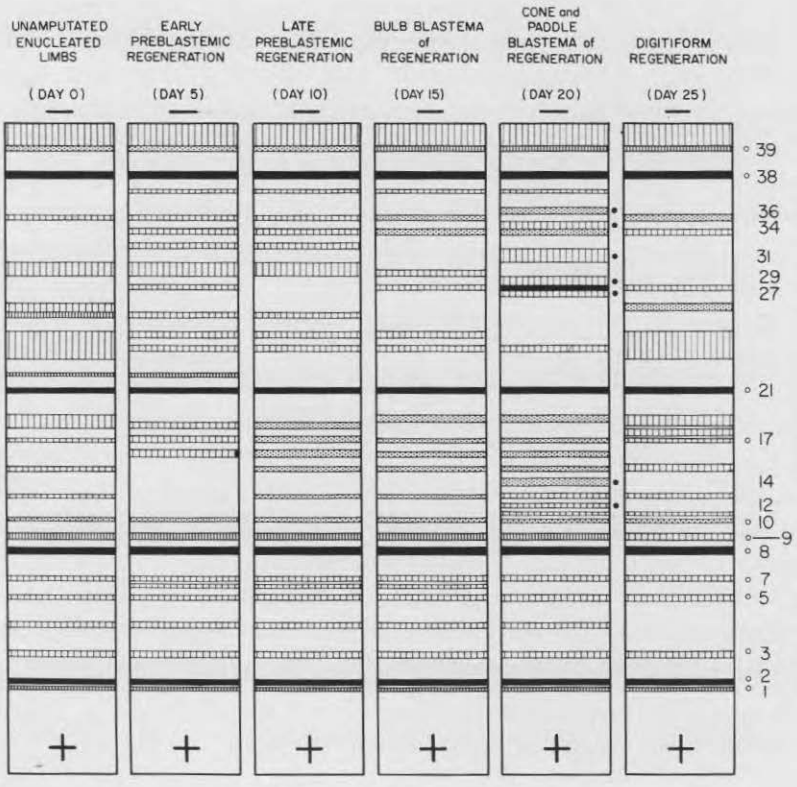
UNAMPUTATED ENUCLEATED LIMBS
(DAY 0)
EARLY PREBLASTEMIC REGENERATION
(DAY 5)
LATE PREBLASTEMIC REGENERATION
(DAY 10)
BULB BLASTEMA of REGENERATION
(DAY 15)
CONE and PADDLE BLASTEMA of REGENERATION
(DAY 20)
DIGITIFORM REGENERATION
(DAY 25)
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ing counterparts in the normal adult tissue. We could not identify with confidence any individual proteins unique to regeneration. However, there were consistent differences in regeneration patterns, which we classified into the following categories.

In the first category are bands seen in normal nonregenerating limb tissues that are absent from the arrays obtained from regenerating tissues (Fig. 5-12). For example, band four, which is in the molecular weight range of approximately 88,000 daltons, is clearly evident in normal limb tissue but absent from regenerating tissue. Band four presence in our preblastemic sample is most likely due to the unavoidable inclusion of limb stump tissue.

Our second category is exemplified by band ten of normal limb tissue within the molecular weight range of approximately 71,000 daltons (Fig. 5-12). This band is absent from regenerating bulb blastema samples, reappears in the following conic blastema, and becomes distinctly visible with differentiation of the paddleform and digitform regenerates.

Gradient gel electrophoresis studies were also conducted on cleanly separated adult limb epidermis and underlying tissues, wound epithelium, and subjacent blastema cells through the course of regeneration (Guy, 1979a, b). Tissues were harvested by mechanically separating the wound epithelium from underlying blastema following incubation in buffered EDTA. Early attempts at separating these tissues (Glade, 1963; Stocum, 1968a; Stocum and Dearlove, 1972; Carlson, 1975; Mescher, 1976; Donaldson and Mason, 1978; Géraudie and Singer, 1978; and others) in several laboratories have given ample evidence to the difficulty in separating these tissues, in that their efforts consistently yielded incomplete or highly cell-contaminated epithelia and/or blastemas. The procedure employed by Guy (1979a, b) provided very clean tissue separations satisfactory for the analysis of their protein profiles. The problem of tissue separation in mature and regenerating urodelean limbs has received extensive and important evaluation by Jasch (1979).

The wound epithelium has been viewed for decades as extremely important if not absolutely essential to the course of regeneration (Salpeter and

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Figure 5-11. Electrophoretic pattern of proteins separated from fresh tissue homogenates of regenerating forelimbs of the adult newt. *A.* Disc electrophoretic patterns obtained on 7.5% polyacrylamide gel columns at 5 mA for thirty minutes in a cold room at 4°C with 0.005*M* Tris-glycine pH 8.2 buffer. (From A. J. Schmidt, *Anat. Rec., 154:*417-418, 1966. Courtesy of the Wistar Institute, Philadelphia.) *B.* Diagram summary of patterns obtained. Twelve (open circle) protein bands appear to coincide for each day of sampling out of a total of forty bands. New protein bands appear at different phases in regeration, as many as seven (solid circle) during late blastema-early morphogenesis stage. (Reprinted from *Cellular Biology of Vertebrate Regeneration and Repair* byA. J. Schmidt by permission of the University of Chicago Press.)

