In vitro studies of the influence of hormones on tail regeneration in adult Diemictylus viridescens

By SWANI VETHAMANY-GLOBUS and RICHARD A. LIVERSAGE

From the Ramsay Wright Zoological Laboratories, University of Toronto

SUMMARY

A total of 260 tail blastemata (13 to 15-day regenerates) of adult newts was cultured, with 170 controls, in medium no. 1760 (modified Parker's Medium, CMRL-1415) for 98 h at 20 ± 0.5 °C. Various hormones, related to the regeneration process, were added individually and in combinations. If hormones were not included in the culture medium, blastema growth and differentiation did not ensue. The results of these in vitro experiments further showed that the presence of insulin was essential in the maintenance and promotion of growth and differentiation of the explanted blastemata. In addition, thyroxine also promoted a limited amount of growth and differentiation of the blastema and maintained a normal epidermis in culture. Furthermore, advanced cartilage differentiation and maximum growth of the blastemata were observed in cultures treated with combinations of insulin, growth hormone and hydrocortisone; insulin, growth hormone and thyroxine or all four of these hormones. The evidence presented indicates that the collective influence of the hormones (insulin, growth hormone, hydrocortisone and thyroxine) is greater than the effect of any of them individually.

The action of insulin on the tissues and the metabolism of vertebrates is discussed and related to the regeneration process. The possible involvement of a multiple hormone action (insulin, thyroxine, hydrocortisone and growth hormone) in regeneration is discussed.

INTRODUCTION

The available data on the endocrine involvement in regeneration of appendages in urodele amphibians has been reviewed by several workers in this field (see Schotte, 1961; Rose, 1964; Schmidt, 1968; Thornton, 1968). Previous workers stressed the role of ACTH-stimulated adrenocorticosteroids, thyroxine, growth hormone and prolactin in regenerating limbs (see Schotté, 1961; Wilkerson, 1963; Connelly, Tassava & Thornton, 1968; Schmidt, 1968). Recent work (Vethamany, 1970) has revealed an insulin involvement in the regeneration process.

The in vivo experiments mentioned above involved one or more of the

1 Author's address: c/o Dr M. Globus, Department of Biology, University of Waterloo, Waterloo, Ontario, Canada.
following procedures: (1) removal of a gland; (2) hormone replacement therapy; or (3) administration of chemical inhibitors of hormone production. Removal of one or more of the hormones from the circulation produces derangements in the metabolism which, in turn, affect the production and action of other hormones, making it difficult to obtain clear-cut answers to the questions posed. Thus, the data available from such in vivo experimentation are, by themselves, inconclusive. However, an in vitro system not only provides the advantage of isolating the regenerating systems from the direct effects of the deranged metabolic conditions, but also makes it feasible to study the direct response and the hormone sensitivity of the regenerate to various combinations of hormones during the different stages of regeneration. Recent studies by Vethamany (1970) have shown that following hypophysectomy, tail regeneration in the adult newt is inhibited. In the current experiments adult urodele tail blastemata were cultured in an attempt to study the effects of hormones on the growth and differentiation phases of regeneration.

Globus (1970), in his study of innervated larval tail and adult limb blastemata, has demonstrated that nerve tissue is essential for the growth and differentiation of the regeneration blastema in vitro. Tail blastemata, unlike the limbs, contain a regenerating spinal cord which provides the blastema explant with a ‘built-in nerve source’. In this way, the nerve influence, which is essential for normal regeneration, remains constant throughout the experiments. Thus, the response of the blastema to various hormones added to the culture medium can be determined with a minimum number of variables.

MATERIALS AND METHODS

Adult newts {Diemictylus viridescens} used in this investigation were obtained from central Massachusetts, U.S.A. They were kept in dechlorinated tap water at 20 ± 1 °C and fed lean, ground beef twice weekly. Animals were allowed a 2-week period to acclimatize to laboratory conditions prior to experimentation. Medium-sized animals of both sexes, weighing between 1-5 and 2 g, were used in all experiments.

The culture medium. In these experiments, CMRL 1415 culture medium (Healy & Parker, 1966) was modified to amphibian salt concentrations of 225 ± 5 m-osmoles and contained 0-5 g/l sodium bicarbonate. This medium was further supplemented with 4 % foetal calf serum, 100 i.u. penicillin G potassium per ml of medium and 50 μg streptomycin sulphate per ml of medium (both supplied by Connaught Medical Research Laboratories, University of Toronto).

