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# Neural Control of Cell Cycle Events in Regenerating Salamander Limbs

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SYNOPSIS. Nerves, wound epidermis, and injury are indispensable for salamander limb regeneration, but their mechanism of action is not understood. A hypothesis has been presented (Tassava and Mescher, 1975) which suggests that injury is important to dedifferentiation and entry of limb stump cells into the cell cycle, nerves are required for one or more $G_2$ events in order that cells can proceed to mitosis, and the wound epidermis maintains the daughter cells in the cell cycle. The resultant cells accumulate to form the blastema.

Complete and partial denervation experiments, which attempted to test this hypothesis, are discussed. Blastema cell cycle parameters, measured after complete denervation, did not vary greatly from innervated controls, even though denervated blastemas were resorbed. Blastema cell cycle parameters of partially denervated limbs, which exhibited delayed regeneration, were likewise not lengthened when compared to completely innervated controls. These results are consistent with the view that after either complete or partial denervation, some blastema cells continue to cycle and reach the M phase in the same time as controls. Other blastema cells block completely, never reach M, and are then removed. A possible mechanism for resorption of denervated blastemas is presented.

## INTRODUCTION

Several species of urodele amphibians are well-endowed with the ability to regenerate amputated limbs. The important aspects of limb regeneration can perhaps be best illustrated by examination of an amputated forelimb of a larval axolotl (*Ambystoma mexicanum*) with regeneration underway. Such a limb, with an early bud stage blastema present (Tank *et al.*, 1976), can be seen in Figure 1 in longitudinal section. $^3$H-thymidine autoradiography has revealed that essentially no cells are undergoing DNA replication in the mesodermal tissues of the unamputated limb stump (Hay and Fischman, 1961; Mescher and Tassava, 1975). Witness, however, the abundant $H^3$-thymidine labelled cells in the blastema of Figure 1. How, then, are cells of the limb stump caused to switch their synthetic activities from those of the reasonably quiescent differentiated cell to those of the active, undifferentiated blastema cell?

It is instructive and of interest to delineate the early events which lead to blastema formation in terms of the cell cycle (Mitchison, 1971) as follows. During the initiation of regeneration, differentiated ($G_0$ phase) mesodermal cells dedifferentiate, enter the cell cycle in $G_1$, replicate DNA in the S phase, continue through $G_2$, and finally undergo mitosis. Continued cell proliferation then results in the establishment of the blastema. These cell cycle events, as they might occur early in regen-

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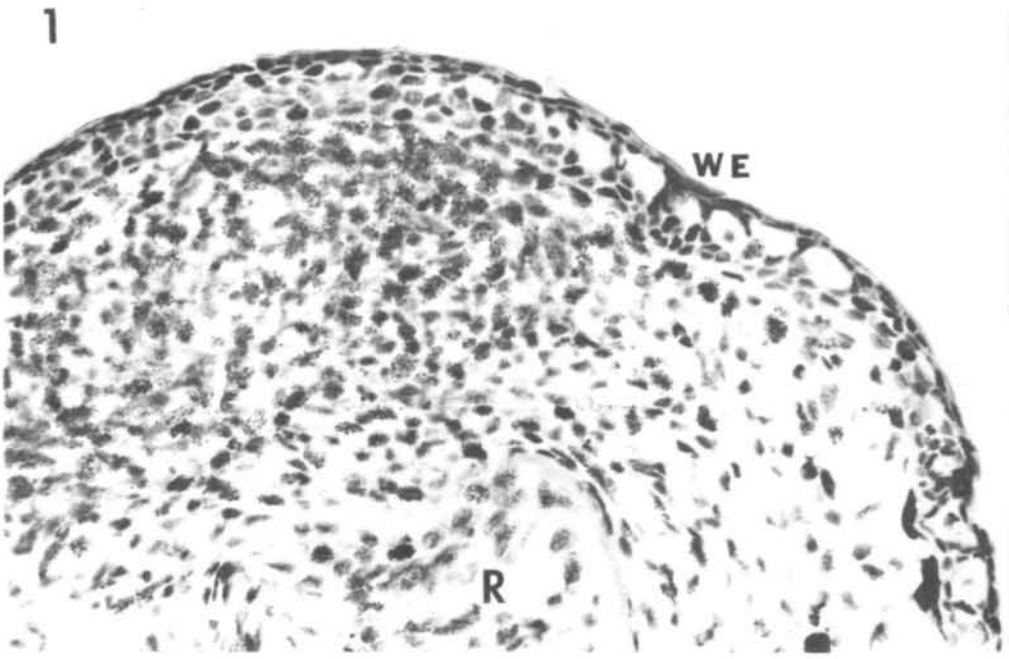