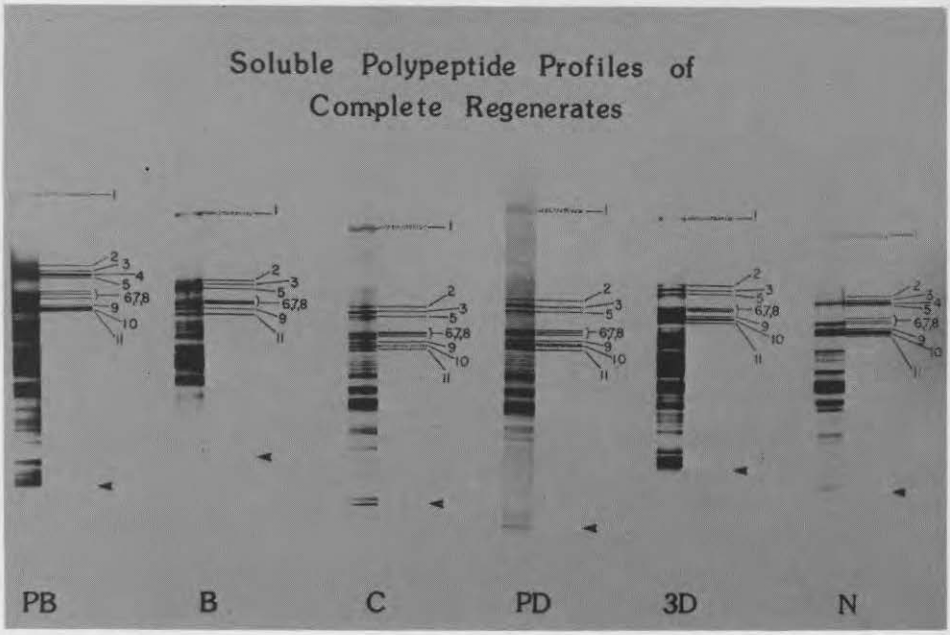


Figure 5-12. Electrophoresis patterns of proteins separated from fresh tissue homogenates of regenerating forelimbs of the adult newt. Gradient 4-15% polyacrylamide SDS gel electrophoresis at 30 mA for three to four hours. Although gels illustrated were not run simultaneously, correct identification of comigrating bands was possible by comparison between experimental tissue array and that of normal adult limb run with every gel. Selected bands were numbered for ease in discussion (see text). PB = preblastemic stage; B = bulb blastema stage; C = conic blastema stage; PD = paddleform stage; 3D = digit stage; N = normal adult limb; arrow = buffer front. (From J. M. Guy, Thesis, University of Illinois Library of the Health Sciences,Chicago, 1979.)

Singer, 1960; Schmidt, 1962; Thornton, 1957, 1960, 1965; and others). Cell contact between the epithelium and subjacent blastema has been described, and molecular uniqueness of each tissue noted (see review by Schmidt, 1968); regeneration is delayed or completely inhibited if wound epithelium does not form or a dermal pad intervenes at the limb amputation surface (Tornier, 1906; Taube, 1921; Godlewski, 1928; Goss, 1956; Mescher, 1976; Tassava and Garling, 1979).

Protein profiles obtained from cleanly separated blastema tissue (Fig. 5-13) appear to be similar to those of the complete intact regenerate (Guy, 1979a, b). Absent from this tissue is the polypeptide band number eight of about 74,000 daltons. However, band eight is found specifically in wound epithelium (Fig. 5-14), where conversely, bands four and ten found in blastemal tissue are absent. Accordingly, these bands are identified as specific to blastema (bands four and ten) and specific to wound epithelium (band eight). The latter tissue has an additional diffuse wide band in the

molecular weight range of 240,000 daltons, which appears in normal mature epidermis as well.

These studies do not confirm the presence of soluble proteins unique to regenerative events described by others, although there does appear to be quantitative protein patterns specific to the course of regeneration. The protein profiles revealed are common to both wound epithelium and blastemal compartments throughout the course of regeneration, albeit there exist proteins that can be localized to wound epithelium, to mature epidermis, and to blastema tissue. Whether these profiles are a reflection of molecular activity essential to regenerative events remains to be explored.

Collagen protein, which exists in multiple forms (Harwood, 1979), has long received attention in mammalian repair where fibrogenesis is a dominant feature (Ross and Benditt, 1962, 1965; and others). On the other hand, cicatrix formation is not evident in the urodele regenerating ap-

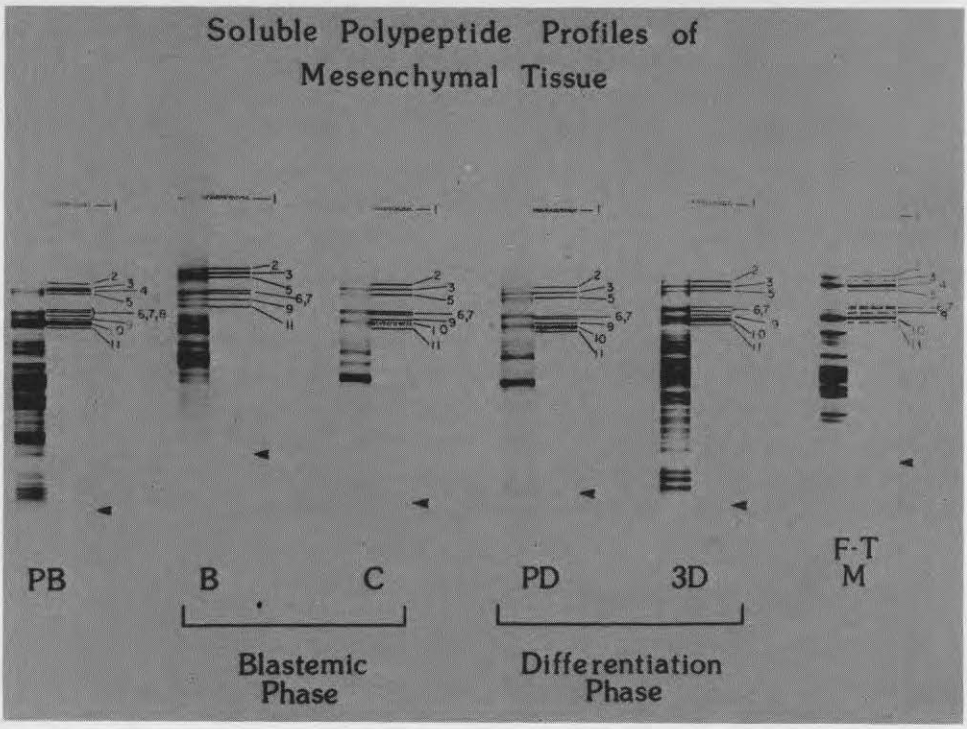


Figure 5-13. Electrophoretic profiles of soluble polypeptides extracted from subepithelial (blastemal) tissue collected over the course of regeneration. Tissues were separated from epithelium following thirty minutes incubation in 10mM EDTA at room temperature. The electrophoretic procedure and abbreviations as in Figure 5-12. F − TM = supepithelial tissue from normal adult limbs separated by freeze-thawing. (From J. M. Guy, Thesis, 1979.)

