Effects of insulin insufficiency on forelimb and tail regeneration in adult *Diemictylus viridescens*

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SUMMARY

Experimental diabetes caused by pancreatectomy resulted in a marked interference in limb and tail regeneration in adult newts. Under pancreatic insufficiency, throughout the entire period of regeneration, limb and tail regenerates (26 days old) consistently showed sparse population of blastema cells, while older regenerates (58–79 days) exhibited severe abnormalities. Newts with these abnormal regenerates always had an atrophied pancreas. In the event that the cauterized pancreas regenerated, there was a subsequent recuperation in the regeneration processes of the appendages. Our data strongly indicates that a correlation exists between the normal (intact) pancreas and regeneration of the limb and tail in the adult newt.

In addition, alloxanization inhibited normal limb and tail regeneration. Again, recuperation of the pancreas from the effects of alloxan was followed by restoration of limb and tail regeneration. These results suggest an insulin role in the hormone control of regeneration. The possible actions of insulin are discussed.

INTRODUCTION

Previous studies have demonstrated that the presence of an intact anterior pituitary gland is essential in adult *Diemictylus viridescens* not only for the survival of the animal but also for the normal regeneration of its appendages (see reviews by Schotte (1961), Rose (1964), Schmidt (1968) and Thornton (1968)). Although considerable interest has been directed toward the study of hormone control of the regenerative processes in urodele appendages, the exact nature of their influence is still not clear.

On the basis of a series of experiments, Schotte and his associates hypothesized that the pituitary gland responds to the stress of limb amputation by releasing ACTH which, in turn, promotes regeneration by stimulating the production of the adrenocorticosteroid hormones (Schotte & Hall, 1952; Schotte & Chamberlain, 1955; Schotte & Bierman, 1956; and Schotte & Christiansen,

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They also postulated that the pituitary-adrenal synergism is probably active in: (a) preventing dermal invasion under the wound epidermis, and (b) supporting tissue dedifferentiation.

However, Wilkerson (1963) obtained limb regeneration when he injected bovine somatotropin into hypophysectomized newts, beginning as late as 15 days post-amputation. He concluded that the loss of regenerative ability in the adult newt limb following hypophysectomy was due to the absence of growth hormone.

Several workers have shown that limb regeneration is thyroxine-dependent. Following thyroidectomy normal limb regeneration is retarded but not completely inhibited. Early removal of the thyroid gland interferes with dedifferentiation and late removal interferes with the growth of the regenerate (Walter, 1910; Richardson, 1940; Schotté & Washburn, 1954). Antuitrin G (a commercially prepared mammalian anterior pituitary extract) in combination with thyroxine was more effective in promoting regeneration in hypophysectomized adult newts than Antuitrin G alone (Richardson, 1945).

Recently, prolactin has been shown to have an effect on limb regeneration (Connelly, Tassava & Thornton, 1968; Tassava, 1969). They showed that prolactin alone increased survival and limb regeneration, but less effectively than did prolactin in combination with thyroxine. From this evidence Tassava (1969) postulated a ‘prolactin-thyroxine’ synergism operating in adult newt limb regeneration.

All of these hormones that have been associated with regeneration in _D. viridescens_, namely prolactin, cortisone, thyroxine and growth hormone, are also known to induce the production of insulin from the islets of Langerhans (Haist, 1959; Lazarus & Volk, 1962; Tepperman, 1965; Bern & Nicoll, 1968). Prolonged treatment of mammals with these hormones can exhaust the islets and, ultimately, cause diabetes; hence, these hormones are called ‘diabetogenic hormones’ (review by Tepperman, 1965). In this connexion, Genuth & Lebovitz (1965) observed that addition of ACTH to culture medium induced the production of insulin in the islets of mouse pancreas _in vitro_. In consideration of these findings, the hormones thus far related to limb regeneration in the adult newt may conceivably act by stimulating the production of insulin. The following experiments have therefore been designed to try to determine whether or not insulin is involved in the hormone control of regeneration.

