

THE ROLE OF PITUITARY HORMONES
IN LIMB REGENERATION AND
SURVIVAL OF THE ADULT NEWT,
NOTOPHTHALMUS VIRIDESCENS

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This is to certify that the

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THE ROLE OF PITUITARY HORMONES IN LIMB

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ADULT NEWT, Notophthalmus viridescens.

presented by

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Ph.D. degree in ZOOLOGY

A handwritten signature in blue ink, appearing to read "Charles L. Brown".

Major professor

Date May 14, 1968







ABSTRACT

THE ROLE OF PITUITARY HORMONES IN LIMB REGENERATION AND SURVIVAL OF THE ADULT NEWT, *NOTOPHTHALMUS VIRIDESCENS*

By Roy A. Tassava

Following hypophysectomy, adult newts do not eat, fail to molt, and usually die within 4 weeks. Limb regeneration of hypophysectomized newts is retarded, abnormal, or does not occur at all. It is clear that pituitary hormones are essential to the normal health of the newt. The exact identity of these hormones is not known, nor is it known how these hormones affect limb regeneration.

Part 1 of this investigation was designed to determine whether the lipid quantity and enzyme activity of the newt adrenal tissue are dependent upon the presence of the pituitary gland. Adrenal lipid content and steroid dehydrogenase activity were ascertained by histochemical tests in normal, hypophysectomized, and hormone-treated hypophysectomized newts. Two weeks after hypophysectomy, with or without ACTH, growth hormone, or prolactin treatment, the adrenal tissue exhibited the same steroid dehydrogenase activity and lipid content as normal newts. ACTH was not effective in prolonging survival of hypophysectomized newts.



Part 2 of this investigation demonstrated that hypophysectomized newts which were fed daily for two weeks prior to hypophysectomy survived significantly longer and regenerated amputated limbs better than hypophysectomized newts which were fasted for two weeks prior to hypophysectomy.

Part 3 of this investigation was designed to determine whether the growth phase of the blastema was influenced by hypophysectomy. At 14 days after limb amputation newts were either hypophysectomized or sham-operated. By comparing statistically the areas of the longitudinal sections of the blastemas, it was found that the blastemas of the sham-operated newts were significantly larger, 8 days after the operation, than were the blastemas of the hypophysectomized newts.

In Part 4 of this investigation, to determine the identity of those pituitary hormones of the newt which are essential to normal limb regeneration and survival, newts were hypophysectomized and treated with various combinations of hormones. Other hypophysectomized newts were grafted with pituitaries from newts or from axolotls (Ambystoma mexicanum).

The results of this investigation led to the following conclusions. (1) Limb regeneration does not depend upon activation of the adrenal gland by ACTH released after the stress of amputation; ACTH and consequently the adrenal hormones, are not the limiting factors for limb regeneration

after hypophysectomy. (2) Survival and limb regeneration after hypophysectomy depend upon the nutritional state of the newt at the time of hypophysectomy; remaining small pituitary fragments exert little or no effect. (3) Hypophysectomy, performed after limb regeneration has progressed through the wound healing and dedifferentiation phases, causes a significant retardation in the growth of the blastema. (4) Prolactin and thyroxine, administered together, effectively prolonged the life of hypophysectomized adult newts, restored their appetite for food, and generally appeared to duplicate the effect of an ectopic pituitary graft. Furthermore, hypophysectomized adult newts given prolactin + thyroxine regenerated amputated limbs in a typical fashion. Thyroxine alone, thyroxine + ACTH, ACTH, or saline were not effective in restoring the health of hypophysectomized newts and were not effective in restoring normal limb regeneration ability.

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INTRODUCTION

Following hypophysectomy, the adult newt (Notophthalmus viridescens) ceases to shed its epidermis, stops eating, and usually dies within 4 weeks (Dent, 1966; Dent, 1967). Amputated limbs of hypophysectomized adult newts regenerate poorly, abnormally, or not at all (Richardson, 1945; Hall and Schotté, 1951). Various injected hormones have served as replacement therapy to alleviate the effects of hypophysectomy on regeneration. These include adrenal steroids and ACTH (Schotté and Bierman, 1954; Schotté and Chamberlain, 1955), a crude growth hormone (Antuitrin G) (Richardson, 1945) and purified growth hormone (Wilkerson, 1963). The ectopically transplanted adult newt pituitary is perhaps the most adequate replacement therapy since hypophysectomized adult newts with ectopic pituitaries not only regenerate amputated limbs but survive for many months (Dent, 1967; Schotté and Tallon, 1960).

Hall and Schotté (1951) observed a failure of adult newt limb regeneration in 72% of the cases (43 of 60 limbs) when hypophysectomy preceded amputation by 5 days, but it should be noted that limb regeneration was seen to be present in approximately 50% of those newts which survived for 20 or more days after limb amputation. When hypophysectomy was

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delayed for 3 or more days after limb amputation, the number of positive cases of regeneration increased as the interval between amputation and hypophysectomy was increased (Schotté¹ and Hall, 1952). Thus, when amputation preceded hypophysectomy by 3 to 7 days, only 22% of the amputated limbs failed to regenerate. Schotté¹ and Hall (1952) concluded from these results that the action of the pituitary was required for subtle events occurring during the wound healing phase of newt limb regeneration. The wound healing phase (0-7 days post-amputation) however, overlaps with the dedifferentiation phase (2-12 days post-amputation) and also with the growth phase, since DNA synthesis in injured stump tissues begins as early as the 4th day after amputation (Hay and Fischman, 1961). It is not clear, therefore, whether pituitary hormones are absolutely required for limb regeneration nor is it clear which stage(s) of limb regeneration are influenced by these hormones.

The hypophysectomized adult newt will survive indefinitely, molt regularly and regenerate amputated limbs if the excised pituitary is implanted autoplastically to an ectopic site (Dent, 1967; Schotté¹ and Tallon, 1960). Ectopic pituitaries from the red eft (land phase) will support regeneration in hypophysectomized adult newts but the pituitary of the brown eft (newly metamorphosed land phase) will not (Schotté¹ and Droin, 1965). Both aquatic adult and red eft stages require the adult-type pituitary for limb regeneration

but the brown eft does not. The brown eft is thus comparable to the larval urodele which can regenerate limbs in the total absence of pituitary hormones (Schotte¹, 1961; Liversage, 1967). It has been suggested by Schotte¹ (1961) that dependence on pituitary hormones is imposed on regenerating limb tissues at the time of maturation of the pituitary during metamorphosis. However, ectopic adult pituitary grafts and injections of ACTH do not modify the regenerative response of limbs of Ambystoma larvae either during treatment or after withdrawal of the pituitary therapy, indicating that larval limb tissues are refractory to pituitary hormones whether these are in excess or absent (Tassava, et al., 1968).