FIG. 1. An autoradiograph of a longitudinal section through an early bud stage blastema of a larval axolotl, 7 days post-amputation. The larva was given 1.5 $\mu$Ci of $^3$H-thymidine 2 hr prior to fixation. WE, wound epidermis. R, radius. The presence of silver grains over a nucleus indicates that the cell was in the S phase of the cell cycle during the labeling (incorporation) period. Rigorous experiments have shown conclusively that the blastema cell originates by dedifferentiation of differentiated mesodermal cells of the limb stump (Steen, 1968). Blastemas which develop after supernumerary limb induction are similar to the one shown here (Bodemer, 1959). × 250.

eration, are illustrated in Figure 2. There are three important prerequisites for blastema formation. The limb must be *injured*, a sufficient number of *nerves* must be present at the amputation surface, and a *wound epidermis*, free of underlying dermis, must cover the amputation surface (Singer, 1952; Hay, 1966; Goss, 1968; Schmidt, 1968; Thornton, 1968). If any one of these three conditions is absent, regeneration fails to occur. Recent experiments have directed attention to the possibility that injury, nerves, and the wound epidermis may influence the entry of limb stump cells into the cell cycle and the subsequent continued cycling of these cells to provide sufficient daughter cells for blastema formation (Tassava and Mescher, 1975).

By denervation, *i.e.*, severing the 3rd, 4th, and 5th spinal nerves at the shoulder, one can readily obtain limbs without nerves at the amputation site. The early recognition of the importance of nerves for regeneration (reviewed by Singer, 1952), as well as the relative ease of denervating limbs, has resulted in numerous attempts to elucidate the role of nerves in regeneration.

When salamander limbs are denervated on the day of amputation, no blastema develops and regeneration does not occur (Butler and Schotté, 1941; Singer, 1952). When a limb with a young blastema is denervated, outgrowth slows, then stops completely within a few days, and the blastema is subsequently resorbed (Schotté and Butler, 1944; Singer and Craven, 1948). However, redifferentiation of the blastema cells into a small limb can occur in the absence of nerves after the first 9 days post-amputation in *Ambystoma maculatum* larvae (Schotté and Butler, 1944) and after 17 days post-amputation in adult newts (*Notophthalmus viridescens*) (Singer and Craven, 1948; Powell, 1969). This transi-

tion of blastemas from nerve-dependence to nerve-independence has been attributed to synthesis of stable mRNAs (Bantle and Tassava, 1974), attainment of a "critical" mass (Dearlove and Stocum, 1974) and to a "critical" level of vascularization (Smith and Wolpert, 1975), but none of these views is conclusively supported by data.

Denervation results in reductions of protein, RNA, and DNA synthesis in both nerve-dependent and nerve-independent blastemas (Dresden, 1969; Lebowitz and Singer, 1970; Morzlock and Stocum, 1972; Bantle and Tassava, 1974). As might be expected, blastema mitotic activity is also adversely affected by denervation, particularly that of nerve-dependent blastemas (Singer and Craven, 1948; McCullough, 1976).

Attempts to extract and purify a "trophic factor" from nerve tissue have met with little success. Denervated limb stumps or denervated nerve-dependent blastemas have been infused with extracts of nerves (Lebowitz and Singer, 1970) or extracts of blastemas (Deck and Futch, 1969) but only slight positive responses were observed. Lebowitz and Singer (1970) have infused nerve extracts into denervated mound stage (nerve-dependent) blastemas and assayed for the neurotrophic factor by measuring isotope incorporation into blastema cell protein. Extracts of nervous tissue were successful in maintaining protein synthesis at the control level for several hours (Lebowitz and Singer, 1970). These experiments, recently extended to examine DNA synthesis (Jabaily and Singer, 1977) and mitotic indices (Foret, 1977), will hopefully better characterize the "active material" in the nerve tissue extracts.

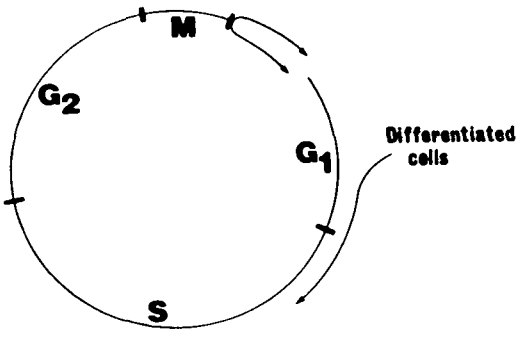


FIG. 2. A model of the cell cycle as applied to limb regeneration. Differentiated cells of the limb mesodermal tissues dedifferentiate, enter the cell cycle in $G_1$, replicate DNA in S, proceed through $G_2$ and undergo mitosis (M). Further cycles provide sufficient cells for blastema formation.

### INJURY, NERVES, AND WOUND EPIDERMIS: A CELL CYCLE HYPOTHESIS

As the above experiments illustrate, investigations into the role of nerves in regeneration have been basically of two types: (1) those in which the primary goal is to identify the chemical agent of the nerve which is important to regeneration, and (2) those in which the primary goal is to clarify the cellular or molecular events under neural control.