**MATERIALS AND METHODS**

Adult newts, _Diemictylus viridescens_ used in this investigation, were obtained from central Massachusetts, U.S.A. Medium sized animals of both sexes, weighing approximately 1.8 g, were used. Prior to experimentation the animals were allowed a 2-week acclimatization period and were kept in dechlorinated tap water at 20 ± 1 °C and fed lean ground beef twice weekly.
Pancreatectomy

Adult newts, previously deprived of food for 3 days, were anaesthetized in M.S. 222 (1 g: 1000 ml aqueous solution, Sandoz), prior to pancreatectomy. An oblique incision, about 3 mm in length, was made in the left ventro-lateral region of the abdomen in order to expose the pancreas. This diffuse gland lies between the duodenum and the stomach and is enclosed in the hepato-gastric and dorsal ligaments.

Pancreatectomy was achieved by cauterizing the gland with a Radiotom (Siemens–Model 614) electro-cautery unit. The pancreas was cauterized until the tissue became opaque; great care was exercised to avoid extensive damage to the blood vessels supplying the gut and the spleen.

Following pancreatectomy, the operated area was rinsed with sterile amphibian Ringer's solution, and the organs which were previously exteriorized were returned to the body cavity; the incision was sutured using no. 6-0 Ethicon eye sutures. Both sham pancreatectomized and control animals served to compare the effects of surgery. All animals were allowed to recuperate for 2 days post-operatively at 15 °C and then returned to 20 ± 1 °C. Their regular feeding schedule was not resumed until 15 days post-pancreatectomy. The water level was maintained at a depth of about 0-5 cm which was sufficient to keep the animals' skin moist until the incision had healed.

Alloxan treatment

Following the method of Falkmer (1961), alloxan was administered to adult newts in an attempt to bring about beta cell destruction and ultimately induce a state of insulin insufficiency. Adult newts, of uniform size (weighing approximately 2 g), were deprived of food for 3 days prior to the injection of alloxan monohydrate (a 5 % solution, freshly prepared in citrate-phosphate buffer at 10 °C and pH 4.0). One group of animals received two intramuscular injections of alloxan (each 0.3 mg per g of b.w.) into the midback region with a 2-day interval between injections. The second group of animals received two injections of 0.2 mg acid alloxan per g b.w. at intervals of 27 days between injections. The controls received an equal volume of buffer solution. The longer interval between injections in the second group was chosen in an attempt to minimize the possible toxic effects of alloxan on the newt. All animals were kept at 10 °C, for 24 h prior to injection and then for 2 additional days following the administration of alloxan, whereupon they were returned to 20 ± 1 °C (see Falkmer, 1961). Urine-sugar analyses were performed on all animals commencing on the fourth day following alloxan treatment.

Daily urine-sugar analyses were made on pancreatectomized, alloxanized and control animals, using Testape (Lilly and Co. Canada Ltd., Toronto) as a method of indicating the amount of glucose (range = 0·1–2 %) in the urine. Thus, the onset and duration of the diabetic state of the experimental animals.
was detected and recorded. The right forelimbs and tails of the pancreatectomized animals were amputated at the onset of diabetes.

Upon termination of the experiments limb and tail regenerates and the pancreatic tissues of the experimental and control animals were fixed in Bouin's fluid. Limb and tail regenerates were decalcified in Jenkins' solution, sectioned at 8 μm and stained with hematoxylin and counterstained with orange G–eosin. Pancreatic tissue was stained with aldehyde fuchsin, Ponceau de xylidine–acid fuchsin and counterstained with fast green (Epple, 1967).

RESULTS

Effects of pancreatectomy on forelimb and tail regeneration

Pancreatectomized animals excreting 0-1 % glucose in the urine survived for periods of up to 52 days, but those excreting 0-25 % glucose died within 15 days after pancreatectomy. It was difficult to keep animals alive in a prolonged state of diabetes; nevertheless, a minimum period of 25 days from the time of amputation is required to observe changes in limb regeneration. Therefore, the majority of cases were fixed 25 days post-pancreatectomy. The results are summarized in Table 1. The regenerates will be considered in two groups: Group A is composed of regenerates fixed 25–35 days post-pancreatectomy; whereas Group B consists of regenerates fixed 58–79 days after pancreatectomy.

Complete pancreatectomy in the newt resulted in death about 4 days after surgery (Table 1, Group A, Series I). Cauterization of approximately 75 % of the pancreas did not result in an observable difference between the control regenerates and those of depancreatized animals, and also glucose was not detected in the urine during the experiment (see Table 1, Group A, Series II). However, when approximately 90 % of the pancreas was cauterized, inhibition of normal limb and tail regeneration was observed (Series III, IV and V).