The ectopic pituitary gland of the adult newt has been shown to produce thyroid stimulating hormone (TSH) in near normal amounts (Dent, 1966). Prolactin is also produced by both the adult newt pituitary and the red eft pituitary (Chadwick, 1941; Reinke and Chadwick, 1939; Masur, 1962). This conclusion is based on evidence from water drive studies with the red eft. The larval newt undergoes a primary metamorphosis to a land form called an eft which lives from 1 to 5 years on land before migrating to water as an aquatic, reproductively mature adult. The "water drive" of the eft has been shown to be induced by prolactin and this migratory behavior and the structural and physiological changes involved have been termed "second metamorphosis" (Grant, 1961). The induced water drive of the eft has been recommended as an



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adequate assay for prolactin(Grant, 1959) and has been used to demonstrate the presence of prolactin in pituitary tissue of Bufo, Fundulus, Cyprinus and Natrix(Chadwick, 1941; Grant, 1961) and adult newts(Reinke and Chadwick, 1939). Antuitrin G will induce water drive(Chadwick, 1940) but LH, ACTH, posterior pituitary tissue, TSH, and Antuitrin S have no water drive activity(Grant and Grant, 1958). The ectopically transplanted eft pituitary will induce a water drive(Masur, 1962) suggesting that in the eft prolactin secretion is under negative control by the hypothalamus (Grant, 1961). Ultrastructural studies of the adult pituitary also suggest that TSH and prolactin are produced by the normal as well as the ectopic gland(Dent and Gupta, 1967). Prolactin will prevent primary metamorphosis of the newt larva while exogenous thyroxine will induce a "land drive" (return to land) in adult aquatic newts(Grant and Cooper, 1965). Thus, there is abundant evidence that prolactin and TSH are produced by the newt pituitary and that these hormones may somehow interact in influencing newt behavior.

Richardson(1945) found that Antuitrin G (a crude growth hormone preparation) supported limb regeneration in hypophysectomized newts but not as well as Antuitrin G when combined with thyroxine. The antuitrin G probably contained prolactin (it induces water drive in the eft, Chadwick, 1940) and also TSH(see Wilkerson, 1963). By injecting growth hormone(NIH), Wilkerson(1963) obtained excellent limb regeneration in



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hypophysectomized adult newts even when injections were begun 14 days after amputation and hypophysectomy. The growth hormone used by Wilkerson contained prolactin as a contaminant in an amount equivalent to that used by Berman et al. (1964) and Bern et al. (1968) which stimulated growth and inhibited metamorphosis in the frog tadpole. The prolactin contamination was also comparable to the water drive dose used by Grant and Grant(1958). That prolactin may play a role in newt limb regeneration is further suggested by the findings of Niwelinski(1958) and Waterman(1965) that limb regeneration is enhanced by prolactin injections into intact newts and the report of Chadwick and Jackson(1948) that prolactin increases mitotic activity in newt epidermis.

Although available endocrinological evidence indicates that prolactin and TSH are produced by the adult newt pituitary and may be involved in limb regeneration and survival, there are also reports that ACTH(adrenocorticotrophic hormone) may be important for newt limb regeneration(Schotté¹ and Lindberg, 1954; Schotté¹ and Chamberlain, 1955; Schotté¹ and Bierman, 1956; Schotté¹ and Wilbur, 1958). Indeed, Schotté¹(1961) has suggested that in typical limb regeneration the pituitary is stimulated to produce ACTH by the stress of amputation, and that the ACTH then activates steroid production by the adrenal gland. The adrenal steroids are thought to be required only during the first 6 days of regeneration and are essential for proper wound healing.

Unfortunately, very little is known about the relationship between the pituitary and the adrenal tissue in urodele amphibians (Gottfried, 1964; Gorbman, 1964). However, a number of reports cast some doubt on the possibility that a stress mechanism is involved in adult newt limb regeneration. A pituitary adrenal axis apparently exists in the bullfrog, Rana catesbiana (Piper and DeRoos, 1967), although Hanke and Weber (1966) found that the adrenal steroid dehydrogenase activity of 14 day hypophysectomized Rana temporaria was comparable to that of intact frogs. The adrenal tissue of the newt Taricha torosus, which is composed of a cord of cells corresponding to the mammalian zona fasciculata, still shows secretory activity 2 months after hypophysectomy (Wurster and Miller, 1960). These authors suggest that the urodele amphibian adrenal shows less dependence on the pituitary than does the adrenal of anuran amphibians.

An hypophysectomized adult newt with an ectopic pituitary gland will regenerate amputated limbs in a normal fashion (Schotté and Tallon, 1960), yet in other vertebrates experiments have shown that the ectopically transplanted pituitary does not respond well to stress. Ectopic pituitaries of the toad, Bufo, secrete little, if any, ACTH and hypophysectomized toads must be supplied with exogenous ACTH to survive (Van Dongen et al., 1966). Mangile et al. (1966) point out that one ectopic pituitary does not maintain adrenal weight in hypophysectomized mammals nor will stress cause an increase

in corticosterone secretion. When 5 ectopic pituitaries are present in the hypophysectomized rat, there is an elevation of plasma corticosterone secretion after stress, but only up to 1/3 that of normal rats(Purnes and Sirett, 1967).

It is known in mammals that the response to stress is nearly immediate, occurring within seconds of the onset of stimulation and, furthermore, that denervation of a limb or ablation of the spinal cord prevents the ACTH response to stimuli applied to the denervated territory(Fortier, 1966). However, when the spinal cord of adult newts is ablated, amputated hind limbs and tail nevertheless regenerate(Liversage, 1959). Furthermore, when limb and spinal cord segments are transplanted to the dorsal fin, followed by spinal cord ablation of the host and later amputation of the limb, normal regeneration follows. Thus neural stimulation of the endocrine activities(especially the pituitary-adrenal synergism) is prevented(Liversage, 1959).

The present investigation was designed to determine

- 1) whether the lipid quantity and enzyme activity of the newt adrenal tissue are dependent upon the presence of the pituitary gland;
- 2) whether the presence of the pituitary is absolutely essential for limb regeneration to proceed;
- 3) whether the normal rate of blastemal growth requires pituitary hormones; and, finally,
- 4) whether prolactin and thyroxine, either alone or combined, may play a role in survival and limb regeneration of adult newts.

MATERIALS, METHODS AND RESULTS

The experiments designed to determine the role of pituitary hormones in limb regeneration and survival of adult newts are described separately below in Series I, II, III, and IV. Each experimental series is individually introduced, described, and the significance of the results are summarized.

SERIES I

HISTOCHEMICAL ANALYSIS OF THE ADRENAL TISSUE OF INTACT ADULT NEWTS, HYPOPHYSECTOMIZED ADULT NEWTS, AND HYPOPHYSECTOMIZED ADULT NEWTS TREATED WITH PROLACTIN, GROWTH HORMONE, AND ACTH.

Because the work of Liversage(1959), Wilkerson(1963), and others (see Tassava et al., 1968) have cast some doubt on the "stress" theory of newt limb regeneration proposed by Schotte(1961), this experimental series was designed 1) to determine whether the lipid content and enzyme activity of the newt adrenal tissue respond to hypophysectomy or pituitary hormone injections and 2) to determine whether ACTH will prolong the survival of hypophysectomized adult newts.