Experiments of the latter type were begun in our own laboratory in 1971 and were specifically designed to determine whether denervation influences any of the events significant to the initiation of regeneration. Since a blastema does not develop until 1 or sometimes 2 weeks after limb amputation, the absence of a blastema on a denervated limb may not be indicative of whether a limb initiated regeneration. Thus the possibility existed that a denervated limb might actually initiate regeneration only to have the process cease before blastema formation. Prior to 1970, comparisons of regenerating limb stumps with non-regenerating denervated limb stumps had been primarily of a histological nature (reviewed by Carlson, 1974). We did not change the basic experimental design of these earlier investigators but merely included autoradiographic analysis of RNA and DNA synthesis and also mitotic index determinations.

Experiments with larval axolotls showed that RNA synthesis increased in the distal limb stump after amputation in both innervated and denervated conditions but mitotic activity increased only in innervated limbs (Kelly and Tassava, 1973). We also noted dedifferentiated cells in the distal limb stumps, with rough endoplasmic reticulum and prominent nucleoli, whether or not nerves were present

(Popiela, 1972; Kelly and Tassava, 1973). Others had earlier noted similar histological changes in denervated and innervated limbs of *Ambystoma* larvae (Butler and Schotté, 1941), adult newts (Powell, 1969) and frog tadpoles (Schotté and Harland, 1943). In all three of these latter studies, dedifferentiation was observed in denervated as well as in innervated limb stumps. From these results, we reasoned that during the initiation of regeneration, *in the presence of nerves*, differentiated mesodermal cells dedi ferentiate, enter the cell cycle in G-1, replicate DNA in the S phase, continue through G-2, and finally undergo mitosis. Continued cell proliferation then results in the establishment of the blastema (see Fig. 2).

*Nerves and $G_2$*

In the case of denervated limbs, which do not form a blastema, we asked where the cells were blocked, *i.e.*, in which phase of the cell cycle. It seemed reasonable that since mitotic activity was almost absent, DNA replication would also not occur, and the cells would thus presumably be "blocked" in G-1. An alternative possibility would be for the dedifferentiated cells of denervated limb stumps to proceed in the cell cycle through G-1, replicate DNA, but become "blocked" in the G-2 phase, *prior* to mitosis. This latter possibility is in fact supported by the data, from experiments with both larval axolotls and adult newts (Tassava *et al.*, 1974; Mescher and Tassava, 1975). DNA synthesis was observed in both innervated, regenerating and denervated, non-regenerating limb stumps. However, the *mitotic index increased significantly only in innervated limbs.* Microspectrophotometric measurements of nuclear DNA contents in the dedifferentiated cells of denervated and innervated limbs revealed that in both cases replication of the entire chromosomal complement occurred (Mescher and Tassava, 1975). The most reasonable explanation of these data, an explanation consistent with the literature, is that limb stumps can initiate regeneration in the absence of nerves, but the dedifferentiated cells are blocked in the G-2 phase of the first cell cycle and consequently do not proliferate to form a blastema. A markedly elongated S phase of the cells of denervated limb stumps or a block in late S are alternative explanations but these are rendered unlikely because of the similar silver grain densities over $^3$H-thymidine labeled nuclei of denervated and innervated limbs (Mescher and Tassava, 1975). Also, Manson *et al.*, (1976) have found that activities of aspartate carbamyl transferase, an enzyme involved in pyrimidine synthesis, are not different in innervated and denervated adult newt limb stumps during the 1st and 2nd weeks post-amputation. Likewise, thymidine kinase and uridine kinase activities are not different in innervated and denervated newt limb stumps. These latter results are consistent with the view that the DNA synthetic phase is not the limiting step in denervated limb stumps.

*The fate of $G_2$ blocked cells in denervated limbs*

There are several possible fates for the cells which replicate DNA in denervated limb stumps. (1) They may break down in the G-2 state and be removed (see Janik, 1974). (2) They may remain in the limb stump in the G-2 phase as undifferentiated cells. (3) They may re-differentiate as G-2 cells and form new tissues to repair the injury, including dermis (Gelfant, 1966). (4) They may finally divide, after an extended G-2 period, and redifferentiate as G-1 cells into new tissues. Additional experiments are needed to distinguish between these possibilities. It should be noted that Butler and Schotté (1941) recognized this problem during observation of dedifferentiation in amputated, denervated *Ambystoma* limbs and stated, "as tissue dedifferentiation proceeds, the resulting cells disappear from the limb region. The manner in which the products of dedifferentiation are removed is incompletely understood." Later, Schotté and Karczmar (1945) described regression of denervated amputated *Ambystoma* limbs as a "breaking down of all the tissues adjacent to the surface of amputation with a subsequent

removal of the broken-down material and a progressive shortening of the limb." In *Ambystoma* limbs, according to the latter observations, possibility (1) above would be reasonable. It would remain to be determined whether the cells are removed whole or broken down first as well as the exact mechanism of removal, *i.e.*, via phagocytes (Bryant *et al.*, 1971) or via the wound epidermis (Singer and Salpeter, 1961) or other means. In adult newts, since regression of denervated limbs is not nearly as extensive, any one or more of the four possibilities would be reasonable.