The hormones. Ten mg of bovine crystalline insulin with a stated activity of 21-2 units/mg, was dissolved in 10-6 ml distilled water acidified with 0-03 ml of

---

1 The culture unit and the medium were developed in collaboration with Dr M. Globus (see Globus, 1970).
Regeneration in vitro and hormones

1N-HCl to yield a stock solution of 20 units per ml. The final concentration of insulin was 0.14 unit per ml of medium. Aqueous solutions of growth hormone (sheep somatotropin – 1·612 i.u. per mg) and hydrocortisone (compound F, cortisol) were added to the culture medium in two different concentrations (5.0 and 0·2 µg/ml of medium). An aqueous solution of L-thyroxine sodium (L-3,3', 5,5'-tetra-iodo-thyronine sodium salt pentahydrate) was added to the medium in concentrations of $1 \times 10^{-4}$ and $1 \times 10^{-8}$ mg per ml of medium. Growth hormone and thyroxine were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, hydrocortisone from British Drug Houses, Toronto, Ontario, and insulin was supplied by Dr R. G. Romans Laboratory, Connaught Medical Research Laboratories, Toronto, Ontario.

The culture unit. The culture unit consisted of a large Petri dish (90 x 25 mm) containing two smaller (35 x 10 mm) Petri dishes (Falcon plastics). One small dish, the culture chamber, contained a stainless steel grid which served to support the explant in the medium. The second dish containing distilled water, served to maintain a humid environment in the chamber.

Explanation. The animals were anesthetized in M.S. 222 (1 g: 1000 ml aqueous solution, Sandoz). The tails were then amputated, initially about one-third of the distance from the distal tip. Prior to culturing the tail blastemata, the animals were fasted for a week and given a daily change of water to reduce the bacterial contamination introduced into the water from gut contents. Since size is a limiting factor in regard to the proper diffusion of nutrients and gases in and out of an explanted tissue or organ (Moscona, Trowell & Wilmer, 1965), small-sized blastemata were selected. Fourteen- or 15-day-old tail regenerates, exhibiting small accumulation blastemata, were selected for explantation. The animals were washed in running water and anesthetized. Then the distal half of the tail (including the young blastema) was immersed in 1 % solution of chloramine (sodium hypochlorite, obtained from Ingram and Bell Ltd., Toronto). Exposure of the tails to this solution for a period of 90 sec resulted in 95–100 % sterility of the cultures (Globus, 1970). Following chloramine treatment, the tail blastemata were excised to include approximately 1 mm of the stump. The explants were then washed in the medium to remove all traces of chloramine solution. Each explant was treated separately during the sterilizing and rinsing procedures in order to prevent cross-contamination.

The explants were then transferred to the grids and the level of the culture medium was adjusted so that the explants were positioned at the air–medium interface. Complete immersion of tail blastemata was avoided in order to allow free access of the tissue to gas exchange. The cultures were incubated at 20 ± 0·5 °C, gassed daily with 5 % CO$_2$ in air and maintained at a pH of 7·2 to 7·4. The medium was replaced every 48 h and the explants were cultured for 4 days.

'Time zero' controls were fixed when the experimental blastemata were explanted and served as a reference for the stage of regeneration at which the
explantations were performed. Thus, all cultured blastemata had corresponding controls which were fixed at the beginning of the culture period. The maintenance, proliferation and further differentiation of all explants in vitro were assessed by comparing the cultured pieces with control regenerates. Pairs or trios of tail regenerates, that closely corresponded in their stage of regeneration, were carefully selected prior to the removal of the regenerates from the animals. One regenerate from each pair or trio was randomly chosen and fixed as a ‘time zero’ control, whereupon the other tail regenerate(s) of the matched pair or trio were explanted.

**Histology.** Upon termination of the experiments the cultured explants and time-zero controls were fixed in Bouins fluid, decalcified in 5 % Versene (EDTA) in 10 % formalin (Pearse, 1968), and serially sectioned at 8 μm. The sections were stained with alcian blue (specific for acid mucopolysaccharides; Pearse, 1968) and counterstained with hematoxylin and orange G–eosin.

**RESULTS**

The results were based on detailed observations of 214 tail blastema explants (13- to 15-day regenerates), cultured with and without hormones for 98 h, and 170 non-cultured controls. The results are summarized in Table 1 and illustrated as a histogram in Fig. 1.