— 1 —

Materials and Methods. Adult newts, Notophthalmus viridescens, were purchased from Lewis Babbitt, Petersham, Mass., and were fasted for at least 2 weeks before the start of the experiments. Hypophysectomy was achieved by removing a portion of the sella turcica directly under the pituitary with the aid of fine-pointed watchmaker's forceps. The entire pituitary gland was removed by gentle suction with a fine glass pipette(Dent, 1966). The excised bone was then replaced. All hypophysectomized newts exhibited darkening of the skin due to improper molting(Dent, 1966). Although this dark, thickened nature of the skin may be an adequate indication of completeness of hypophysectomy(Dent, 1966; Myers et al., 1961), heads of those newts whose tissue was sampled for histochemical analysis were examined for pituitary fragments. Heads were prepared for staining by the method of Stone(1967). Sections (10 microns thick) of heads were stained by the PAS technique(Pearse, 1960).

The adrenal tissue of the urodele amphibian exists in small islets composed of cords of cells, partially embedded in the ventral surface of the kidneys lateral to the vena cava (Figure 1). Entire kidneys containing the interrenal tissue were removed from the newts and either frozen immediately (solid CO₂-acetone) for cryostat sectioning, or fixed for lipid staining (formol-calcium; Pearse, 1960) of paraffin embedded tissues.

Tissues were fixed at 14 days post-hypophysectomy.



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Paraffin embedded tissues were cut serially in cross section at 10 microns. Frozen tissues were cut serially in cross section at 8 microns and mounted on coverslips. Paraffin-embedded tissues were stained with Sudan Black B(Pearse, 1960). For sectioning on the cryostat, kidneys were first wrapped in ventral abdominal skin of the same newt and tissues from intact and hypophysectomized newts were placed on the same cover slip for incubation and/or staining. Staining reactions in adrenal, skin and kidney tissue could therefore be compared between hypophysectomized and intact newts on the same slide. Frozen sections were stained with Sudan Black B for 1 hour or used for histochemical determinations of steroid dehydrogenase activity(Levy et al., 1959). Dehydrogenation of dehydroepiandrosterone, a 3 α -hydroxysteroid, is demonstrable in all types of steroid-producing cells in the adrenal gland, ovary and testis, when sections are incubated in a phosphate buffer (pH 7.1-7.4) solution containing substrate, propylene glycol, DPN, nicotinamide and the tetrazolium salt, Nitro-BT. No activity towards this substrate is evident in liver or kidney(Levy et al., 1959).

To demonstrate steroid dehydrogenase (SDH) activity, frozen sections were first placed for 5 minutes in 0.1 M phosphate buffer, pH 7.1-7.4, at room temperature to remove endogenous substrates. They were then incubated in a Columbia jar containing the dehydroepiandrosterone medium (Table 1) at room temperature for periods of 2, 4, and 6



hours. After incubation, the sections were fixed for 30 minutes in a mixture containing 50% ethanol and 10% formalin, and mounted in glycerol gelatin. Steroid dehydrogenase activity was determined in newt adrenal tissue of 6 intact newts and 6 hypophysectomized newts. Mouse adrenal tissue and frog adrenal tissue (Rana pipiens) prepared for cryostat sectioning in the same way, served as a control to insure that all ingredients in the reaction mixture were functioning properly.

Sudan Black staining was done on adrenal tissue of 3 intact newts and 2-week hypophysectomized newts treated with saline (0.1 ml/newt/2 days, 3 newts), prolactin (0.05 U/newt/0.1 ml/2 days, 3 newts), ACTH (1 U/newt/0.1 ml/2 days, 3 newts), and growth hormone (0.3 mg/newt/0.1 ml/2 days, 3 newts). Hormone doses were comparable to those used by Connelly(1968), Schotte¹ and Chamberlain(1955) and Wilkerson(1963). The hormone (Armour ACTH) reported by Schotte¹ and Chamberlain(1955) to enhance limb regeneration in hypophysectomized adult newts probably had other pituitary hormone contaminations since 1 mg. of the preparation contained only 1.14 U ACTH (see Evans et al., 1966). The ACTH used in the present experiments was essentially pure ACTH, obtained from the UpJohn Co., Kalamazoo, Michigan, and contained 46.3 U ACTH/mg. Injections were made every 2 days beginning two days after hypophysectomy and continued until the day of fixation. The value of ACTH and growth hormone in prolonging survival of

hypophysectomized newts was also determined. ACTH, growth hormone, and saline were injected into hypophysectomized adult newts (same quantities as above) and the number of newts surviving was recorded at various days post-hypophysectomy. Fifteen hypophysectomized adult newts were injected with saline, 15 with growth hormone (NIH), and 14 with ACTH (UpJohn, 46.3 U/mg.).

Results. Adrenal tissues of the adult newt exist as either randomly distributed compact cords of cells or as rounded islets of tissue with diameters varying from 100-150 microns, adjacent to or near the vena cava (Figure 1). Unstained tissues, when examined with a low power lens system, are yellowish orange in color and occur as intermittent masses of cells extending along the entire ventral surface of the kidney. Individual cells are either round or oblong with oval nuclei. Concentration of lipid is approximately the same and uniformly distributed in all tissue masses.

The intensity of Sudan Black reactions (paraffin sections) in adrenal tissues of adult newts two weeks subsequent to hypophysectomy, was essentially the same as that of the controls (Figures 1 & 2). The administration of ACTH, prolactin, or growth hormone to hypophysectomized newts did not visibly modify intensity of Sudan Black reactions or alter either the size or shape of cells or their nuclei (Figure 3). Growth hormone significantly increased the survival time of

hypophysectomized adult newts whereas ACTH or saline were without effect (Chi Square Test, 0.01 level, Figure 21).

Sudan Black staining of frozen sections again showed no difference in lipid quantity (Figures 4 & 5) between adrenal tissue of hypophysectomized and intact newts. Mouse adrenal stained with Sudan Black (Figure 6) exhibited the zonation characteristics of the mammalian adrenal (Levy et al., 1959) and a greater amount of lipid (Table 2) than newt adrenal tissue.

Cryostat cut sections of tissues that were incubated in the dehydroepiandrosterone medium showed that the reaction product (formazan deposit) increased from two hours to reach a maximum at four hours and then remained fairly uniform in concentration until the experiment was terminated at six hours. Formazan was abundantly deposited in the adrenal tissue of the adult newt (Figures 7 & 8). Careful examination of cross sections of kidneys revealed formazan deposition only in the adrenal tissue. The tissue exhibiting SDH activity could be located in the ventral portion of the kidney in close proximity to the vena cava and areas of formazan deposition corresponded to Sudan Black stained areas in paraffin sections. No SDH activity could be demonstrated in kidney tissue or in the skin of the newt (Table 3) while mouse adrenal again exhibited the characteristic zonation (Figure 9). Adrenal tissue of Rana also exhibited abundant enzyme activity. While steroid dehydrogenase

activity is present only in the adrenal tissue of the newt, malate dehydrogenase and lactate dehydrogenase, although present in the adrenal, are also present in the kidney and in the skin of the newt (Table 3). Adrenal tissue of intact newts and newts hypophysectomized for two weeks exhibited essentially equal steroid dehydrogenase activity (Table 3). Although differences in SDH activity could be noted between individual adrenal islets, the same apparent range of SDH activity was found in adrenal tissue of both hypophysectomized and intact newts.