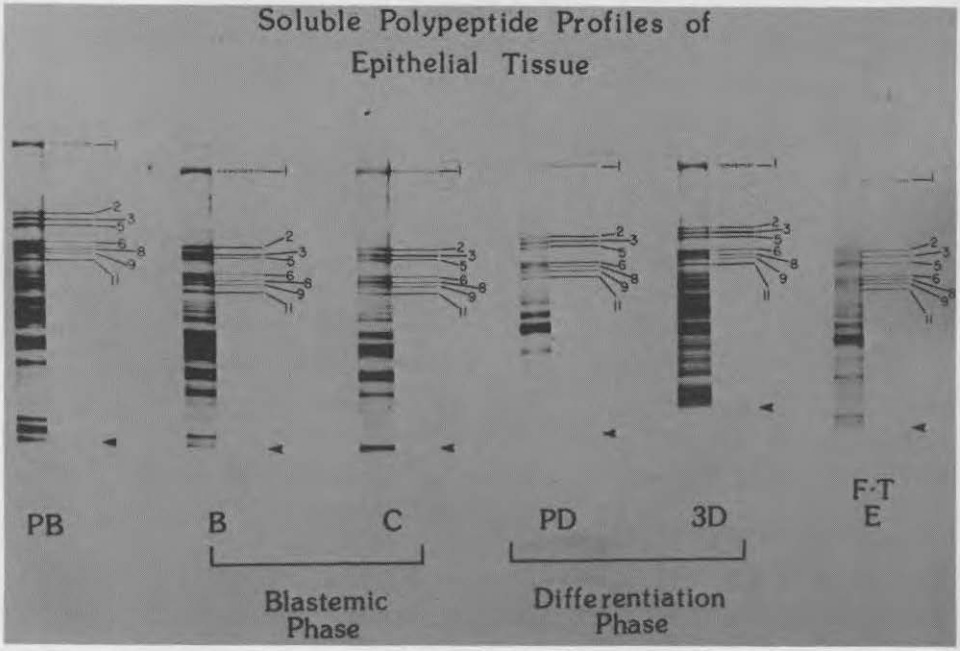


Figure 5-14. Electrophoretic profiles of soluble polypeptides extracted from limb epidermis and from wound epithelium collected over the course of regeneration. Separation procedure as in Figure 5-13. Electrophoretic procedure and abbreviations as in Figure 5-12. F − TE = epidermis from normal adult limb separated by freeze-thaw. (From J. M. Guy, Thesis, 1979.)

pendages, and it is known that precocious scarring or the placement of a dermal pad over an amputation wound inhibits regeneration (vide ante). Fibrogenesis has been identified within the differentiative stages of regeneration (Revel and Hay, 1963; Salpeter, 1968; Schmidt, 1969), but the question raised by Revel and Hay (1965) as to whether or not fibrogenesis might even occur in the regeneration blastema has since resulted in some interesting experiments (Schmidt, 1969, 1970; Johnson and Schmidt, 1974; Mailman and Dresden, 1976, 1979). Light and electron microscopic observations (Schmidt, 1962a; Norman and Schmidt, 1967a) have described thin, amorphous fibrils and filaments scattered within the interstitial space throughout the regeneration blastema of the adult newt limb. Revel and Hay (1963) employed tritiated proline as a metabolic tracer in autoradiographic studies on the synthesis of collagen in regenerating limbs of larval *A. maculatum* and reported an absence of isotope uptake by the forming blastema. In the latter investigations as well as those in the adult newt (Salpeter, 1968; Schmidt, 1969), a very high uptake of tritiated proline provided suggestive evidence of collagen synthesis within the regenerating cartilage of differentiating limbs. In these studies, purified

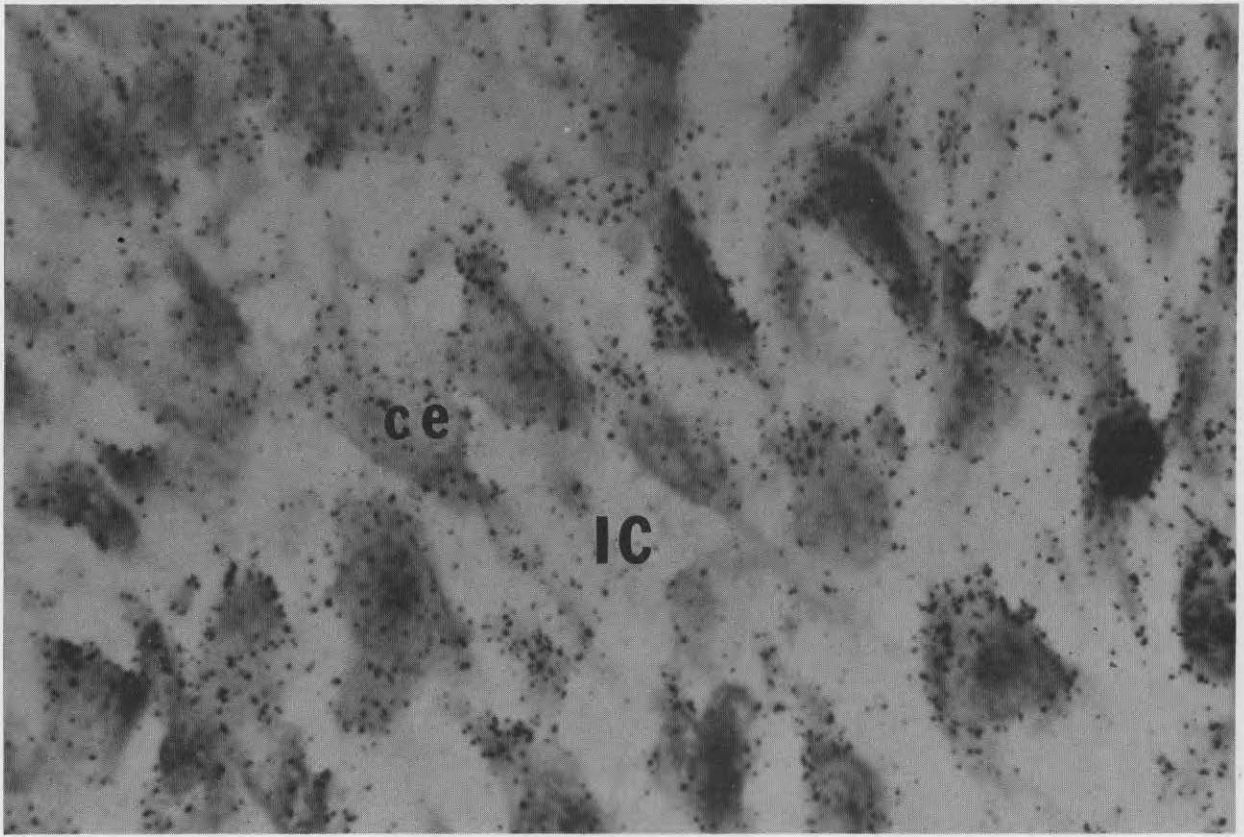


Figure 5-15. Autoradiograph of a twenty day limb regeneration blastema fifteen minutes after intrapleuroperitoneal injection of 50 μc ^{3}H-proline. The cells (Ce) average 22.4 grains/sq CM, and the intercellular (IC) space approximates the background count of 4 grains/sq cm. (Giemsa, × 100).