By 15 days post-amputation (the time at which blastema explantations were made), the tail regenerates exhibited a dense population of blastema cells largely localized ventral to the regenerating spinal cord. However, at the time of explantation, neither blastema cell condensation nor cartilage differentiation was detected in histological preparations of the ‘time zero’ controls (Fig. 2). In studying the histological changes of the tail blastemata in organ culture in response to hormones, the following parameters were considered: (a) proliferation of blastema cells; (b) the extent of cartilage differentiation; (c) regeneration of the spinal cord; and (d) the condition of the epidermis.

A point that needs emphasis is that the events following the initial amputation of the tail, namely, wound healing, dedifferentiation and the accumulation of blastema cells, have already occurred in the tail regenerates in vivo, prior to explantation. The results from our in vitro experiments pertain only to the proliferation and tissue differentiation phases.

**Experimental controls (Series I, Table 1)**

When explants were cultured in medium without the addition of exogenous hormones, growth and differentiation were totally absent; there was a noticeable lack of blastema cells and cartilage condensation was not observed. In addition, growth was not observed in the spinal cord and the epidermis had undergone heavy molting (Figs. 2, 3).

It is conceivable that the foetal calf serum in the medium may contain trace
<table>
<thead>
<tr>
<th>Series no.</th>
<th>Hormone combinations</th>
<th>Hormone conc. per ml of medium</th>
<th>No. of explants controls (cultured for four days)</th>
<th>No. of explants included in results</th>
<th>Degree of cartilage differentiation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>No hormones</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>II</td>
<td>Insulin</td>
<td>5 µg</td>
<td>20</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>Hydrocortisone</td>
<td>0.2 µg</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>IV</td>
<td>Hydrocortisone</td>
<td>5 µg</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>V</td>
<td>Growth hormone</td>
<td>0.2 µg</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>VI</td>
<td>Growth hormone</td>
<td>5 µg</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>VII</td>
<td>L-Thyroxine</td>
<td>10⁻⁸ mg</td>
<td>20</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>VIII</td>
<td>L-Thyroxine</td>
<td>10⁻⁴ mg</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>IX</td>
<td>Insulin</td>
<td>5 µg</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>X</td>
<td>Growth hormone</td>
<td>0.2 µg</td>
<td>0.2 µg</td>
<td>0.2 µg</td>
<td>L-Thyroxine 10⁻⁸ mg</td>
</tr>
<tr>
<td>XI</td>
<td>Insulin</td>
<td>5 µg</td>
<td>20</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>XII</td>
<td>Insulin</td>
<td>5 µg</td>
<td>20</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>XIII</td>
<td>Growth hormone</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>Hydrocortisone 5 µg</td>
</tr>
<tr>
<td>XIV</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>0.2 µg</td>
<td>L-Thyroxine 10⁻⁴ mg</td>
</tr>
<tr>
<td>XV</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>L-Thyroxine 10⁻⁸ mg</td>
</tr>
<tr>
<td>XVI</td>
<td>Insulin</td>
<td>5 µg</td>
<td>0.2 µg</td>
<td>0.2 µg</td>
<td>L-Thyroxine 10⁻⁴ mg</td>
</tr>
<tr>
<td>XVII</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>Hydrocortisone 5 µg</td>
</tr>
<tr>
<td>XVIII</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>L-Thyroxine 10⁻⁴ mg</td>
</tr>
<tr>
<td>XIX</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>L-Thyroxine 10⁻⁸ mg</td>
</tr>
<tr>
<td>XX</td>
<td>Insulin</td>
<td>5 µg</td>
<td>0.2 µg</td>
<td>0.2 µg</td>
<td>L-Thyroxine 10⁻⁴ mg</td>
</tr>
<tr>
<td>XXI</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>XXII</td>
<td>Insulin</td>
<td>5 µg</td>
<td>5 µg</td>
<td>5 µg</td>
<td>Growth hormone</td>
</tr>
</tbody>
</table>

* Cartilage differentiation: 0 = none; + = cartilage cell alignment (no matrix); ++ = beginning cartilage differentiation; +++ = moderate cartilage differentiation; ++++ = advanced cartilage differentiation.
amounts of hormones. However, it appears from our results that the medium alone, without the addition of exogenous hormones, is insufficient to promote growth and differentiation of the adult newt tail blastema.