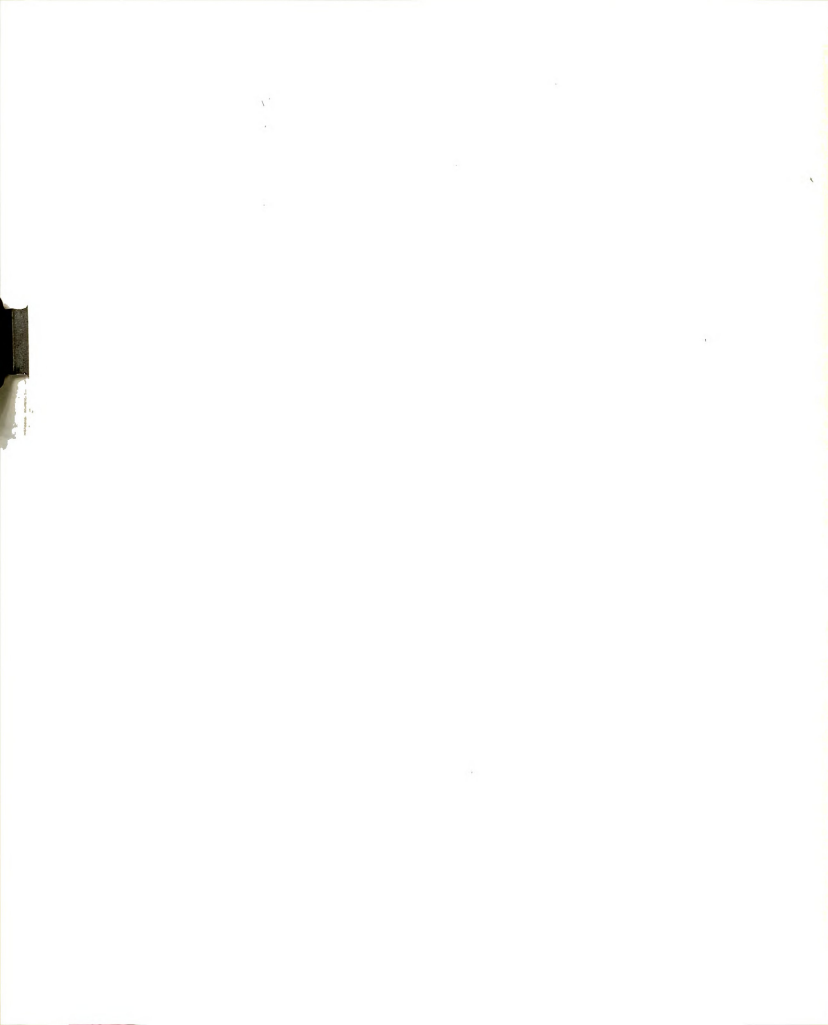
In summary, the steroid dehydrogenase activity and the lipid quantity of the adrenal tissue of the adult newt do not change significantly either after hypophysectomy or after hormone treatment. Growth hormone significantly enhances survival of hypophysectomized adult newts, whereas ACTH was ineffective in this regard. From the histological and histochemical observations it can be concluded that the adrenal tissue of the adult newt is active in synthesizing steroid hormones but these hormones are not limiting in the hypophysectomized newt.

SERIES II

COMPARISON OF THE SURVIVAL AND LIMB REGENERATION OF FED HYPOPHYSECTOMIZED ADULT NEWTS, FASTED HYPOPHYSECTOMIZED ADULT NEWTS, AND FASTED PARTIALLY HYPOPHYSECTOMIZED ADULT NEWTS.

After hypophysectomy, some adult newts survive for only 10 days while others survive for over 4 weeks (Dent, 1967). Some amputated limbs of hypophysectomized newts will regenerate and the amount of limb regeneration observed can be correlated, to some extent, with the length of time the newt survives (Hall and Schotté, 1951). However, Schotté and Hall (1952) have concluded that amputated limbs of completely hypophysectomized adult newts do not regenerate. It is difficult to attribute this variation in survival and limb regeneration to fragments of pituitaries (Hall and Schotté, 1951), or to complete lack of adrenal hormones (Gorbman, 1964 and Series I results). Newts appear to differ considerably in their nutritional state, however, which is suggested by casual observations of differences in size, eating habits, and behavior, and even more by variation in body weight. This experimental series was designed to determine whether the presence of the pituitary is absolutely essential for limb regeneration to proceed and whether the nutritional state of the newt at the time of hypophysectomy has any bearing on survival and/or limb regeneration.

Methods and Materials. Fifty-three adult newts, weighing from 1 to 2 grams, were randomly selected from planted aquaria. Of these fifty-three newts, 25 were fed beef liver daily for two weeks. The remaining 28 newts were fasted for the same two week period. Sixteen of the fed newts and 16 of the



fasted newts were then randomly selected and their body weights determined. The individual body weights of the newts of the two groups were compared statistically by the non-parametric Mann-Whitney U Test. The individual weights of the fed newts were significantly greater (0.01 level of significance). On the average, each fed newt weighed 0.318 grams more than each fasted newt. All twenty-five of the fed newts and 16 of the fasted newts were then hypophysectomized. The forelimbs of the 16 fasted newts were amputated 5 days post-hypophysectomy. The forelimbs of the 16 fed newts that were weighed were amputated 5 days post-hypophysectomy. The forelimbs of the other 9 fed newts were amputated 2 days post-^{hypophysectomy} ~~amputation~~. The remaining 12 fasted newts were partially hypophysectomized with care being taken to remove all but a small fragment of the anterior lobe of the pituitary, with or without the posterior lobe. It was hoped that this partial hypophysectomy series would determine whether small pituitary fragments could support survival and/or limb regeneration in adult newts. The forelimbs of the newts were amputated 2 days post-^{hypophysectomy} ~~amputation~~. Amputated limbs of newts of all three series were selected at various times during the experimental period and examined for the presence of a regeneration blastema. Limbs were fixed within a few hours after death of the newt or randomly sampled from the surviving newts. Both forelimbs of the 8 surviving fed hypophysectomized newts were fixed 26 days post-hypophysectomy.

(21 days post-amputation) and examined histologically. Limbs were fixed in Bouin's Fluid, decalcified, dehydrated, cleared in methyl salicylate and embedded in paraffin. Sections of limbs, cut at 10 microns, were stained with either hematoxylin-eosin, iron-hematoxylin or Masson's Trichrome (Merchant, et al., 1964).

Results. Of the hypophysectomized newts which were fed daily for two weeks prior to hypophysectomy, 17 of 25 were surviving at 20 days post-hypophysectomy (12 of 16 of those fed newts whose body weights were determined). Of the 16 hypophysectomized newts which were fasted for 2 weeks prior to hypophysectomy, none were surviving at 20 days post-hypophysectomy. Of the 12 fasted partially hypophysectomized newts, 2 of 12 were surviving at 20 days post-hypophysectomy. Figure 22 compares the percent survival of these three groups. The 17 of 25 (or 12 of 16 of the newts whose body weights were determined) surviving newts which were fed prior to hypophysectomy is a significantly greater number than the 0 of 16 fasted hypophysectomized newts or the 2 of 12 fasted partially hypophysectomized newts (X^2 Test, 0.01 level of significance).