*Injury and the wound epidermis—The complete hypothesis*

These early observations of dedifferentiation in amputated, denervated limbs (Butler and Schotté, 1941) and particularly in injured (fractured) denervated limbs, which possess neither a wound epidermis nor nerves (Thornton, 1953) strongly suggested that injury alone is sufficient to cause dedifferentiation. This view has been extended recently to include entry of the dedifferentiated cells into the cell cycle at $G_1$ and passage through S (DNA replication). Supporting evidence has come from autoradiographic observations of $^3$H-thymidine incorporation into dedifferentiating cells of injured or amputated limbs of both *Ambystoma mexicanum* larvae and adult newts without nerves and without wound epidermis (Tassava and Bennett, unpublished; Loyd, 1978). These experiments were designed after those of Goss (1956), which involved inserting freshly amputated limb stumps into the body cavity, and those of Thornton (1953), which involved injuring (fracturing) limbs without amputation. However, we included comparisons of DNA labeling indices and mitotic indices in the presence and absence of wound epidermis and/or nerves. These comparative data, along with results recently reported by Mescher (1976), led to the hypothesis shown in Figure 3 (see also Tassava and Mescher, 1975). The essence of the hypothesis is as follows: *injury* causes dedifferentiation of stump tissue cells which enter the cell cycle in $G_1$ and replicate DNA (S phase). In the presence of *nerves*, all $G_2$ events can be completed and mitosis (M) occurs. The *wound epidermis* insures continued passage of these progeny cells through further cell cycles, thus resulting in blastema formation. While this hypothesis may prove to be incorrect, it has most certainly stimulated much thought and experimentation.

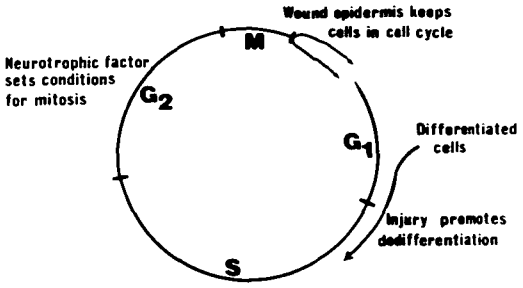


FIG. 3. A hypothesis explaining the roles of injury, nerves, and the wound epidermis in the initiation of limb regeneration. The hypothesis represents a generalized cell cycle of a dedifferentiating cell of the limb stump.

## TESTING THE HYPOTHESIS

To gain further insight into the role of nerves during regeneration, we have recently measured $^3$H-T labeling indices, mitotic indices, and cell cycle parameters of larval axolotl regeneration blastemas with and without denervation (McCullough and Tassava, 1975; McCullough, 1976; McCullough and Tassava, unpublished). Nerve-dependent, early bud stage blastemas were chosen so that $^3$H-T labeling indices, mitotic indices and cell cycle parameters could be measured just prior to and during resorption. In close agreement with previous studies with newts (Singer and Craven, 1948), mitotic indices of denervated axolotl blastemas followed the levels of controls for 3 days but then dropped off sharply as resorption occurred (Fig. 4). $^3$H-thymidine labeling indices followed the same patterns (Fig. 5) except that labeling indices never fell below 30%, even at 7 days post-denervation, at which time only small blastemas remained.

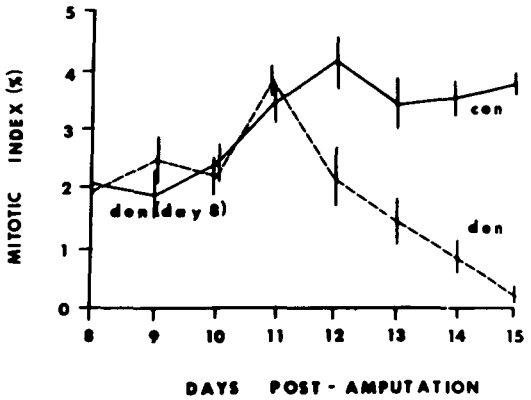


FIG. 4. Mitotic indices of innervated and denervated axolotl blastemas through 7 days post-denervation. All left limbs were denervated on the 8th day post-amputation. The mitotic indices of the denervated blastemas are significantly lower ($P < 0.01$, t-test) than the control, right limb blastema indices at 4 days post-denervation and thereafter. Con, control innervated blastemas. Den, denervated on day 8 post-amputation. At 7 days post-denervation, left limb blastemas were almost completely resorbed while right blastemas were in palette stages. Vertical lines represent the standard error of the means of three to six blastemas sampled at each point.