Clostridium histolyticum collagenase (Clostridiopeptidase H-EC 3.4.24.3) was employed in digesting denatured collagen in order to distinguish the collagenous from noncollagenous protein incorporating tritiated proline. Autoradiographs (Schmidt, unpub.) revealed a rapid incorporation of tritiated proline by several tissues of the amputated regenerating newt limb, including the blastema cells (Fig. 5-15). Intracellular isotope label was present as early as fifteen minutes after injection of 50 microcuries of tritiated proline, its incorporation increasing during the following six hours when extracellular label appeared and accumulated during the remaining twenty-four hours of the test. *C. histolyticum* collagenase digested all typical collagen fibers (trypsin resistant fibers; Schmidt, 1962b) in the dermis, mysial sheaths, cartilage matrix (Fig. 5-16), and elsewhere, removing concentrations of isotope in these tissues as well as depleting the extracellular response of the blastema (Fig. 5-17).

In view of the failure of light microscopic and especially electronmicrographic studies to demonstrate distinguishable banded collagen polymers in the regeneration blastema, we (Schmidt, 1969) considered the possibility that these cells were synthesizing and releasing proline-rich hydroxproline-deficient collagen monomers susceptible to rapid degradation in the extracellular environment. We initiated quantitative assays for neutral saline (0.45M NaCl) soluble collagen (Schmidt, 1970; Johnson and Schmidt, 1974), representing newly synthesized collagen protein (Jackson and Bentley, 1960). Others since investigated an acid soluble fraction (Mailman and Dresden, 1976, 1979) which represents older, mature collagen tightly bound through intra- and intermolecular crosslinkages (see reviews by Martin, Byers, and Piez, 1975; Fessler and Fessler, 1978).

These quantitative studies agreeably demonstrated that indeed there is a collagen protein being synthesized within the regeneration blastema, albeit at a level well below that of noncollagen protein (Figs. 5-18 and 5-19). Synthetic activity continues at increasingly higher levels with parallel accretion of collagen fibers as the differentiation of the regenerate progresses. Aging of the formed regenerates is accompanied by a fairly steady state of synthetic activities and fibrogenesis. It was also interesting to note in these studies some indication of a higher proline to hydroxproline ratio in collagen extracts of regenerates than found in the adjacent limb stump tissues, and this differential is even more pronounced when compared with untraumatized normal control tissues (Johnson and Schmidt, 1974).

If not directly hostile, certainly the environment of the early pre- and blastemic stages of regeneration appeared incompatible with the requirements for accretionary growth of collagen fibers. Attempt to influence collagen synthesis and fibrogenesis by introducing an essential precursor, such as proline, or by increasing oxygen tension as high as a partial pressure of 683 mmHg (Schmidt and Jasch, 1971) had little appreciable