Limb regenerates

Group A: Series III and IV, Table 1, 25–28 days. By 25 days post-amputation, the control regenerates showed advanced cone-shaped blastemata (Fig. 1). Bone dedifferentiation had occurred and a dense population of blastema cells had accumulated distal to the stump bone. Mitoses were frequently observed and procartilage condensation was apparent in the blastema region (see reviews by Schotte (1961), Rose (1964), Schmidt (1968) and Thornton (1968)).

A total of 110 adult animals (controls = 50; experimental = 60) was included in series III of which 31 experimental animals survived. Limb and tail regenerates from these experimental animals were fixed 25–28 days following pancreatectomy. Histological examination of the regenerates from these pancreatectomized animals showed 11/31 cases with bone protrusion through the wound epidermis, 5/31 cases with epidermal blisters and 28/31 cases exhibiting sparse populations of cells in the regeneration area. The remaining three cases showed near normal
Table 1. Effects of pancreatectomy on forelimb and tail regeneration in adult newt at 20 °C.

<table>
<thead>
<tr>
<th>Group and series</th>
<th>Total no. of animals</th>
<th>Survival at end of expt.</th>
<th>Total no. of days of regeneration at fixation</th>
<th>Control</th>
<th>Abnormal</th>
<th>Experimental</th>
<th>Control</th>
<th>Abnormal</th>
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<th>Tail regenerates</th>
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<td>Gr. A. Ser. I</td>
<td>20</td>
<td>40</td>
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<td>20</td>
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<td>48</td>
<td>31</td>
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<td>Ser. IV</td>
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<td>D4</td>
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Abbreviations (included in Table 1): AF = abnormal fin; Bp = bone protrusion; CO = cone stage; D3 = early three-digit regenerate; D4 = four-digit regenerate; Dab = digital abnormalities; EB = epidermal blister; PAT = atrophied pancreas; PNR = pancreas regeneration; RC = sparse blastema cell population; S = stump (no regeneration).
**Figures 1, 2.** The arrows indicate the level of amputation

Fig. 1. A longitudinal section through a 26-day forelimb regenerate of a control adult newt showing a normal advanced cone-shaped blastema: $b$, blastema cells, $e$, epidermis.

Fig. 2. A similar section through an experimental 26-day forelimb regenerate of a partially pancreatectomized adult newt. The blastema cells ($b$) are sparse; growth is inhibited as compared to the control. The stump bone lies close to the epidermis. Magnification about $\times 75$. 
cone regenerates when fixed at 28 days post-amputation. These latter three cases excreted sugar for the first 10-15 days post-amputation, and then stopped. Neither bone protrusion nor epidermal blisters were observed in the control animals. Procortilage cell condensation was not detected in any of the experimental cases, indicating a delay in the rate of regeneration (Fig. 2).

In series IV partial pancreatectomy (90%) was performed 4 days following amputation on 30 animals; by this time the epidermis had completely covered the amputation surface. Out of the 14 animals that survived for 26 days, two cases had regenerates in which the humerus protruded through the epidermis; three cases exhibited stumping (no regeneration); and nine showed markedly reduced blastema cell populations compared to the controls.

**Group A: reamputation experiments.** Reamputation of 10 pancreatectomized animals from series III was performed and subsequent regeneration over the additional 25 days was compared with the original 25-day regenerates. The diabetic state of these animals was monitored by daily urine-sugar analyses, as previously described. Although the survival rate among these re-amputees amounted to only two cases, interesting results were obtained. The first and second limb regenerates from one diabetic animal were similar in their morphological and histological appearance and in their rate of regeneration. That is to say, both regenerates exhibited reduced blastema cell populations compared to control regenerates and the diabetic state persisted throughout the experiment (total of 50 days). However, the second regenerate of the other case showed a considerably advanced limb morphogenesis over the first regenerate. Interestingly enough this was preceded by the recovery of the animal from diabetes. This animal excreted sugar in the urine for 35 days and thereafter the urine was free of sugar. The first regenerate showed a sparse population of cells and resembled the delayed regenerate of the diabetic animals previously described; cartilage condensation was not apparent. This was in sharp contrast to the second regenerate of this case which showed cartilage differentiation, dense blastema cell aggregation and numerous mitotic figures. (Figs. 3-5). Restoration of normal limb regeneration in the second regenerate was correlated with a correction in the diabetic condition of the animal, as evidenced by the sugar-free urine and the regenerated pancreas.