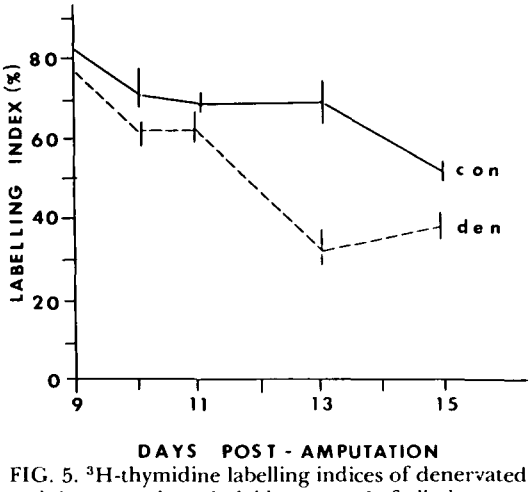


FIG. 5. $^3$H-thymidine labelling indices of denervated and innervated axolotl blastemas. Left limbs were denervated on day 8 post-amputation. At noon on each day indicated, 3 larvae were each given 1.5 $\mu$Ci of $^3$H-thymidine and fixed after a 2 hr incorporation period. Vertical lines represent the standard error of the mean of 3 blastemas sampled at each point. The labelling indices of denervated blastemas were significantly lower than the controls at day 5 and 7 post-denervation ($P < 0.02$, t-test).

*Cell cycle parameters do not change markedly in denervated, resorbing blastemas*

To measure cell cycle parameters, we administered to each larva a single short pulse of $^3$H-thymidine and fixed blastemas at 2 hr post-injection and at 8 hr post-injection intervals thereafter. By autoradiography, it was then possible to examine mitotic cells (late prophase through telophase) for silver grains and thus determine percent labeled mitotic figures for a 72 hr period after the pulse (Takahashi, 1966; McCullough and Tassava, 1975). Denervations of left limbs were at 8 days after amputation but, prior to giving the $^3$H-T pulse, either 24 hr or 48 hr were allowed to elapse to allow time for the blastema cells of left limbs to respond to the denervation. Right limb blastemas thus were 9 and 10 day controls respectively at the time of $^3$H-thymidine injection. These cell cycle parameters are given in Tables 1 and 2. What is immediately apparent from this comparison is that the cell cycle parameters, specifically $G_2$, of denervated blastemas are not greatly lengthened, as compared to controls. Yet the mitotic indices and $^3$H-T indices of these denervated blastemas are decreasing

TABLE 1. *Cell cycle parameters of larval axolotl blastemas as measured from the 9th to the 12th day post-amputation.**

| Blastema | Cell cycle parameters (hr) $G_1$ | S | $G_2$ | M | Cell cycle time (hr) |
|---|---|---|---|---|---|
| Control | 1.87 | 32.00 | 5.07 | 1.06 | 40.00 |
| Denervated (day 8) | 15.99 | 30.50 | 5.79 | 1.22 | 53.50 |

* Left limbs were denervated on the 8th day post-amputation. Each larva was given a single pulse of H$^3$-thymidine on the 9th day after amputation and three left limb blastemas and three right limb blastemas were then fixed and sampled for labeled mitotic figures at 8 hr intervals through 80 hr. Cell cycle parameters for adult newts (Grillo, 1971) and larger axolotls (Wallace and Maden, 1976) have also been reported but denervations were not included in these latter studies.

TABLE 2. *Cell cycle parameters of larval axolotl blastemas as measured from the 10th to the 13th day post-amputation.**

| Blastema | Cell cycle parameters (hr) $G_1$ | S | $G_2$ | M | Cell cycle time (hr) |
|---|---|---|---|---|---|
| Control | 1.44 | 38.00 | 5.94 | 1.12 | 46.50 |
| Denervated (day 8) | 3.44 | 34.00 | 11.44 | 1.12 | 50.00 |

* Left limbs were denervated on the 8th day post-amputation.

and resorption has begun. What these observations indicate is that, after denervation, many blastema cells continue to reach mitosis (M), and thus traverse the cell cycle, in nearly the same time as controls. Other cells, instead of requiring more time to traverse the cycle, do not cycle at all, block completely, and are somehow lost from the population, thus accounting for resorption. The data of Figures 4 and 5, which show that the labeling indices of denervated blastemas are still above 30% when the mitotic indices are almost zero, are consistent with the view that the block is in $G_2$. It is important to note that resorption of denervated, amputated limbs (Butler and Schotté, 1941) and denervated, injured (fractured) limbs (Thornton, 1953) of *Ambystoma* larvae can be accounted for in a similar manner (see also Tassava and Mescher, 1975).