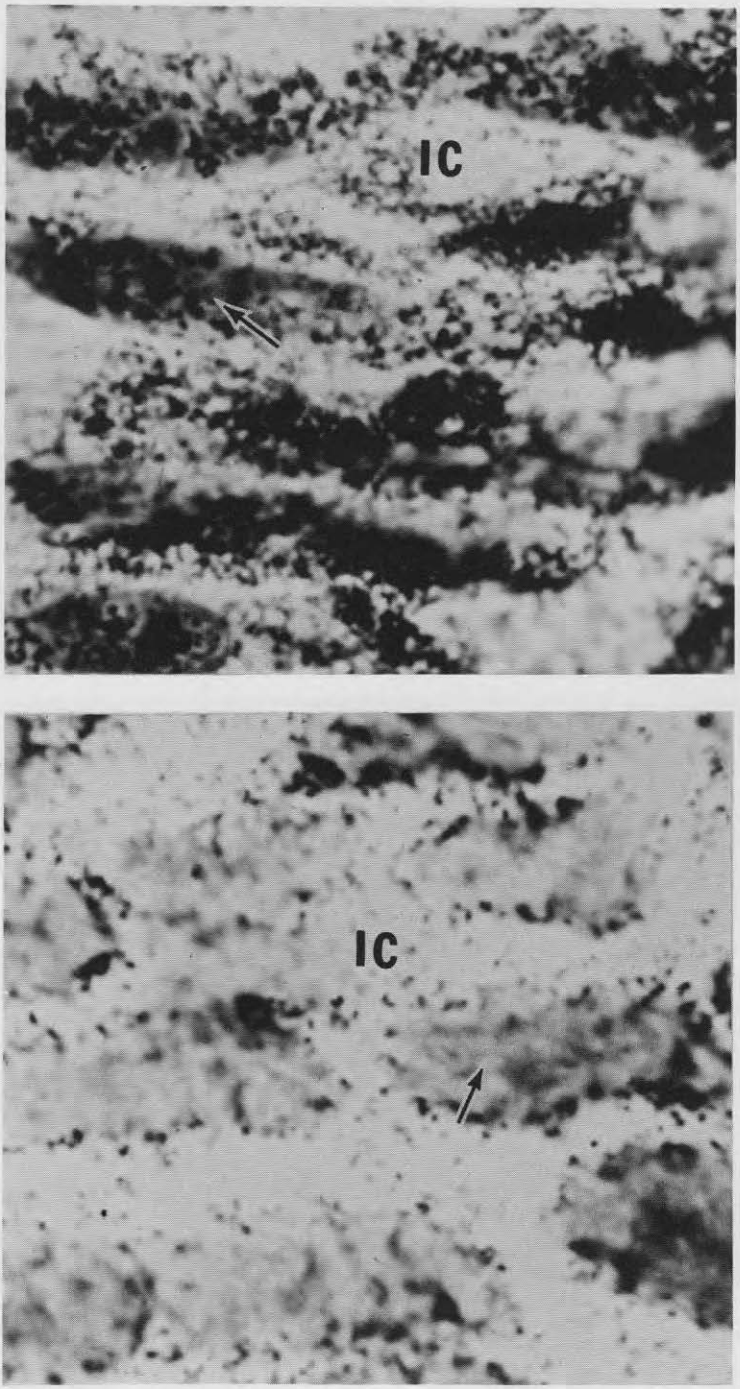


Figure 5-16. Autoradiographs of skeletal cartilage of a thirty day limb regenerate twenty-four hours after intrapleuroperitoneal injection of 50 μc ^{3}H = proline. *A.* Chondrocytes (arrow) are heavily labeled, as is the intercellular (IC) space, averaging 25.2 grains/sq cm. *B.* An adjacent section to *(A.)* following four hours' hydrolysis by collagenase, illustrating about 50 percent loss in label with the intercellular (IC) space averaging 10.6 grains/sq cm. This loss in label is presumably due to the digestion of newly synthesized collagen protein. (Van Gieson, ×400).

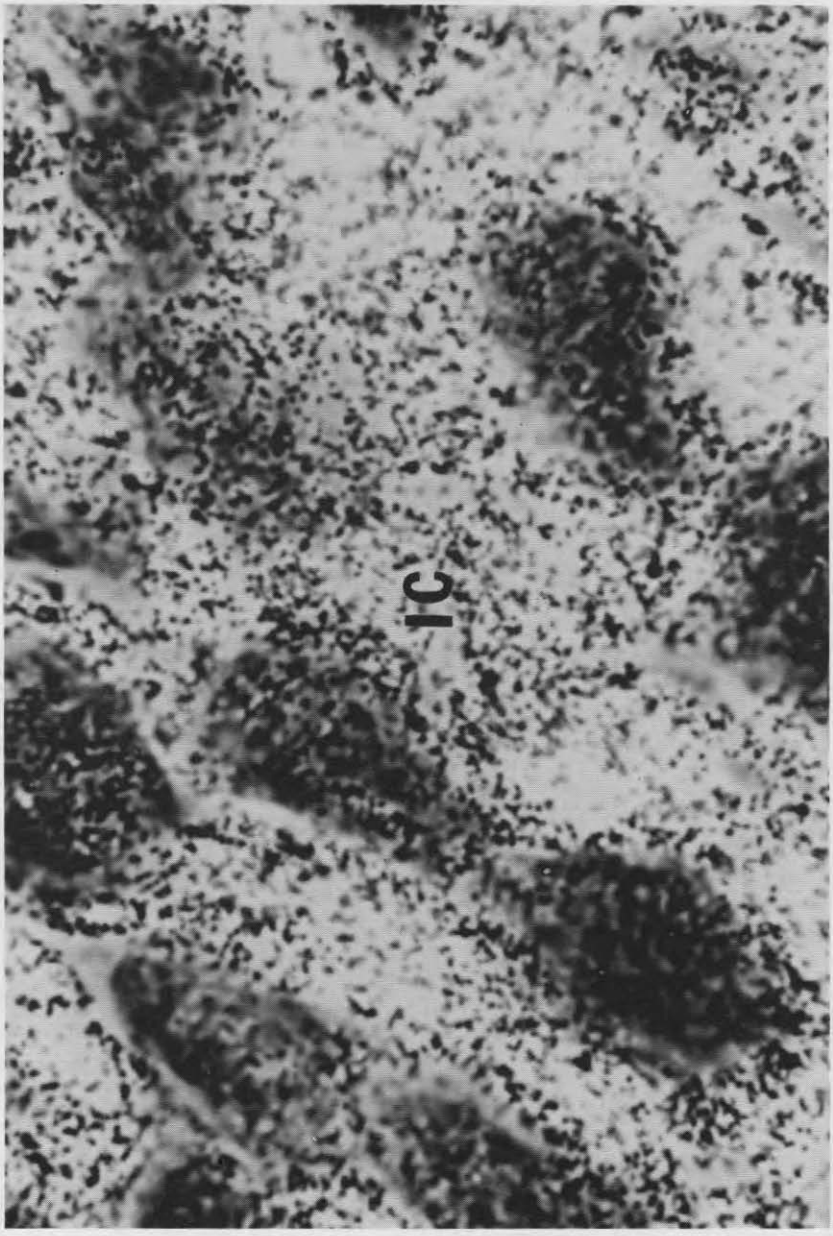
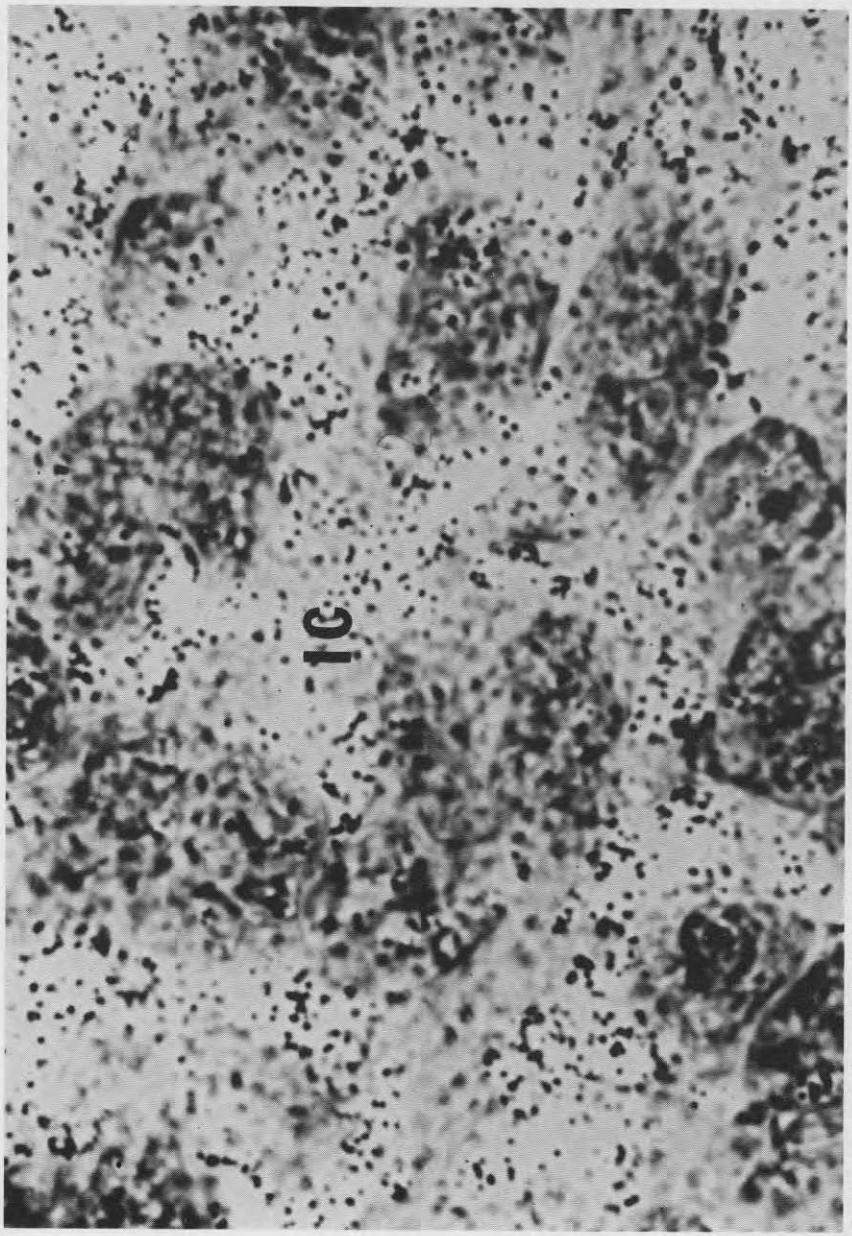