**Group B: series V (Table I), 58- to 79-day regenerates.** Control limb regenerates from all 20 newts exhibited four, well-formed cartilaginous digits by 79 days post-amputation and the morphological pattern of the regenerates resembled that of normal forelimbs.

Twenty-five pancreatectomized animals that stopped excreting sugar within 10 days post-pancreatectomy were allowed to regenerate for 58-79 days. Out of the 25 experimental animals, eight regenerated normal limbs and tails. These eight animals appeared healthy and accepted food readily. An examination of their viscera revealed a partial regeneration of the pancreas. The histological preparations showed the presence of normal acinar and islet tissues.
The remaining 17 pancreatectomized animals, however, exhibited either abnormal or no regenerates. These animals refused food, lost weight and became extremely thin. Limb regeneration was totally absent in two cases, even after 58 days post-amputation and abnormal in nine others. Of these nine cases four exhibited spike-like regenerates, whereas five showed a lack of digital components and considerable retardation of regeneration. Histological examination of these four spike-like regenerates revealed complete absence of the radius and the regenerates had only one digit giving a spike-like form to the new limb (see Figs. 6, 7). Also, muscle and connective tissue development was considerably reduced. The five cases that exhibited a lack of digital components were short, thin and stunted. Histologically they showed one digital cartilage condensation in only one of the five regenerates; the others were digitless. In addition to the above-mentioned abnormal cases, extremely delayed regeneration was observed in three cases exhibiting only a cone stage at 60 days. The remaining three animals showed no signs of limb regeneration up to 32 days post-amputation whereupon small cone-like limb regenerates began to appear, 58 days after amputation.

The cauterized pancreas of the animals that exhibited abnormal limb and tail regeneration was considerably reduced in bulk and severe atrophy was observed in the acinar as well as in the islet tissue. The nuclei were pycnotic and surrounded by only scanty amounts of cytoplasm; the latter was significantly lacking in zymogen granules.

Tail regenerates

Group A: Series III and IV (Table 1), 25- to 28-day tail regenerates. The control regenerates were approximately 2-2 mm long by 25 days post-amputation, and showed well-differentiated dorsal and ventral fins, spinal cord and muscles. Maturing cartilage and vertebral segmentation were evident. A comparison between the control and experimental tail regenerates revealed considerable delay in the rate of regeneration in the pancreatectomized animals. Mitotic figures were infrequent in the sparse populations of blastema cells.
Group B: Series V, 58- to 79-day tail regenerates. The control tail regenerates showed advanced growth and differentiation by 79 days post-amputation, and exhibited well-defined vertebral arches (4 to 7 in number). Also spinal cord, sympathetic and spinal ganglia, skin glands, blood vessels, tail musculature and fins were well-differentiated at this time. In contrast, regeneration in the 17 experimental animals was extremely delayed, as evidenced by the small, retarded regenerates (Figs. 8, 9). Moreover, growth of the fins was delayed in relation to the central axis of cartilage and spinal cord; this resulted in a spike-like appearance. (This type of regeneration was also observed in hypophysectomized newts by Vethamany, 1970.) Mitoses were less frequently seen and some rudimentary cartilage differentiation was evident. Tail regeneration in the remaining eight experimental cases, however, was quite normal as indicated in Table 1 and as similar to Fig. 8.

Effects of alloxan treatment

Twenty animals received two injections of 0.3 mg of alloxan per g of b.w. at 2-day intervals. The right forelimbs and tails were amputated 2 days following the second injection. However, four animals survived up to 55 days. The amputated forelimbs and tails of these four animals showed abortive regenerates; that is to say, they had permanent blastemata showing sparse populations of cells. The distal tip of the stump bone was enveloped by a collar of cartilagenous tissue and osteoclastic erosion of the bone was still much in evidence at 55 days post-amputation. Mitotic activity was minimal, extensive vacuolization was noticeable in the cells, and there was no trace of cartilage differentiation even up to 55 days following amputation. However, the control animals (10), on the other hand, exhibited well-formed forelimb regenerates by this time and showed normal regrowth of the humerus, radio-ulna and four digits and had well-developed limb musculature (Figs. 10, 11).

In the second set of animals (20 = experimentals and 10 = control) the

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Fig. 6. A longitudinal section through a 62-day forelimb regenerate of a control animal. Note the well-formed cartilaginous limb skeleton and musculature. Magnification about × 25.