Histological examination of the limbs of the 8 surviving fed hypophysectomized newts on day 21 post-amputation (26 days post-hypophysectomy), revealed regeneration blastemas on every limb (16 limbs) (Figure 10). Examination of serial

sections (10 microns in thickness) of the heads of these 8 surviving newts (PAS stain) revealed no pituitary fragments (Figure 11). On the contrary 10 of 12 of the heads of the partially hypophysectomized newts contained small pituitary fragments (Figure 12), however, a significantly smaller number of these newts survived than did newts which were completely hypophysectomized and previously well fed. Histological examination of 4 limbs of the fed hypophysectomized newts and 4 limbs of the fasted partially hypophysectomized newts, which were amputated just 2 days after hypophysectomy and fixed 20 days post-hypophysectomy, revealed that nearly all of these limbs had regeneration blastemas although retarded as compared to normal regenerates (Compare Figures 13 & 16).

In summary, limbs of newts (fed prior to hypophysectomy) which were amputated 2-5 days post-hypophysectomy exhibited regeneration in the great majority of cases (20 of 21 limbs) when examined histologically 20 days post-hypophysectomy. Thus, limbs of newts when amputated after hypophysectomy, in the absence of pituitary hormones, nevertheless exhibit positive regeneration. Furthermore, the length of time hypophysectomized newts survive depends more upon the nutritional state of the newts at the time of hypophysectomy than on the completeness of the hypophysectomy operation.

SERIES III

THE EFFECT ON LIMB REGENERATION WHEN HYPOPHYSECTOMY IS PERFORMED AFTER THE WOUND HEALING AND DEDIFFERENTIATION PHASES ARE ESSENTIALLY COMPLETE

This experimental series was designed to determine whether pituitary hormones are influential during the growth phase of regeneration. Schotte¹ and Hall(1952) concluded that pituitary hormones (specifically ACTH) are required for only the first 6 days of regeneration, while Hay(1966) has suggested that hormones may act on growth of the blastema, as is true of nerves(Singer and Craven, 1946). According to Hall and Schotte¹(1951) the wound healing and dedifferentiation phases of limb regeneration are over at approximately 12 days post-amputation. Therefore, in this series, hypophysectomy was performed at 14 days post-amputation at which time a mound blastema is present (Figure 14).

Methods and Materials. Either the right or left forelimb (chosen randomly) of each of 36 newts previously fasted for 2 weeks, was amputated through the distal portion of the humerus. At 14 days post-amputation these newts were randomly divided into two groups. The newts in one group (n=20) were hypophysectomized while the remaining 16 newts were sham-operated. The sham operation consisted of removing a portion of the bone over the sella turcica without removing

the pituitary. The bone was then replaced. At 8 days post-hypophysectomy or post-sham (22 days post-amputation), 6 of the newts from each group were randomly selected, sacrificed and the amputated limb and the head of each was fixed for histological examination. Limbs were prepared for histology as in Series II. Limbs were serially sectioned longitudinally at 10 microns, and stained with hematoxylin and eosin. The area and the length of the regeneration blastema was determined for each of 5 sections of each limb. The image of the section to be measured was projected onto graph paper from a constant height. The image of the blastema of each section sampled was then traced with a fine pointed pencil. The number of squares representing the blastema was counted for each section sampled (5 sections for each limb). The 5 sections to be sampled were determined by selecting the approximately largest section (usually in the center of the limb), and measuring the area of the blastema of that section. Two sections 80 microns apart on either side of this largest section were also sampled. Thus the 5 sections sampled, spaced at 80 micron intervals, covered a total distance through the blastema of 400 microns (approximately $\frac{1}{2}$ mm.). By trial and error this technique was shown to sample the largest portion of each blastema.

The limbs of 6 additional newts from each group were sampled 16 days post-hypophysectomy (28 days post-amputation) and the blastema area for each section was determined. Heads

of 6 of the hypophysectomized newts were randomly chosen and prepared for staining as in Series I. Serial sections of heads cut at 10 microns were stained with the PAS stain.

Results. The results are summarized in Figures 23 & 24, and Table 4. The mean blastema area for each limb was determined (mean of 5 sections) and the means compared statistically for the two groups (6 limbs in each group) at 8 days post-operation (hypophysectomy or sham) with the Mann-Whitney U Test. The mean areas of the sham operated newts were significantly greater than the mean areas of the hypophysectomized newts at 8 days post-operation (15.90 square v.s. 9.25 square) and at 14 days post-hypophysectomy (22.47 square v.s. 15.22 square)(significant at the 0.01 level). Comparison of Figures 19 & 20 reveals these size differences. The blastemas of the sham-operated newts were also significantly longer (17.5 units) than the blastemas of the hypophysectomized newts (11.5 units)(Mann-Whitney U Test, 0.01 level of significance). Histological and gross observations also revealed that the regenerates of the sham-operated newts were in more advanced stages of differentiation. Digit differentiation was apparent in 4 of the 6 regenerates of the sham-operated newts (28 days post-amputation) whereas only 1 of the 6 regenerates of the hypophysectomized newts exhibited digit differentiation 14 days post-hypophysectomy. Cells in mitosis could be observed in the regenerates of the hypophy-

sectomized newts (8 days post-hypophysectomy) and growth was therefore not completely stopped. Furthermore, the mean blastema areas were larger at 14 days post-hypophysectomy than at 8 days post-hypophysectomy suggesting that some growth did occur. (Compare Figure 15 and Figure 17; see Figure 24).

In summary, these results demonstrate that the normal growth rate of the blastema requires pituitary hormones. Hypophysectomy influences the rate of regeneration even after the wound healing and dedifferentiation phases are complete. The suggestion by Hay(1956, 1966) that pituitary hormones may act on growth was therefore a cogent one. If the early growth of the blastema, DNA synthesis and subsequent cell division(Hay and Fischman, 1961), is also dependent upon pituitary hormones, one would expect, especially in fasted newts, a more adverse effect on limb regeneration when hypophysectomy is performed concomitantly or shortly after limb amputation. The data of Schotte¹ and Hall(1952) support this interpretation.

SERIES IV A

THE EFFECT OF PROLACTIN + THYROXINE, PROLACTIN, THYROXINE, AND SALINE ON SURVIVAL AND LIMB REGENERATION OF HYPOPHYSECTOMIZED ADULT NEWTS.