**Experimental series – single hormones (Series II–VIII, Table 1)**

**Insulin.** Explants cultured with insulin showed an increase in the overall size of the blastema and exhibited cartilage formation just distal to the stump bone (Figs. 4, 5). Also blastema cells aggregated loosely, distal to the newly differentiated cartilage and ventral to the spinal cord. Thus a thin strand of cells was formed in a pattern unlike that characteristic of procartilage cell alignment; the cells were oriented with their long axes parallel to the longitudinal plane of the tail instead of the usual perpendicular cell alignment. Mitotic figures were frequently observed in the spinal cord of the stump, as well as in the regeneration area. The regenerated spinal cord extended close to the inner border of the epidermis. The latter maintained a normal appearance with moderate molting activity. Compared to untreated explants, an increase in fin growth was observed in the insulin treated explants.

**Hydrocortisone.** Procartilage cell condensation and cartilage differentiation
were absent in all explants cultured with hydrocortisone only (Fig. 6). These blastemata exhibited a scarcity of cells and further growth of the regenerating spinal cord or fin was not observed. The epidermis became thickened and squamous and was accompanied by heavy molting. The results obtained with hydrocortisone treatment were comparable to those in which hormones were omitted from the medium.

**Growth hormone.** Explants cultured with growth hormone showed a small amount of cartilage differentiation. The regeneration area was largely made up of connective tissue cells and fibers and was characterized by a marked reduction in the density of blastema cells compared to time zero controls (Figs. 7, 8). The spinal cord, however, exhibited continued growth in culture.

**Thyroxine.** Procartilage cell condensation and new cartilage differentiation were readily detectable in the thyroxine-treated explants especially at the higher concentration. However, the cartilage differentiation was localized in the proximal part of the regenerate and all cells distal to this differentiated area remained loosely arranged to form a thin strand of cells. These results were very similar to those obtained in the insulin-treated explants. However, the higher concentration of thyroxine promoted better cartilage differentiation than insulin alone; the latter favored cell proliferation. The spinal cord was densely packed with cells undergoing mitosis, and extending distally to the inner border of the epidermis. The latter was maintained with minimum molting in the presence of L-thyroxine in the medium.

**Experimental series – combinations of hormones**

The results which follow suggest that combinations of growth hormone, hydrocortisone and thyroxine with insulin provide the optimum environmental conditions to promote normal growth and differentiation of the tail blastemata in culture.

**Insulin, growth hormone and hydrocortisone** (Series IX, Table 1). A combination of insulin, growth hormone (0-2 µg/ml) and hydrocortisone (0-2 µg/ml) resulted in a marked increase in the growth of the explanted regenerates. Advanced cartilage differentiation was also observed at the proximal part of the regenerate, tapering off distally where cell proliferation predominated (Fig. 9). Mitotic figures were seen in the spinal cord which had regenerated extensively, to the distal tip of the blastema.

**Insulin, growth hormone and thyroxine.** When a combination of insulin, growth hormone (0-2 µg/ml) and thyroxine (10⁻⁸ mg/ml) was used (series XIV) advanced, somewhat excessive cartilage differentiation occurred but only limited blastema cell proliferation was observed (Fig. 10). This combination did, however, support mitosis and growth of the spinal cord. In addition, the epidermis was maintained in a normal condition with minimum molting activity when thyroxine was present in the medium.

When higher concentrations of thyroxine (1 x 10⁻⁴ mg/ml) and growth hor-
Regeneration in vitro and hormones

mone (5 μg/ml) were used in combination with insulin the results were less pronounced (series XV). Although procartilage cells aggregated ventral to the spinal cord forming a thin strand of cells, cartilage differentiation was stimulated to a lesser degree (Fig. 11).

Insulin, growth hormone, thyroxine and hydrocortisone (Series IX, Table I). Culture medium containing these four hormones promoted: cartilage differentiation; regeneration of the spinal cord; and a general increase in the growth of the regenerate in a manner comparable to that observed with the above-mentioned three hormone combinations.

Growth hormone, hydrocortisone and thyroxine. However, when insulin was excluded from the hormone combinations and only growth hormone, hydrocortisone and thyroxine were added to the medium, the condition of the explants was altered considerably. There was a marked decrease in the blastema cell population and cartilage differentiation was not initiated. From these results, it is apparent that the presence of insulin is essential for growth and differentiation of the cultured tail regenerates.

Insulin plus growth hormone. A combination of insulin and growth hormone resulted in a conspicuous sparsity of cells in the blastema region; neither cartilage differentiation nor procartilage cell condensation was observed. In these explants the spinal cord exhibited few mitotic figures and the regeneration of the cord was retarded.