Fig. 7. A similar section through a 62-day forelimb regenerate of a partially pancreatectomized adult newt showing an abnormal spike-like regenerate. Note the absence of radius and many digital components. Only one digit (d) is seen. Muscles and connective tissue in the regenerate are considerably reduced as compared to the control. Magnification about × 25.

Fig. 8. A mid-sagittal section of a 62-day tail regenerate of a control newt showing well-differentiated regenerate with six vertebral arches (v), spinal cord (s) differentiation of centra in the cartilage (c) and fins (f). Magnification about × 40.

Fig. 9. A similar section of a 62-day tail regenerate of a partially pancreatectomized adult newt exhibiting extremely small and retarded regenerate as compared to the control above. Magnification about × 50.
Insulin and regeneration

experimental received two injections of 0.2 mg acid alloxan/g of b.w. at intervals of 27 days between injections; the right forelimbs and tails were amputated 2 days following the second injection. Five of these alloxanized animals survived for 47 days post-amputation. Urine-sugar excretion (0.1 %) continued for 25 days following amputation and thereafter sugar was not detected in the urine. The amputated limbs and tails did not exhibit external signs of regeneration until 33 days post-amputation and they had the appearance of non-regenerating stumps. However, small cone-like blastemata were seen breaking through the stump region by 47 days post-amputation (Figs. 12, 13). The forelimb and tail regenerates as well as the pancreas of three animals were fixed at this time; the remaining two cases were fixed 57 days post-amputation. The amputated limbs and tails of the control cases regenerated quite normally, and by 47 days post-amputation they displayed normal advanced four-digit regenerates (see Fig. 12).

Histological examination of the three 47-day cone-shaped limb regenerates from the second set of alloxanized animals revealed an accumulation of blastema cells and profuse vascularization of the region. Cell proliferation was active as evidenced by the abundance of mitotic figures among the epidermal and blastema cells. However, there was no detectable cartilage differentiation by 47 days post-amputation. Interestingly enough, the appearance of cone-shaped blastemata and the sudden burst of mitotic activity was preceded by the disappearance of glucose from the urine. The two limb regenerates which were fixed 57 days post-amputation were heteromorphic but exhibited some cartilage differentiation. The tail regenerates from these alloxanized animals exhibited a delay in the regeneration; the 47- and 57-day-old regenerates resembled controls of 22 days of regeneration in their morphological and histological appearance.

The acinar cells of alloxanized pancreas (first set of animals) were packed with dense zymogen granules that stained a deep purple with basic-fuchsin; this is indicative of normally functioning exocrine tissue. However, compared to

**Figures 10-13**

Fig. 10. A longitudinal section of a 55-day forelimb regenerate from an adult newt exhibiting normal regenerate with four digits. Magnification about × 30.

Fig. 11. A similar section of a 55-day forelimb regenerate of an alloxanized adult newt exhibiting arrest in regeneration. Note the permanent blastema (b) and the thick apical cap (e). Magnification about × 30.

Fig. 12. A longitudinal section through a 47-day forelimb regenerate of an alloxanized adult newt exhibiting restoration of regeneration. Note the small cone-blastema; extremely delayed as compared to the control (Fig. 13). The pancreas of this animal exhibited hypertrophy of the islets (see Fig. 16). Vacuoles (v) are seen near the distal end of the stump bone. Note the accumulation of blastema cells (b). Magnification about × 85.

Fig. 13. A longitudinal section through a 47-day forelimb regenerate of an adult newt which served as a control for the alloxanized animal (Fig. 12). The regenerate is well-differentiated with four digits. Magnification about × 30.
controls only a few islets were ever observed in the alloxanized pancreas. Beta granules were not easily discernible and vacuoles were seen in the islet cells (compare Figs. 14 and 15). The pancreas from the second set of alloxanized animals was conspicuously larger and more dense than those of the controls. Cells of the exocrine part of the gland were normal and heavily packed with zymogen granules. The most striking feature was the extensive hyperplasia observed in the islets. The islets were conspicuously larger in size and were found to extend the whole width of the gland (Fig. 16). This hyperplastic activity of the islets, which was seen in the second set of alloxanized animals, suggests that the islets had recuperated from the alloxan damage and the animals recovered from alloxan diabetes (urine-sugar not detected after 25 days following amputation).