The hypophysectomized adult newt will survive and regenerate amputated limbs in a normal fashion (Schotté and Tallon, 1960). It is known that the ectopic pituitary produces prolactin and TSH (Masur, 1962; Dent and Gupta, 1967; Dent, 1966). Growth hormone injections also maintain the health and regenerative ability of hypophysectomized adult newts (Wilkerson, 1963). The growth hormone (NIH) contained both prolactin (0.015 U prolactin/0.3 mg. GH/newt/2 days) and TSH as contaminants (Berman et al., 1964). This experimental series was designed to determine whether prolactin in the amount contaminating Wilkerson's GH, and in larger amounts, either alone or combined with thyroxine, would enhance survival and limb regeneration of hypophysectomized newts. Thyroxine alone was also tested since Dent (1966) suggested that the cause of death of hypophysectomized newts is due to the build up of the epidermis which interferes with respiration through the skin.

Methods and Materials. One hundred fasted newts were hypophysectomized and randomly divided into 4 groups. Group I (30 newts) received intraperitoneal injections of prolactin, 1.2 U/newt/2 days; Group II (30 newts) received intraperitoneal injections of prolactin, 0.015 U/newt/2 days, and continuous thyroxine treatment (1×10^{-7} conc. in the aquarium water; 0.1 mg. thyroxine/1000 c.c. H_2O); Group III (20 newts) received thyroxine treatment alone (1×10^{-7}

conc. in the aquarium water), and Group IV (20 newts) received intraperitoneal injections of saline (0.9%) every other day. The volume of each injection was 0.1 c.c. Prolactin was suspended in 0.9% saline. Plastic disposable syringes with 27 gage hypodermic needles were used for injections. Injections were continued until day 20 post-hypophysectomy. Limbs were amputated and injections were begun 5 days after hypophysectomy. Limbs and heads of newts from all four groups were sampled for histological examination at various times during the experiment. The number of surviving newts of each group was recorded each day until day 23 post-hypophysectomy.

Results. The results, expressed as percent survival, are summarized in Figure 25. On day 19 and also on day 23 post-hypophysectomy, a significantly greater number of prolactin and prolactin-thyroxine treated hypophysectomized newts were surviving than were saline or thyroxine treated hypophysectomized newts (X^2 Test, 0.01 level of significance). In addition, over 95% of the prolactin-thyroxine treated hypophysectomized newts survived to day 30 post-hypophysectomy. The surviving prolactin-treated hypophysectomized newts (Group I) and the prolactin-thyroxine treated newts (Group II) all exhibited typical limb regeneration (Figures 18 & 19). Limbs of thyroxine and saline treated hypophysectomized newts exhibited lack of a regeneration blastema earlier

than day 16 post-amputation. However, limbs of these newts (Group III and IV) which survived longer than 17 days post-hypophysectomy revealed a small blastema and delayed regeneration (Figure 20). Hypophysectomized adult newts treated with the prolactin-thyroxine combination appeared healthy, had smooth normal appearing skin, and were active. Although the newts in this experiment were not fed, other experiments had shown that prolactin-thyroxine treated newts had good appetites and readily accepted food. The appearance and behavior of prolactin-thyroxine treated hypophysectomized adult newts resembled those of hypophysectomized adult newts with ectopic pituitary grafts. Hypophysectomized newts treated with thyroxine alone appeared normal in that the epidermis did not become cornified and the skin retained the olive-green color of normal newts. However, thyroxine treated hypophysectomized newts were not active and would not feed. Hypophysectomized newts treated with prolactin alone appeared more healthy (greenish color) than hypophysectomized newts treated with saline (black). However, in both of these latter groups, molting clearly did not occur and the skin darkened.

In summary, administering prolactin and thyroxine together to hypophysectomized adult newts appears to be an adequate replacement for the pituitary. Prolactin-thyroxine treated newts survive for up to 30 days post-hypophysectomy or longer if hormone treatment is continued, regenerate

amputated limbs in a normal fashion, have good appetites, are active and in all respects resemble normal newts. Prolactin alone, even in large doses, does not prolong survival as well; however, limb regeneration was apparent in all survivors. Thyroxine alone had no more survival benefit than saline when administered to hypophysectomized adult newts.

SERIES IV B

THE EFFECT OF DELAYED PROLACTIN AND THYROXINE, ACTH, AND WATER ON SURVIVAL AND LIMB REGENERATION OF HYPOPHY-SECTOMIZED ADULT NEWTS.

The normal health and regenerative ability is restored to hypophysectomized newts even when growth hormone injections are begun 14 days after hypophysectomy (Wilkerson, 1963). It was found in Series I, II, and III that by 10 days post-hypophysectomy, previously fasted newts were beginning to die in relatively high frequency. This experimental series was designed to test whether the amount of prolactin contamination in Wilkerson's GH combined with thyroxine, given 10 days post-hypophysectomy, would effectively prevent additional deaths of hypophysectomized newts from occurring. ACTH and water were also tested in this regard.

Methods and Materials. Sixty fasted newts were hypophysecto-

mized and on day 10 post-hypophysectomy, both forelimbs of the surviving newts were amputated. The newts were then randomly divided into 4 groups. Group I (12 newts) received prolactin (0.015 U/newt/2 days) plus continuous thyroxine treatment (1×10^{-7} conc. in the aquarium water). Group II (12 newts) received ACTH (1 U/newt/2 days). Group III (12 newts) received distilled water (0.1 cc/newt/2 days). Group IV (10 newts) received no treatment. The heads of two newts of each group were examined histologically for verification of completeness of hypophysectomy. The number of surviving newts in each group was recorded daily until 25 days post-hypophysectomy. Treatments were continued until day 20 post-hypophysectomy.

Results. On day 25 post-hypophysectomy, all 12 of the prolactin-thyroxine treated newts (Group II) were surviving, a significant difference compared to the number of surviving newts in the other three groups (X^2 Test, 0.01 level of significance). ACTH had no more survival value than did water alone (Figure 26). Histological examination of limbs of the prolactin-thyroxine treated hypophysectomized newts revealed typical regeneration. At 16 days post-amputation, limb regeneration of newts in Groups II, III, and IV was either not progressing at all or very retarded.

These results illustrate that prolactin and thyroxine, administered together at a time when hypophysectomized fasted

newts were beginning to die, restored the health of the newts and prevented death. Hypophysectomized newts given ACTH continued to die as did untreated or water treated hypophysectomized newts. Furthermore, limbs of the prolactin-thyroxine treated newts, even though amputated 10 days post-hypophysectomy, exhibited typical regeneration.

SERIES IV C

THE EFFECT OF PROLACTIN AND THYROXINE, PROLACTIN, GROWTH HORMONE, AXOLOTL PITUITARY GRAFTS, ACTH, AND SALINE ON SURVIVAL AND LIMB REGENERATION OF HYPOPHYSECTOMIZED ADULT NEWTS.

The growth hormone which was obtained from NIH for these investigations contained 10 times as much prolactin/mg. of preparation as did the growth hormone (NIH) used by Wilkerson. This experimental series was designed to test whether 0.03 mg. growth hormone, 1/10 the quantity administered by Wilkerson, would still support limb regeneration and survival of hypophysectomized adult newts. Newts receiving this smaller amount of growth hormone nevertheless received 0.015 U prolactin per injection. Prolactin alone, in the same amount, was also tested, as was ACTH and the same prolactin-thyroxine combination used in Series IV A. It is known that Ambystoma mexicanum (axolotl) larvae do not require their pituitary for regeneration or survival (Tassava et al., 1968). However, because prolactin has been found

in the pituitary of another neotenic amphibian, Necturus (Nicoll et al., 1966) and because of the goitrogenic effect of prolactin (Gona, 1967), axolotl pituitary grafts were tested as to their survival and limb regeneration value in hypophysectomized adult newts.