### *The mechanism of blastema resorption*

The exact mechanism by which blastema cells or stump tissues are removed during resorption has not been clarified. Ultrastructural observations of denervated, resorbing larval *Ambystoma* and adult newt blastemas (Bryant *et al.*, 1971; Popiela, 1972) have revealed 4 different cell types: normal blastema cells, vacuolated cells, pale cells, and phagocytes (macrophages).

Using larval axolotl blastemas, we have recently developed preparative techniques which preserve cellular morphology, enhance staining characteristics, and thus enable us to identify macrophages, blastema cells, and mitotic cells with the light microscope (Fig. 6; C. Little and Tassava, unpublished). Early bud blastemas of larval axolotls (7 days post-amputation) were denervated on the left limb, prepared as above, and examined for macrophages, blastema cells, and mitotic figures. Opposite, innervated right limb blastemas were counted as controls. These results, shown in Table 3, indicate that an increase in macrophage indices occurs during resorption, concomitant with a leveling off and then precipitous drop in mitotic indices. In contrast, innervated control blastemas have few macrophages and the mitotic indices increase as the blastemas progress in regeneration (Table 3). From these results it is tempting to speculate that in denervated blastemas the cells block in some phase of the cell cycle and are removed by macrophages. The blocked cells may be the vacuolated and/or pale cells

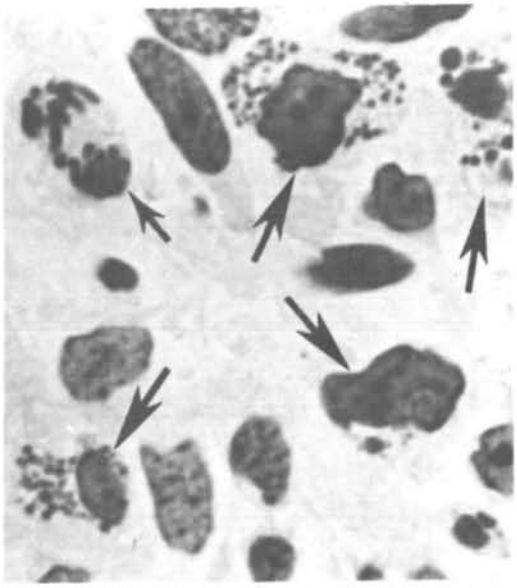

FIG. 6. A light micrograph of a portion of a longitudinal section of a denervated, nerve-dependent axolotl blastema, 5 days post-denervation. Blastema cells, a mitotic figure (small arrow), and macrophages (larger arrows) can be recognized. Polychrome stain (Jha, 1976). Blastemas were embedded in plastic (Spurr's resin) and sectioned with a glass knife at 5 μicrons. × 1,000.

TABLE 3. *Macrophage indices and mitotic indices ± standard deviation of axolotl blastemas at 7 days post-amputation (denervation day 0) and at 3 day intervals thereafter.**

| Day post-denervation | | Blastema macrophage index | Blastema mitotic index |
|---|---|---|---|
| 0 | | 3.10 ± 3.8 | 1.01 ± 0.16 |
| 3 | Left | 11.52 ± 0.14 | 0.94 ± 0.77 |
| | Right | 2.39 ± 1.94 | 2.90 ± 2.31 |
| 5 | Left | 12.55 ± 2.98 | 0.92 ± 0.15 |
| | Right | 1.31 ± 0.88 | 2.44 ± 1.38 |
| 8 | Left | 14.20 ± 1.40 | 0.04 ± 0.05 |
| | Right | 1.39 ± 1.31 | 1.33 ± 1.11 |

* All left limbs were denervated on the 7th day post-amputation at which time early bud stage blastemas were present. Innervated control right limbs regenerated in a typical fashion, but denervated left limb blastemas were resorbed. Each number is the mean of determinations for two blastemas. Over 4500 cells were sampled per blastema. Indices represent percent of that cell type present. The macrophage indices of left limb blastemas on days 3, 5, and 8 post-denervation were significantly greater than indices of right limb blastemas ($P < 0.001$, Chi-squared Test).

observed by Bryant *et al.* (1971). However, according to Bryant *et al.* (1971) macrophage indices do not increase during resorption of denervated newt blastemas and remain less than 5%. Furthermore, we are unable to identify pale cells with the light microscope. Further experiments, perhaps including prior labeling of blastema cells with $^3$H-thymidine, are obviously important to solving this problem.