Insulin plus hydrocortisone; insulin plus thyroxine. However, if insulin and hydrocortisone or insulin and thyroxine were added to the medium, cartilage differentiation was evident but it was less extensive than that observed with the three-hormone combinations with insulin.

Growth hormone plus hydrocortisone. When growth hormone and hydro-

Figures 2–5

The arrows indicate the level of amputation

Fig. 2. Mid-sagittal section through a 15-day tail blastema of adult newt (fixed at the time of explantation) which served as ‘time-zero’ control for explants cultured without the addition of hormones to the medium. There is an accumulation of blastema cells (b), ventral to the spinal cord (s). Cartilage differentiation is not detectable. Magnification about ×140.

Fig. 3. Mid-sagittal section through a 15-day tail blastema explant from adult newt which was cultured for 4 days with no hormones added to the medium. Note the sparse cell population (b) in the blastema region; heavy molting of the epidermis; no growth and differentiation of either cartilage or spinal cord. Magnification about ×120.

Fig. 4. Mid-sagittal section through a 15-day tail blastema of adult newt which served as ‘time-zero’ control for the explant cultured with insulin in the medium. Magnification about ×100.

Fig. 5. Similar section through a 15-day tail blastema explant from adult newt which was cultured for four days with 5 μg/ml of insulin added to the medium. Note the cartilage differentiation (c) posterior to the level of amputation. v, enlarged blood sinuses. Magnification about ×100.
Fig. 6. Mid-sagittal section through the tail blastema explant from adult newt which was cultured for 4 days with 0.2 μg/ml hydrocortisone (Hydro) added to the medium. Note that the population of blastema cells is very sparse (b); heavy molting of the epidermis (e). No spinal cord regeneration; no cartilage differentiation. Vesicles (v) are seen in the blastema region. Magnification about × 100.

Fig. 7. Mid-sagittal section through a 15-day tail blastema of adult newt which served as 'time-zero' control for explants cultured with growth hormone in the medium. Magnification about × 150.

Fig. 8. Similar section through a 15-day tail blastema explant from adult newt which was cultured for 4 days with 0.2 μg/ml of GH added to the medium. A small amount of cartilage condensation (c) can be seen at the proximal end of the blastema. Large spaces (v) are seen in the blastema. Magnification about × 120.
cortisone were added to the cultures and insulin was excluded, the explants showed no cartilage differentiation and a sparse cell population in the blastema region. These results indicate that growth hormone and hydrocortisone in combinations do not promote growth and differentiation of the tail blastemata in culture. Whereas, the addition of insulin to this two-hormone combination, as seen above, promotes extensive growth and differentiation of the explants.

**DISCUSSION**

The results of the current experiments show that the presence of insulin is essential for the promotion of growth and differentiation of tail blastema in culture, thus corroborating the recent findings from the in vivo experiments (Vethamany, 1970) that demonstrated that insulin is involved in limb and tail regeneration. Furthermore, the evidence indicates that the collective influence of the hormones (insulin, growth hormone, hydrocortisone and thyroxine) is greater than the effect of any individually.

If hormones were not included in the culture medium, blastema growth and differentiation did not ensue. Thus, our results show that hormones are required for proliferation and tissue differentiation in vitro. Stocum (1968) observed the continuation of cartilage differentiation in limb blastemata of the larval Ambystoma maculatum in culture without the addition of exogenous hormones to the medium. However, this was probably due to the fact that larval limb regeneration (at least in vivo) is not dependent upon the presence of pituitary gland (Liversage, 1967); the dependency of newt limb regeneration on the pituitary gland does not develop until the red eft stage (Schotte & Dronin, 1965). However, the possibility exists that trace amounts of some hormones were present in the medium Stocum used which contained 2% beef embryo extract and 10% foetal calf serum. This possibility gains support from the experiments of Globus (1970). Although the role of hormones in regeneration was not his objective, Globus found in his in vitro studies of the influence of nerves in regeneration that, in addition to the nerves, the presence of insulin in the culture medium was essential for promotion of growth and differentiation of adult limb and larval tail blastemata in vitro.