Figure 5-17. Autoradiographs of twenty day limb regeneration blastema twenty-four hours after intrapleuroperitoneal injection of 50 μc ^{3}H-proline. *A*. The blastema cells and intercellular (IC) are heavily labeled. The intercellular space averages a count of 28 grains/sq cm.

effect. There appears to be an ample amount of ascorbic acid present, this reportedly increasing to twice normal levels in regenerating axolotl limbs (Ryvkina, 1940), and an interstitial matrix of proteoglycans derived from overlying wound epithelium (Chapron, 1974) as well as a rapid accumulation of hyaluronate (Toole and Gross, 1971). On the other hand, the pre- and blastemic regenerates become acidic, dropping from a normal pH 7.2



B. A section adjacent to *A* following four hours of hydrolysis by collagenase, illustrating about 25 percent loss in label with the intercellular (IC) space averaging 20.8 grains/sq cm. This loss in label is presumably due to the digestion of newly synthesized collagen protein. (Van Giesen, ×400).

to pH 6.7 (Okuneff, 1928). The acidity is likely due to the accumulation of two and one-half times the normal levels of lactic acid (Okuneff, 1933) possibly as a result of anaerobic glycolysis (Wolfe and Cohen, 1965; Schmidt and Weidman, 1964; Johnson and Singer, 1964; Jasch and Schmidt, 1974) principally fueled by glycogen released from injured muscle fibers (Schmidt, 1960). There is a marked increase in proteolytic activ-

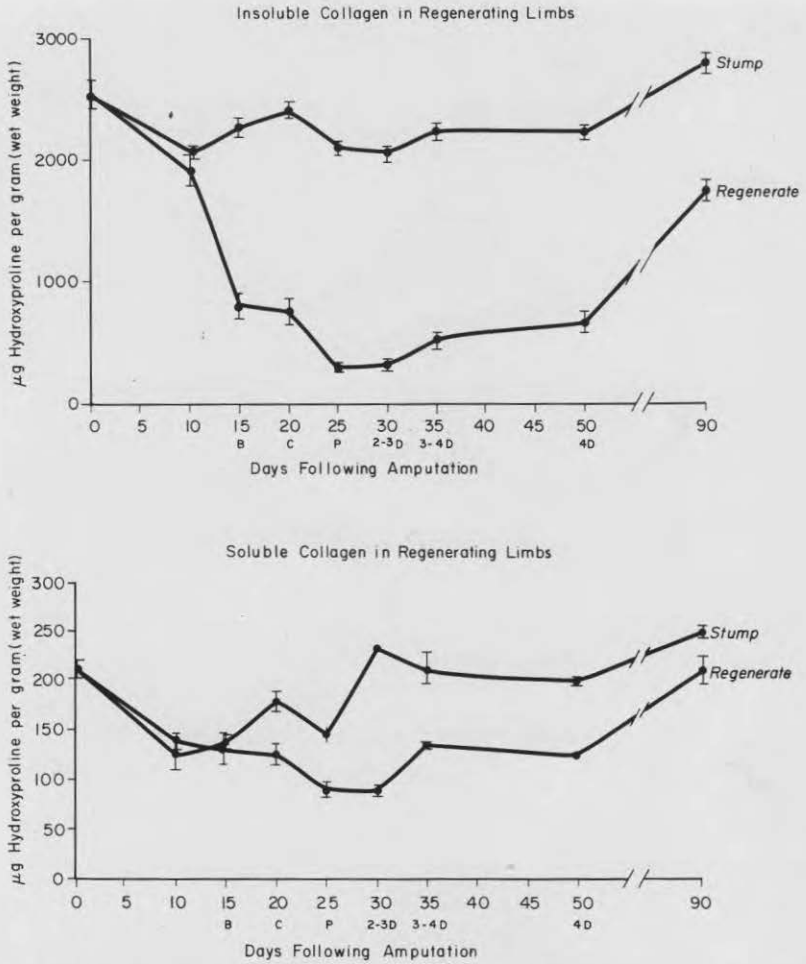


Figure 5-18. Saline soluble and insoluble collagen fractions in regeneration limbs. *A.* Insoluble collagen obtained from limb stump and regenerating tissues. The regenerate exhibits little insoluble collagen until differentiation of the new limb is advanced, whereas in the limb stump, the quantity of insoluble collagen is high over the test period. *B.* Soluble collagen in regenerating limbs is extractable in only very small amounts, and significantly ($p = <0.01$) less than the quantity of soluble collagen obtained from the limb stump. B = bulb blastema; C = conic blastema; P = paddleform; D = digitform. Vertical bars = S.E. (From M. C. Johnson and A. J. Schmidt, *J. Exp. Zool., 190:*185-198, 1974. Courtesy of the Wistar Institute, Philadelphia.)

ity at this time, including significant rises in such proteases as cathepsin C (Deuchar et al., 1957), cathepsin D (Slattery and Schmidt, 1975) (Fig. 5-20), collagenase (Grillo et al., 1968; Dresden and Gross, 1970; Mailman and Dresden, 1970) and other lysosomal hydrolases (see reviews by Schmidt, 1966, 1968) that could conceivably contribute to the degradation of collagen within the prevailing environment.