*Predictions from the cell cycle hypothesis*

Based on the cell cycle model presented above (Fig. 3; Tassava and Mescher, 1975), we predicted that after a blastema is denervated and the neurotrophic factor becomes limiting, blastema cells will require longer and longer times to traverse the $G_2$ phase of the cell cycle and will finally block completely. This hypothetical possibility is depicted in Figure 7. As pointed out above, however, this prediction did not hold true. Instead, cells were seen to continue cycling in denervated blastemas in times close to those of control blastemas (Tables 1 and 2) and led to the suggestion that while some cells cycled in the normal time, other cells completely blocked and were subsequently removed. Another experimental situation exists, however, in which a lengthened $G_2$ period could be visualized (Fig. 7), this situation being established by *partial* denervation. From an innovative and elaborate series of experiments, Singer and his colleagues (Singer and Egloff, 1949; Singer, 1952) concluded that for adult newt limb regeneration to occur, a threshold quantity of axoplasm must be present at the amputation surface. Limbs with variations of axoplasm above this threshold, but below that of complete innervation, exhibited various degrees of delay in the regeneration process (Singer and Egloff, 1949). Karczmar (1946) and also Deck (1961) did similar but less extensive experiments with *Ambystoma* larvae.

*Partial denervation does not markedly increase the length of $G_2$ nor other cell cycle parameters*

Initially, we repeated these early partial denervation experiments with *Ambystoma mexicanum* (axolotl) larvae (C. Whiteside and Tassava, unpublished). Axolotl forelimbs were amputated and the left limbs were immediately partially denervated by severing the 3rd spinal nerve at the shoulder level. Left forelimbs were thus deprived of 30% of their total nerve supply (Deck, 1961). Blastemas were staged through time according to Tank *et al.* (1976) and, from the results shown in Table 4, we concluded that partial denervation indeed delays axolotl regeneration to a considerable degree. The question we next set out to answer was: Is the delayed regeneration of partially denervated limbs caused by an increase in the cell cycle time and, if so, which phase is lengthened?. Our prediction, that the increased time would be in the $G_2$ phase of the cycle, was consistent with the cell cycle hypothesis above (Fig. 3 and Fig. 7). The various cell cycle parameters obtained in this experiment can be compared in Table 5. The significant observation is that the overall cycle time of cells of partially denervated blastemas is not longer than that of con-

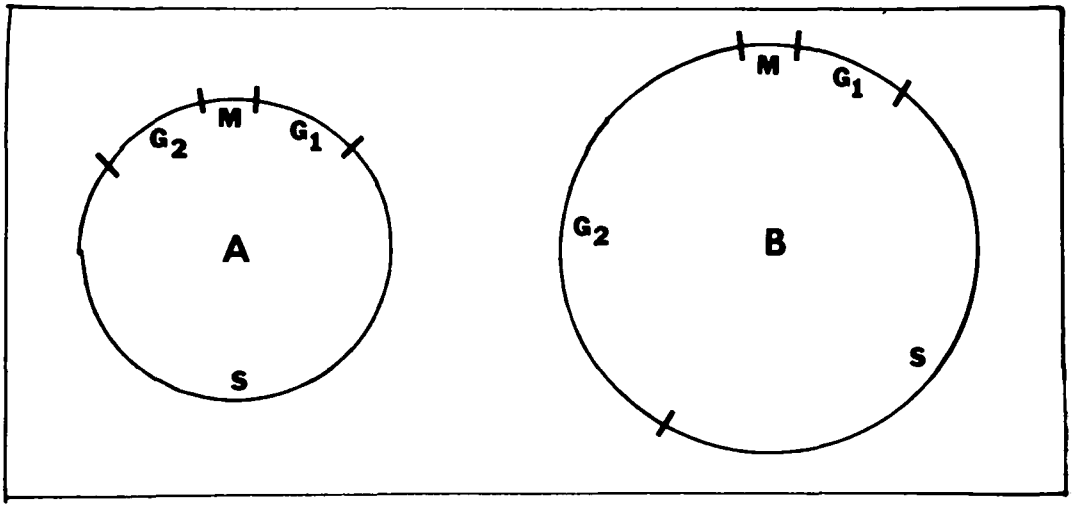


FIG. 7. A prediction of the effect of denervation or partial denervation on the $G_2$ phase of the cell cycle. The $G_1$, S, and M phases would not change in length but the $G_2$ time, and consequently the total cell cycle time, would increase. Compare cell cycle A with cell cycle B. This prediction is based on the view that nerves regulate one or more $G_2$ events of blastema cells and that, after removal of some or all of the limb nerves, the $G_2$ events would take more time to be completed (see Tassava and Mescher, 1975).

trol, innervated blastemas. We have also measured the length of the $G_2$ phase of blastema cells of partially denervated adult newt limbs and have found, although regeneration is significantly delayed by partial denervation, the length of $G_2$ is not increased (B. Stover and Tassava, unpublished). The prediction of Figure 7 clearly does not hold and an alternative explanation for delayed regeneration after partial denervation is therefore necessary. This alternative is actually as would be expected from the above results of the complete denervation experiments (Tables 1 and 2; McCullough, 1976) and is as follows. In partially denervated limbs, blastema cells either cycle in the normal time or are completely blocked and removed. Figures 8A and 8B depict the mechanism by which this might occur. Thus consider three blastema cells in a control innervated limb (Fig. 8A). All three cells (A, B, and C) would be provided, or would be able to sequester, the necessary supply of the neurotrophic factor to complete cell cycle events and undergo mitosis. Thus six