An explanation of the precise mode of action of insulin in regenerating systems is beyond the scope of this paper. However, speculations can be made on the basis of known insulin action in other systems. When the source of insulin is removed from the animal, a marked sequence of events occurs which is intricately interconnected involving not merely carbohydrate metabolism, but also fat, protein and nucleic acid metabolism as well as electrolyte and water balance (see review by Tepperman, 1965). Regeneration processes 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 are secreted during the first 24–48 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 of the adult newt prior to differentiation, and are probably utilized, later, as a substrate reservoir for metabolic events essential to the cells in regeneration. Presumably, insulin plays a role in this initial synthesis of glycogen and lipids.

There is a significant amount of DNA synthesis during regeneration (Hay & Fischman, 1961). Chalkley (1954, 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 colchicine (Thornton, 1943) in regenerating systems, have shown that cell proliferation is a prerequisite for normal regeneration to ensue.

In the current experiments, absence of insulin resulted in a reduced blastema cell population and the addition of insulin to cultures enhanced cell proliferation. Since the production of new cells involves DNA synthesis presumably insulin is involved in DNA synthesis. 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) used chloramphenicol and puromycin respectively to inhibit protein synthesis which resulted in retarded and abnormal limb regeneration. Since insulin is known to increase the protein synthetic capacity in diabetic rats (Wool et al. 1968), it is possible that insulin is also involved in protein synthesis during regeneration. Insulin is also known to

Figures 9-11

Fig. 9. Mid-sagittal section through a 15-day tail blastema explant from adult newt which was cultured for 4 days with 0.2 μg GH, 0.2 μg Hydro and 5 μg of insulin added per ml of the medium. Compare this to 'time-zero' control (Fig. 7). Note the advanced cartilage differentiation (c); extensive growth of the blastema and the regenerated spinal cord (s). Cell proliferation (b) continues at the distal tip. v, enlarged blood sinuses. Magnification about ×100.

Fig. 10. Mid-sagittal section through a 15-day tail blastema explant of adult newt which was cultured for 4 days with 5 μg/ml of insulin, 0.2 μg/ml of GH and 10⁻⁶ mg/ml of Thy added to the medium. Note the extensive cartilage differentiation (c) ventral to the regenerated spinal cord (s). e, Epidermis is normal. Compare it to 'time-zero' control (Fig. 7). Magnification about ×120.

Fig. 11. Mid-sagittal section through a 15-day tail blastema explant of adult newt which was cultured for 4 days with 5 μg/ml of insulin, 5 μg/ml of GH and 10⁻⁴ mg/ml of Thy (higher concentration of hormones) added to the medium. Note the increase in growth in the blastema region. Cartilage differentiation (c) is limited, instead the blastema cells (b) form a thin strand of cells, ventral to the regenerated spinal cord. The epidermis (e) is normal. Magnification about ×110.
increase RNA synthesis in mammals (Steiner & King, 1966; Wool et al. 1968); and may also exert an influence on RNA synthesis in newt regeneration. Future work along these lines will elucidate the exact role played by insulin in the metabolic processes involved in regeneration.

While the presence of insulin in the medium enhances cell proliferation in our explants, thyroxine favors cartilage differentiation. Similar experiments by Fell & Mellanby (1955) have shown that thyroxine accelerates the normal hypertrophy of cartilage cells in cultured chick long bone rudiments. The effects of thyroxine on cartilage differentiation in our explants are consistent with similar effects of the hormone observed by Richardson (1940, 1945). She reported that the injection of thyroid hormone enhanced the growth-promoting effects of anterior pituitary gland extracts, particularly in the maturation of the skeleton in both hypophysectomized and thyroidectomized newts. Also blastema formation is accelerated but differentiation is inhibited in Rana pipiens tadpoles with a thyroxine insufficiency (Peadon, 1953).

When growth hormone alone was added to the medium, the growth and differentiation of our blastema explants were extremely limited. Leslie (1952) also found that growth hormone alone, in concentrations of 0.1–0.5 mg per ml of medium, inhibited cell growth in chick heart fibroblasts by 25%, while the cell composition remained unchanged. In the present investigation, when growth hormone was used in combination with insulin, growth and differentiation were not observed. Overell, Condon & Petrov (1960) found that growth hormone and insulin were antagonists; they suggested that growth hormone selectively inhibits the glucokinase reaction, whereas insulin promotes the reaction.