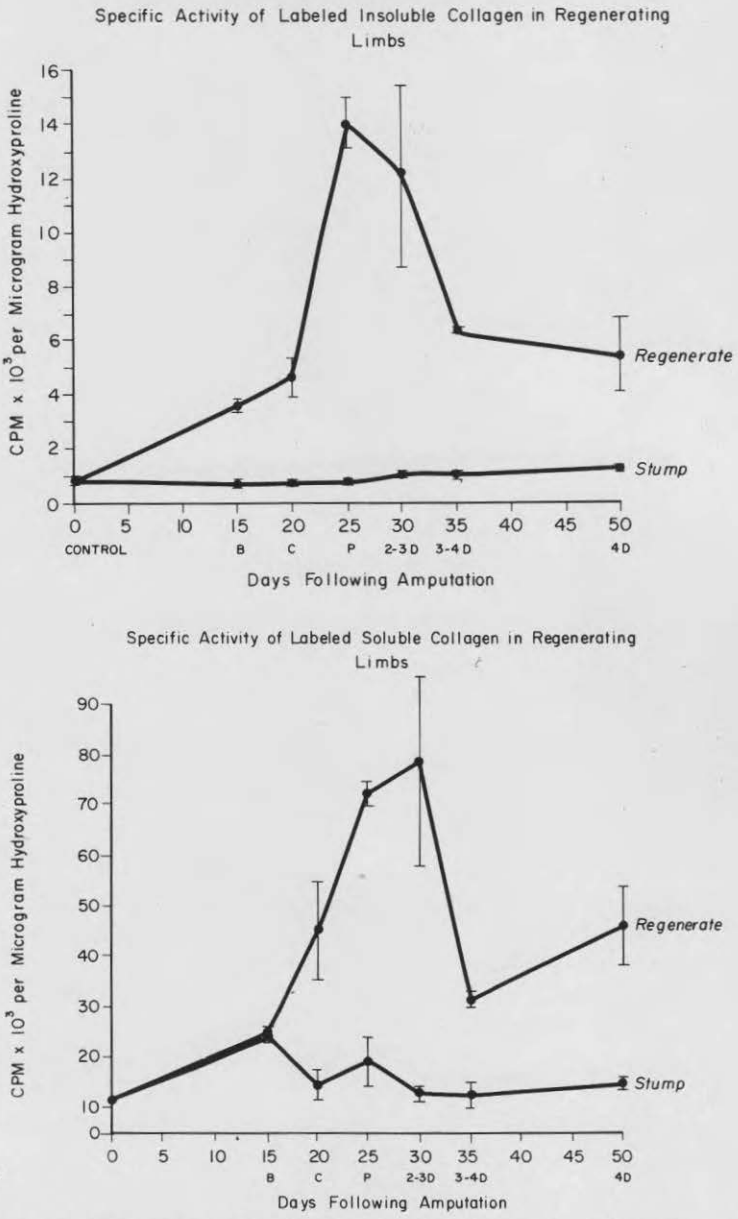


Figure 5-19. Specific activity of ^{3}H-labeled saline insoluble and soluble collagen fractions in regenerating limbs. *A*. Insoluble collagen.This illustrates that the regenerate is more actively engaged in collagen synthesis than is the limb stump, and that this activity is greatest during the paddleform and early digitform stages. *B*. Soluble collagen. The regenerate is more actively engaged in collagen synthesis than is the limb stump, and this activity is greatest during paddleform and digitform stages. The saline soluble fraction is considered representative of collagen synthesis and is more highly labeled on a percentage basis than is the insoluble collagen illustrated in *A*.Vertical bars = ranges. (From M. C. Johnson and A. J. Schmidt, *J. Exp. Zool., 190:*185-198, 1974. Courtesy of the Wistar Institute, Philadelphia.)

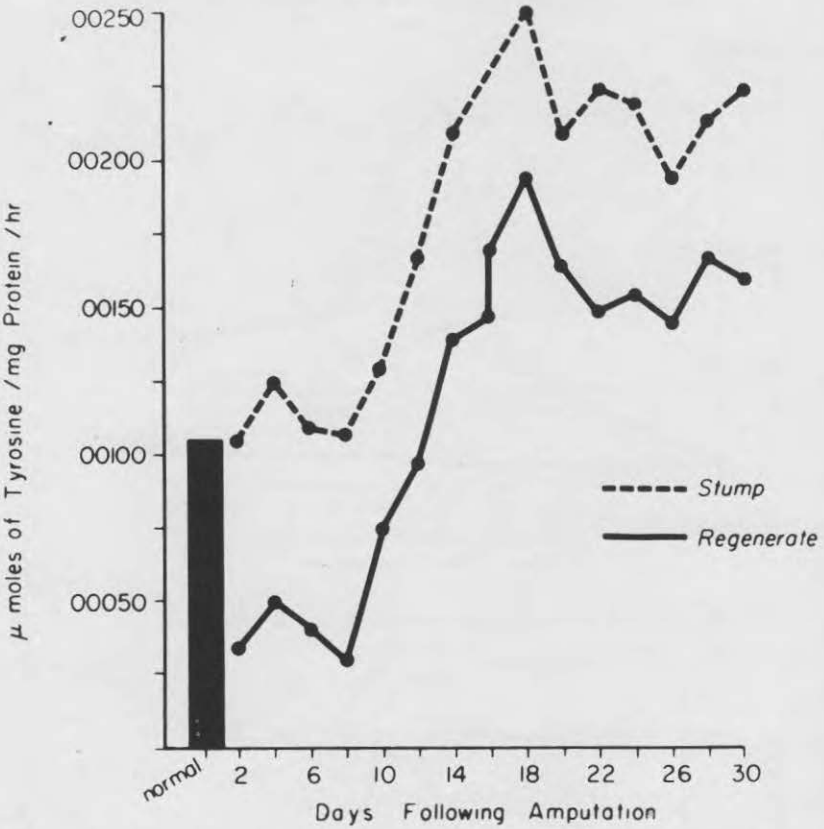


Figure 5-20. Cathepsin D activity in the newt limb regeneration process. The enzyme activity of the limb stump tissue is greater than that of the regenerating tissues, although there are clearly marked increases in cathepsin D activity in both the limb stump and regenerate following amputation through the newt forelimbs. (From G. G. Slattery and A. J. Schmidt, *Ann. N.Y. Acad. Sci., 243*:257-268, 1975.)