**DISCUSSION**

In these experiments, the effects of insulin insufficiency are primarily manifested in: (1) the failure of the blastema to accumulate a sufficient population of cells in the regeneration area; (2) reduced cartilage formation in the limb and tail regenerates; and (3) delayed growth and differentiation of the fins and spinal cord in the tail regenerates.

Some of the partially depancreatized animals maintained normal health, recovered from glucosuria, ate well and regenerated normal limbs and tails. Paralleling these results was the fact that the pancreas regenerated in these animals, whereas the cauterized pancreas of other animals exhibited extensive atrophy of the entire gland. These latter animals lost weight gradually, ate poorly and exhibited stunted, abnormally small regenerates. The factors responsible for regeneration of the cauterized pancreas in some cases and atrophy in others are not clear. Nevertheless, animals with an atrophied pancreas consistently produced abnormal regenerates, whereas animals that regenerated the pancreas also regenerated normal limbs and tails. This lends support to the possible existence of a direct relationship between the functional state of the pancreas and limb and tail regeneration in newts.

In addition to partial pancreatectomy, alloxanization was utilized as an
Fig. 17. Hormone interactions in appendage regeneration in adult *Diemictylus*. 

---, Established relationships; ---, present investigation; -----, possible additional pathways involving insulin.

Additional approach to study the effects of insulin insufficiency on regeneration processes. Alloxan selectively causes lesions in the beta cells, thereby leading to diabetes mellitus in mammals. Lukens (1948) suggested that the action of alloxan on the islets of the pancreas is direct and the resultant pathological changes are the consequence of an immediate injury to the beta cells. In the alloxanized newts, limb and tail regeneration was inhibited in most cases, and the effects were more drastic than in the partially pancreatectomized cases. However, some alloxanized newts showed recuperation of the pancreas from alloxanization. This was followed by the subsequent restoration of limb regeneration. The above evidence strongly indicates that a relationship exists between the functioning pancreas (more specifically insulin) and the regeneration of appendages in adult newts.

Corroborating these findings, are the recent in vitro experiments of Vethamany (1970) which demonstrated that the presence of insulin is essential in promoting
growth and differentiation of the blastema in culture. This *in vitro* work further indicates that the collective influence of the hormones (insulin, growth hormone, hydrocortisone and thyroxine) is greater than the effect of any of them individually.

In order to appreciate the role insulin might play and to gain more insight into the hormone control of regeneration, the inter-relationships between insulin and other hormones should be considered. Removal of a hormone from the circulation produces widespread derangements involving the whole metabolism of the animal which in turn will undoubtedly affect the production, the feedback mechanisms and the action of other hormones. Thus, the interactions and interdependence of hormones cannot be overemphasized. Figure 17 is a diagrammatic representation of possible pathways of hormone interactions in regeneration of appendages in adult newt. The dark, thick lines represent the current investigation which provide evidence to link insulin to regeneration. Previous work regarding the involvement of other hormones in regeneration are depicted by thin unbroken lines, whereas the dotted lines represent the speculations put forth by the authors (presently under investigation) as to the possible pathways of hormone interaction in the regeneration process.

According to the hypothesis of Schotte (1961) and co-workers the pituitary gland responds to the stress of amputation by releasing ACTH which, in turn, stimulates the production of the adrenocorticoid hormones. In addition, they suggest that the pituitary-adrenal synergism is probably active in the early phases of limb regeneration by preventing dermal invasion of the subapical space. They also propose that the pituitary-adrenal synergism is not active in the later differentiative and morphogenetic phases of regeneration.

Although Schotte and his group stressed the importance of an anterior pituitary-adrenal synergism in the regeneration processes of the limb, they have not excluded other possibilities. To quote Schotte & Bierman (1956): ‘in view of our ignorance of the pathways, particularly in amphibia, of hormonal interactions, one must remain hesitant in assuming as self-evident that the action of ACTH in regeneration can be explained by its stimulatory role upon the adrenals.’

It is known that corticosteroid-induced hyperglycemia causes morphological changes in the pancreatic islets; this is indicative of increased insulin output which restores blood sugar homeostasis in mammals (see Volk & Lazarus, 1963). Wurster & Miller (1959) have also shown, in the salamander (*Taricha torosa*), that hydrocortisone injections produce hyperglycemia and subsequent beta cell degranulation. Although the results of the present investigation do not preclude the possibility of a direct effect of ACTH-stimulated corticosteroids on regeneration, it is equally possible that corticosteroids also promote regeneration indirectly by stimulating the production of insulin.