Hydrocortisone alone did not promote growth and differentiation of our explants. It has been previously reported that corticosteroids inhibit the proliferation of cells, in particular fibroblasts isolated from the mouse embryo heart (Von Haam & Cappel, 1940). In addition, retardation of growth was observed in treated chick embryonic limb-bone rudiments (Buno & Goyena, 1955; Sobel & Freund, 1958; Fell & Thomas, 1961). Whitehouse & Lash (1961), while studying the action of hydrocortisone on chick somite chondrogenesis in vitro, found that the amount of cartilage in the treated somites was reduced severely and suggested that the disturbance in cartilage formation was brought about by the inhibition of chondroitin sulphonation. However, Leslie (1952) showed that if cortisone was applied in combination with growth hormone, it counteracted the growth inhibitory effects of cortisone and produced a stimulation of growth instead. He also showed that cortisone increased the growth-promoting effect of insulin. In the present work, while the combination of insulin and hydrocortisone promoted growth and cartilage differentiation in the explants, hydrocortisone in combination with growth hormone failed to induce a growth response.

Our results support the existence of a multiple hormone control of growth and differentiation in adult newt tail blastemata. Although insulin was found
to be essential for the maintenance, growth and differentiation of tail blastemata in culture, thyroxine also evoked a limited amount of growth and cartilage differentiation in culture. Combinations of two hormones, namely insulin and hydrocortisone or insulin and thyroxine, increased growth and promoted better differentiation than either insulin or thyroxine added individually to the culture medium. However, maximum growth and advanced cartilage differentiation were obtained when all four hormones or combinations of either insulin, growth hormone and hydrocortisone or insulin, growth hormone and thyroxine were added to the cultures.

The existence of a multiple hormone action has been reported in other tissues. When isolated tadpole (*Rana catesbiana*) muscle was treated with either insulin or thyroxine, the incorporation of \(^{3}H\)leucine nearly doubled. But, the greatest rates of hormone stimulated incorporation of \(^{3}H\)leucine, relative to the control muscles receiving no hormone treatment, were observed in muscles exposed briefly to a combination of thyroxine, insulin and growth hormone (Hosick, Strohman & Bern, 1969).

Leslie (1952) showed that cortisone, growth hormone and insulin in combination resulted in a marked stimulation of growth in chick heart explants and total synthesis of lipid phosphorus, DNA phosphorus and RNA phosphorus increased by 50–100%. Stockdale *et al.* (1966) proposed that insulin-mediated DNA synthesis and mitosis occurs in the presence of hydrocortisone to promote the induction of casein synthesis by prolactin in mouse mammary gland *in vitro*. They proposed that cell division may make a cell especially susceptible to environmental factors (hormones) capable of eliciting changes in cell function.

The probability of a multiple hormone requirement existing in limb and tail regeneration can be appreciated when one considers the metabolic events leading to regeneration. During the early stages of regeneration, proteolysis occurs, followed by an initial build-up of glycogen and lipids. Later, these are utilized and there is a definite increase in protein synthesis in the differentiation phase of regeneration, preceded by an increase in DNA and RNA synthesis (Schmidt, 1968). Insulin and other hormones related to regeneration processes may influence any one of these major syntheses. It is also likely that these hormones do not act in isolated pathways but interact interdependently through their effects on the metabolism.

This work shows that it is not any one hormone that single-handedly promotes regeneration of the limb and tail in adult urodeles, but rather that a multiple hormone control exists. Presumably, insulin, growth hormone, hydrocortisone, thyroxine and prolactin are present in the blood vascular system and are available to the blastema cells throughout regeneration. Then the responding tissue, namely the blastema cells and the pre- and post-blastemal regenerating system, probably varies in its response to the hormones according to the metabolic events predominant at a particular phase in regeneration. Or, possibly, the level of these hormones may fluctuate during regeneration if the regenerating
system exerts a pronounced demand on the general metabolism. On the basis of the available data, it would seem unlikely that hormones provide a specific factor for regeneration; they probably contribute, at least in part, to the environmental milieu that supports growth and differentiation of the blastema cells of limb and tail regenerates in *D. viridescens*. The hormones may act at the level of the cell surface, cytoplasm and/or gene control.

We wish to express our appreciation to Dr M. Globus for his interest and valuable discussions throughout this work and his assistance in the preparation of this manuscript. This paper was prepared from a portion of a Ph.D. thesis submitted (by S. V. G.) to the Department of Zoology, School of Graduate Studies, University of Toronto. This investigation was supported by grants from the Province of Ontario (to S. V. G.) and National Research Council of Canada (to R. A. L.).

**REFERENCES**


Regeneration in vitro and hormones


(Received 9 February 1973)