Methods and Materials*. One hundred fasted adult newts were hypophysectomized and at 5 days after hypophysectomy, were randomly divided into 6 groups and both forelimbs of each newt were amputated. Group I (10 newts) received prolactin (0.015 U/newt/2 days) with continuous thyroxine treatment (1×10^{-7} conc. in the aquarium water). Group II (15 newts) received growth hormone (NIH) (0.03 mg/newt/2 days). This quantity of growth hormone, 1/10 the quantity used by Wilkerson (1963), nevertheless contained the same prolactin contamination (0.015 U prolactin/0.03 mg. GH/newt/2 days). Group III (20 newts) received prolactin alone (0.015 U/newt/2 days). Group IV (15 newts) received ACTH (1 U/newt/2 days). Group V (20 newts) received 0.9% saline (0.1 cc/newt/2 days). Of the 15 saline injected newts, 6 received grafts of axolotl brain tissue. Group VI (16 newts) received axolotl (Ambystoma mexicanum) pituitary grafts (2 axolotl pituitaries per newt). Newts containing the axolotl pituitary grafts

* I am indebted to John DeFazio for doing the growth hormone portion of this series.

were considerably darker due to the melanophore expansion caused by MSH secretion from the ectopic pituitary. It was felt that these pituitary grafts, although xenoplastic, would survive for at least 2 months because axolotl skin and limb grafts to the newt readily survive for over 2 months (Tassava, unpublished). Heads of the newts with axolotl pituitary grafts were examined to determine the health of the grafted pituitary and the completeness of hypophysectomy of the newt. Some heads of the newts of the other series were also examined to insure completeness of hypophysectomy. It should be pointed out here that examination of serial sections of over 40 heads (Series I-IV) revealed only 2 cases of partial hypophysectomy (less than 5%).

Results. Hypophysectomized newts survived to day 24 post-hypophysectomy in significantly greater numbers when given prolactin + thyroxine, (10/10 survived), GH (14/15 survived, and axolotl pituitary grafts (13/16 survived), than when given prolactin alone (0.015 U/newt/2 days)(4/20 survived), ACTH (2/15 survived) or saline (3/15 survived)(χ^2 Test, 0.01 level of significance). The percent survival of newts after the various treatments is illustrated in Figure 27. Limb regeneration on day 24 post-hypophysectomy was typical on newts in Groups I, II and VI, which received prolactin + thyroxine, GH and axolotl pituitary grafts respectively. It is somewhat surprising that the axolotl pituitary supported

survival and limb regeneration of hypophysectomized adult newts. Hypophysectomized axolotls will regenerate limbs and survive indefinitely (Tassava et al., 1968). It is important to note that the axolotl pituitary is not known to produce TSH (thyroid stimulating hormone) (Blount, 1950). When the grafted axolotl pituitary was removed from 8 of these newts, typical hypophysectomy symptoms resulted: lack of molting, sluggishness and finally death. These results also demonstrate that prolactin alone (0.015 U/newt/2 days) is not effective in enhancing survival and limb regeneration of hypophysectomized adult newts. The same amount, combined with thyroxine, is very effective in this regard. The growth hormone given contained this same amount of prolactin as contamination. It is also likely that the growth hormone contained enough TSH to activate the newts thyroid since molting was occasionally seen and the epidermis did not build up as in hypophysectomized newts treated with prolactin alone. However, an effect of growth hormone cannot be ruled out.

SERIES IV D

THE EFFECT OF DELAYED PROLACTIN + THYROXINE, THYROXINE, AND SALINE ON SURVIVAL OF HYPOPHYSECTOMIZED ADULT NEWTS WHEN TREATMENT IS BEGUN 10 DAYS AFTER HYPOPHYSECTOMY.

This series is similar to Series IV B except that thyroxine alone was tested since Wilkerson(1963) claimed that TSH treatment of hypophysectomized newts prolonged their life. Furthermore, the prolactin + thyroxine combination in Series IV B had such an immediate effect on restoring the health of hypophysectomized newts, it was decided to repeat that portion of Series IV B to be certain of the results.

Methods and Materials. Forty-five fasted newts were hypophysectomized and, 10 days after hypophysectomy, the surviving 28 newts were randomly divided into 3 groups. Group I (10 newts) received prolactin (0.015 U/newt/2 days) and continuous thyroxine treatment (1×10^{-7} conc.), Group II (9 newts) received thyroxine alone (1×10^{-7} conc. in aquarium water), and Group III (9 newts) received saline (0.1 cc/newt/2 days). The number of surviving newts in each group was recorded each day until day 23 post-hypophysectomy. Injections were continued until day 20 post-hypophysectomy.

Results. Figure 28 summarizes the survival results. A significantly greater number of prolactin-thyroxine treated hypophysectomized adult newts survived to day 23 post-hypophysectomy than did thyroxine or saline treated hypophysectomized adult newts (χ^2 Test, 0.01 level of significance). Thyroxine alone had no beneficial effect, in fact, on day 19

post-hypophysectomy (7 days after hormone treatment was begun), no thyroxine treated newts were surviving (0 of 9) while 6 of 9 saline treated newts were still surviving and 9 of 10 prolactin-thyroxine treated newts. This result suggests that thyroxine administration, begun 10 days post-hypophysectomy, may be detrimental to the hypophysectomized newt unless given with prolactin.

SERIES IV E

THE EFFECT OF PROLACTIN + THYROXINE AND ACTH + THYROXINE ON SURVIVAL OF HYPOPHYSECTOMIZED ADULT NEWTS.

The previous series clearly show that a prolactin-thyroxine combination maintains the health and regenerative ability of hypophysectomized adult newts. Since prolactin alone (0.015 U) and ACTH alone are not effective in this regard, this experimental series tested whether thyroxine + ACTH would also be an effective combination.

Methods and Materials. Twelve fasted hypophysectomized newts were treated with prolactin (0.015 U/newt/2 days) and thyroxine (1×10^{-7} conc. in aquarium water) and 12 fasted hypophysectomized adult newts were treated with ACTH (1.0 U/newt/2 days) and thyroxine (1×10^{-7} conc.) beginning 4 days post-hypophysectomy. The number of surviving newts in each of the two groups was recorded daily until day 23

post-hypophysectomy. Treatment was continued until day 20 post-hypophysectomy.

Results. On day 23 post-hypophysectomy a significantly greater number of prolactin-thyroxine treated hypophysectomized newts were surviving (12 of 12) than ACTH-thyroxine treated hypophysectomized newts (2 of 10) (X^2 Test, 0.01 level of significance). Thus, thyroxine is effective in increasing survival of hypophysectomized newts when combined with prolactin but not when combined with ACTH.