In addition, ACTH has been shown to stimulate directly the release of insulin. At this point, mention should be made of Schotte’s experiments, in which he
obtained better results, in terms of animal survival, the rate of regeneration and the degree of morphogenesis, when he injected ACTH rather than cortisone acetate into hypophysectomized animals. Thus, it is possible that ACTH promotes limb regeneration in hypophysectomized animals by directly stimulating the production of insulin in addition to its effect on the adrenals.

In addition to the corticosteroids, other hormones which have been shown to support limb regeneration are also known to stimulate insulin production. For example, growth hormone injected into hypophysectomized adult newts promotes normal limb regeneration (Wilkerson, 1963). There are also data available which show that limb regeneration is dependent upon the presence of an intact thyroid gland (Schmidt, 1968; Schotte & Washburn, 1954). Connelly et al. (1968) have shown that prolactin, in combination with thyroxine, promotes limb regeneration in hypophysectomized animals.

There is an interesting correlation between the hormones mentioned above and the production of insulin. Growth hormone, prolactin, thyroxine, corticosteroids and ACTH are known to stimulate the production of insulin (Bern & Nicoll, 1968; Haist, 1959; Lazarus et al. 1962; Tepperman, 1965). It has also been shown that hypophysectomy in addition to inhibiting limb and tail regeneration in adult newts, results in the atrophy of the pancreas (Vethamany, 1970). Thus the possibility exists that all of these hormones promote limb and tail regeneration either directly and/or indirectly by stimulating the production of insulin (see Fig. 17). Since hypophysectomy or pancreatectomy brings about widespread derangements in the metabolism, it is highly unlikely that only one specific hormone is involved in regeneration. It is more likely that these hormones do not act in isolated pathways but interact interdependently through their effects on the metabolism.

When the source of insulin is removed from an animal, a marked sequence of events occurs which is intricately interconnected. These events involve not only carbohydrate metabolism, but also fat, protein and nucleic acid metabolism as well as electrolyte and water balance (see review by Tepperman, 1965). Regeneration processes in adult newts also involve carbohydrate, lipid, protein and nucleic acid syntheses (see review by Schmidt, 1968). In response to severe injury in mammals, large quantities of corticosteroids have been found in the circulation during the first 24 to 48 h and, as a result, hyperglycemia ensues (Schmidt, 1968). If this is true in urodeles, hyperglycemia is likely to stimulate insulin production. Schmidt (1968) has reported that glycogen and lipids are synthesized in the blastema cells prior to differentiation; these are probably utilized, later, as a substrate reservoir for metabolic events essential to the cells in regeneration. Insulin might play a role in this initial synthesis of glycogen and lipids.

Chalkley (1959) has shown that mitotic activity during regeneration reaches its maximum level around 25–31 days after amputation. Also studies concerning the inhibition of mitotic activity, using X-irradiation (Butler, 1933) and colchi-
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cine (Thornton, 1943) in regenerating systems, have shown that cell proliferation is a prerequisite for normal regeneration to ensue. There is also evidence for increased DNA-synthesizing activity of blastema cells during regeneration (Hay & Fischman, 1961). Insulin insufficiency, in the current experiments, resulted in a reduced blastema cell population and extremely retarded regeneration. Further, the addition of insulin to cultures enhanced cell proliferation (Vethamany, 1970). Since the production of new blastema cells must surely involve DNA synthesis one can invoke a DNA synthesis-stimulating role to insulin. Similar enhancement of DNA synthesis was also reported in the alveolar epithelial cells of mammary gland tissue in vitro when insulin was added to the medium (Stockdale, Juergens & Topper, 1966).

Schmidt (1968) has shown, through quantitative estimations of total nitrogen in regenerating forelimbs (D. viridescens), that there is active protein synthesis during the initiation as well as the differentiation phases of regeneration. Burnet & Liversage (1964) and Liversage & Colley (1965) obtained retarded and abnormal limb regeneration when they used chloramphenicol and puromycin to inhibit protein synthesis. Since insulin is known to increase the protein synthetic capacity in diabetic rats (Wool et al. 1968), it is possible that insulin is involved in protein synthesis during regeneration. Insulin is also known to increase RNA synthesis (Steiner & King, 1966; Wool et al. 1968) and may well exert an influence on RNA synthesis in limb regeneration. Future work along these lines will elucidate the exact role played by insulin in the metabolic processes involved in regeneration.

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