TABLE 4. *Blastema stages of right limb control blastemas with full innervation and left limb, 3rd nerve denervated, blastemas with only 70% of the full innervation.**

| Larva | Day post-amputation | Stage | |
|---|---|---|---|
| | | Left | Right |
| 1 | 9 | Early Bud | Late Bud |
| 2 | 9 | Early Bud | Mid Bud |
| 3 | 9 | Mid Bud | Late Bud |
| 4 | 9 | Late Bud | Late Bud |
| 5 | 9 | Mid Bud | Palette |
| 6 | 9 | Mid Bud | Late Bud |
| 7 | 12 | Palette | 2 Digit |
| 8 | 12 | Early Bud | 2 Digit |
| 9 | 12 | Palette | 2 Digit |
| 10 | 12 | Late Bud | Palette |

* Partial denervations of the left limbs were performed 1 day after amputation. Stages according to Tank *et al.* (1976).

TABLE 5. *Cell cycle parameters of control completely innervated axolotl blastemas and experimental, 3rd nerve denervated blastemas as measured between 6 and 9 days post-amputation.**

| Blastema type | Cell cycle parameters (hr) $G_1$ | S | $G_2$ | M | Total cell cycle time |
|---|---|---|---|---|---|
| Control | 4.5 | 34.0 | 5.5 | 1.0 | 45 |
| Partially denervated | 2.0 | 30.5 | 7.5 | 1.0 | 41 |

* Left limbs were partially denervated by severing the 3rd spinal nerve at the shoulder at 1 day post-amputation. Each larva was given a short pulse of $^3$H-thymidine on the 6th day post-amputation and two left and two right limb blastemas were then fixed and sampled for labelled mitotic figures at each 8 hr interval through 56 hr.

daughter cells would result. However, in a partially denervated limb (Fig. 8B), only two of every four cells can sequester the necessary supply of neurotrophic factor. These two cells (A and B) complete cell cycle events in the normal time and divide, resulting in four daughter cells. Cell C, without sufficient neurotrophic factor, blocks, before reaching M, and is removed. Thus from three cells only four cells result, accounting for the delay in regeneration but with no increase in the cell cycle time.

Recent *in vitro* studies have appeared (Globus and Liversage, 1975; Globus and Vethamany-Globus, 1977; Foret, 1977) which report data consistent with the cell cycle hypothesis of Tassava and Mescher (1975). Additional *in vitro* and *in vivo* experiments will undoubtedly finally clarify the chemical identity of the trophic factor as well as its mechanism of action during regeneration.

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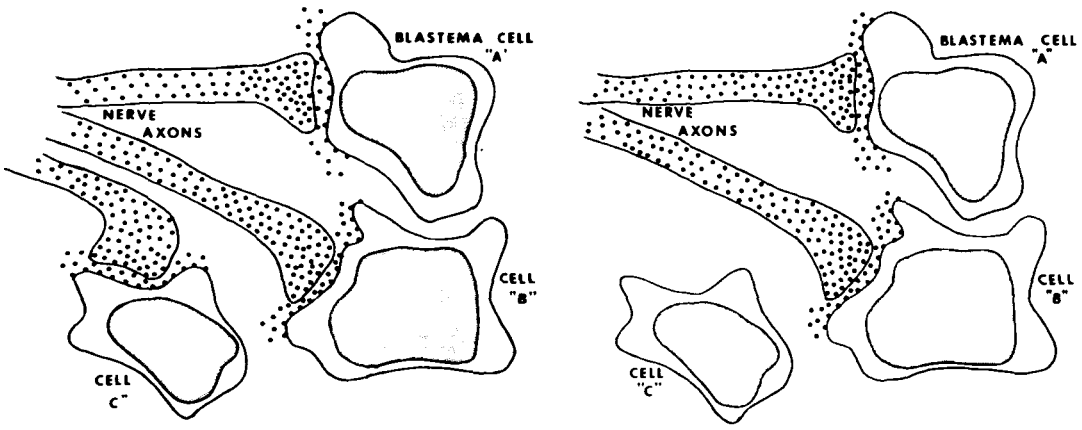


FIG. 8. A. A hypothetical view of the sequestering of nerve trophic molecules by blastema cells. All three cells shown (A, B, and C) are able to sequester sufficient trophic molecules to complete cell cycle events and undergo mitosis. B. A hypothetical view of the inability of all cells to sequester trophic molecules after partial denervation. Cell C, without sufficient trophic molecules, is blocked and removed from the blastema. Cells A and B are able to complete cell cycle events and undergo mitosis.

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