Finally, there is an observation reported by Norman and Schmidt (1966, 1967) that, during blastemic growth between the fifteenth and twenty-second days after limb amputation, fibroblastic cells were identified at the stump-regenerate interface containing numerous collagenlike fibrils within their cytoplasm, lying free or enclosed by smooth membranes (Fig. 5-21.) At that time, we considered this observation reflecting either phagocytosis or intracellular condensations of newly synthesized collagen (see also Usuku and Gross, 1965). However, more recent evidence based on a number of fibroblastic populations surveyed by Ten Cate and Deporter (1975) leads them to conclude that this phenomenon illustrates phagocytosis and collagen degradation by the same fibroblasts responsible for synthesizing this protein.

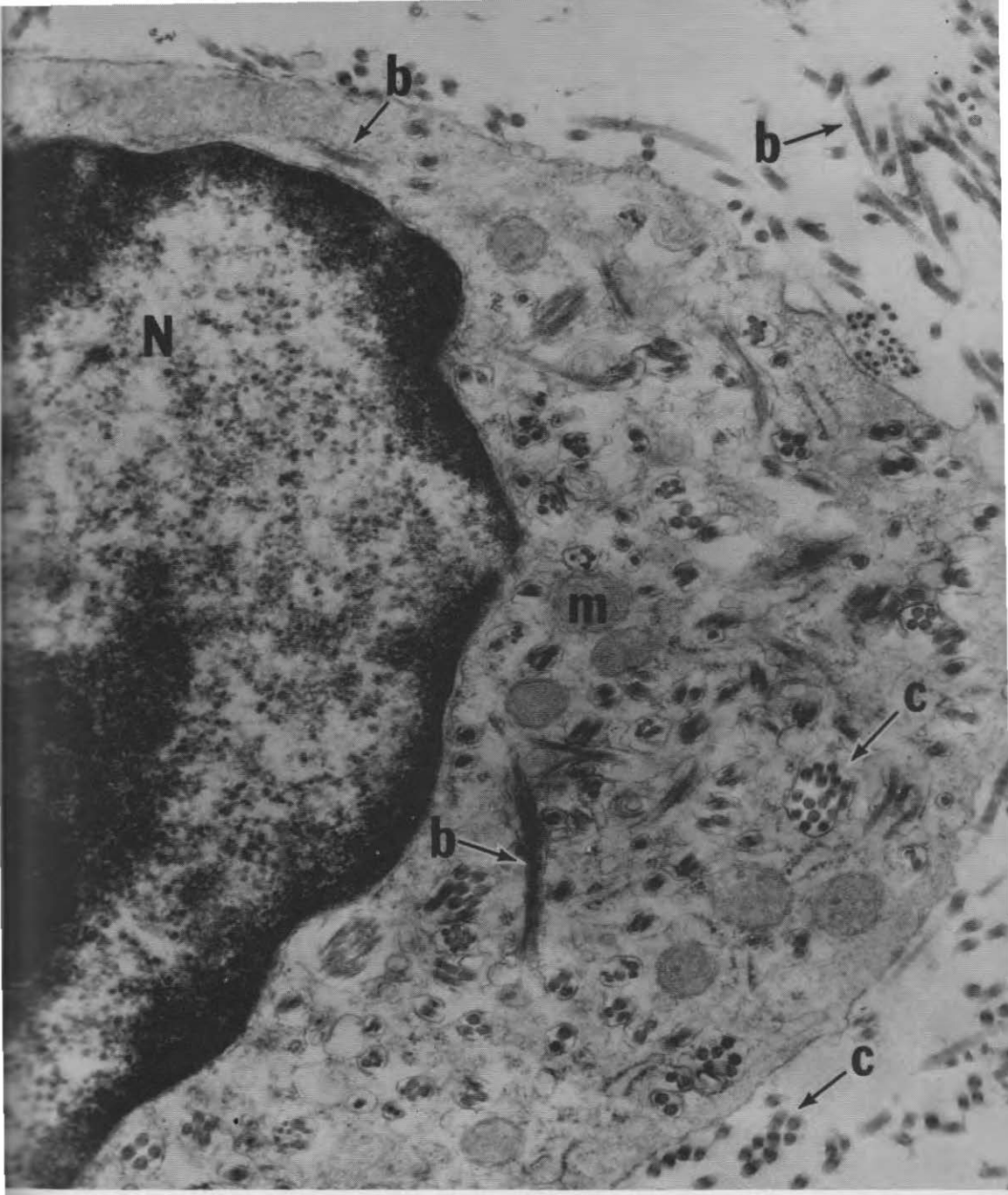


Figure 5-21. Electronmicrograph of a seventeen day limb regeneration blastema. A cross section of a fibroblast at the blastema-limb stump interface illustrating banded (b) fibrils both within the cell cytoplasm and extracellular space. Membrane-bound bundles of fibrils in cross section (c) are also indicated in both loci. One of the several mitochrondria (m) is identified. N = nucleus. ×50,000.

In summary, we have learned that the postamputational environment of the urodele appendage does change significantly from that of the untraumatized normal limb. Protein synthesis is extremely active even in the very early stages of regeneration; that there are changes in protein profiles with the advances in regenerative events even though a specific regeneration protein has not been substantiated. The wound epithelium and underlying blastema cell population do reveal some identifying molecular individuality. There is an avid uptake of proline into the regeneration blastema cells that is incorporated into newly synthesized proteins, and there is evidence some is converted to hydroxproline, indicating the presence of collagen protein in spite of the lack of any visible evidence of collagen polymers in electromicrographs. The environment of the regeneration blastema appears suited for collagen synthesis, although it is incompatible for fibril accretion in the presence of significant amounts of proteolytic enzymes, including collagenase, and for phagocytic activity not only by macrophages but also by fibroblastic cells, which assist in removal of collagen fragments.

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