SERIES IV F

THE EFFECT OF ACTH TREATMENT ON LIMB REGENERATION OF INTACT NEWTS.

Schotté¹ and Chamberlain(1955) using crude Armour ACTH reported that 1 U ACTH/newt/2 days caused a temporary inhibition of intact newt limb regeneration. Since the effects of ACTH reported by Schotté¹ and Chamberlain(1955) on hypophysectomized newts may have been due to contamination hormones (Series I) it is possible that the effect on intact newts was also due to contaminants. This experimental series was designed to determine whether pure ACTH (UpJohn) would influence intact newt limb regeneration.

Methods and Materials. ACTH (1 U/newt/2 days), dissolved in water, was administered to 8 intact newts for a period of 24 days. Water (0.1 c.c.) was administered to another 8 intact newts. Four days after beginning the injections both forelimbs of all 16 newts were amputated. The status of regeneration was observed grossly until day 30 post-amputation.

Results. ACTH (UpJohn) injected newts became dark in color and remained dark during the 24 days of treatment due to the MSH effect of ACTH(Geschwind, 1967). All 8 newts in each group survived the experiment. Newts of both groups regenerated their amputated limbs in a comparable manner; 100% of the amputated limbs of all 16 newts showed typical regeneration on day 20 post-amputation. This finding is contrary to the finding of Schotté¹ and Chamberlain(1955) who found that ACTH (Armour) administered to intact newts resulted in temporary inhibition of limb regeneration.

SERIES IV G

THE EFFECT OF GROWTH HORMONE AND ECTOPIC PITUITARY GRAFTS ON SURVIVAL OF HYPOPHYSECTOMIZED ADULT NEWTS.

The purpose of this series was to attempt to confirm the findings of Schotté¹ and Tallon(1960) and Wilkerson(1963) that ectopic pituitary grafts and growth hormone (0.3 mg/

newt/2 days), respectively, would support limb regeneration and enhance survival of hypophysectomized adult newts.

Methods and Materials. Twenty-six adult newts were hypophysectomized. The pituitary of 10 of these newts was transplanted to an ectopic site (lower jaw), 6 newts received growth hormone (NIH-0.3 mg/newt/2 days) for 14 days beginning 1 day post-hypophysectomy. The remaining 10 hypophysectomized newts received saline (0.1 cc/newt/2 days) for 14 days beginning 1 day post-hypophysectomy. Both forelimbs of all 16 hypophysectomized newts were amputated through the radius and ulna 3 days after hypophysectomy. Survival and limb regeneration were noted daily.

Results. All of the growth hormone treated hypophysectomized newts and the hypophysectomized newts with ectopic pituitary grafts were surviving and exhibited typical limb regeneration at 22 days post-hypophysectomy. Only 2 of the 10 saline-treated hypophysectomized newts were surviving on day 22 post-hypophysectomy. These two newts exhibited limb regeneration although retarded compared to that of GH treated hypophysectomized newts or hypophysectomized newts containing ectopic pituitary grafts. These results support the findings of Schotte¹ and Tallon(1960) and Wilkerson(1963) that the ectopic pituitary or growth hormone serve as replacement therapy for the missing pituitary hormones of hypophysecto-

mized newts. When the ectopic pituitary of 6 of these hypophysectomized newts was removed, typical hypophysectomy symptoms resulted: lack of molting, sluggishness, and finally death.

SERIES IV H

THE EFFECT OF PROLACTIN + THYROXINE AND PROLACTIN + NaCl (100% AND 10% HOLTFRETER'S SOLUTION) ON SURVIVAL OF HYPOPHYSECTOMIZED ADULT NEWTS.

Prolactin is known to be instrumental in controlling water balance in fish (Ball and Ensor, 1967). Prolactin prevents the decrease of plasma sodium after hypophysectomy and prolongs life of the hypophysectomized fish (Ball and Ensor, 1967). As a crude attempt to determine whether a similar role of prolactin is required for newt survival, hypophysectomized newts were treated only with prolactin and provided NaCl in the aquarium water.

Methods and Materials. Thirty-six fasted newts were hypophysectomized and randomly divided into 4 groups. Group I (10 newts) received prolactin (0.015 U/newt/2 days) beginning on day 1 post-hypophysectomy and were kept in aerated water containing 3.5 g. NaCl/liter (equivalent to 100% Holtfreter's solution). Group II (10 newts) were given prolactin (0.015 U/newt/2 days) beginning 1 day post-hypophysectomy and kept

in aerated water containing 0.35 g. NaCl/liter (equivalent to 10% Holtfreter's solution). Group III (8 newts) received prolactin (0.015 U/newt/2 days) and continuous thyroxine treatment (1×10^{-7} conc. in the aquarium water) beginning 1 day post-hypophysectomy. Group IV (8 newts) were injected with prolactin (0.015 U/newt/2 days) and treated with thyroxine (1×10^{-8} conc. in the aquarium water). Treatment was continued for 14 days (15 days post-hypophysectomy). The number of surviving newts in each group was recorded daily. An additional 6 hypophysectomized newts were maintained in 3.5 g. NaCl/liter but were not treated with hormone.

Results. The percent survival is summarized in Figure 29. A significantly greater number of prolactin-thyroxine treated newts (at either thyroxine conc.) were surviving 20 days post-hypophysectomy than were prolactin treated hypophysectomized newts maintained in either NaCl solution. NaCl treatment without hormone injections likewise did not prolong survival.

These results indicate that providing Na^+ to hypophysectomized newts with prolactin is not effective in prolonging survival whereas prolactin with thyroxine, even 1×10^{-8} conc., effectively prolongs the life of hypophysectomized newts.

DISCUSSION

The results of the four series of experiments presented in this paper can be interpreted in the following manner:

1) limb regeneration does not depend upon activation of the adrenal gland by ACTH released after the stress of amputation; ACTH and consequently the adrenal hormones, are not the limiting factors for limb regeneration after hypophysectomy; 2) survival and limb regeneration after hypophysectomy depend upon the nutritional state of the newt at the time of hypophysectomy; remaining, small pituitary fragments exert little or no effect; 3) hypophysectomy, performed after limb regeneration has progressed through the wound healing and dedifferentiation phases, causes a significant retardation in the growth of the blastema; 4) prolactin and thyroxine, administered together, effectively prolong the life of hypophysectomized adult newts, restore their appetite for food, and generally appear to duplicate the effect of an ectopic pituitary graft. Furthermore, hypophysectomized adult newts given prolactin + thyroxine regenerate amputated limbs in a typical fashion. Thyroxine alone, thyroxine + ACTH, ACTH, or saline are not effective in restoring the health of hypophysectomized adult newts and are not effective in restoring normal limb regeneration ability.

The results of the histochemical analysis of the adrenal tissue of intact, hypophysectomized and hormone treated hypophysectomized adult newts suggest, but do not prove, that pituitary hormones other than ACTH are the essential hormones for survival and limb regeneration of the adult newt. The small islets of adrenal tissue of intact newts and of newts hypophysectomized for two weeks exhibited essentially equal steroid dehydrogenase activity. SDH activity was found only in adrenal tissue while LDH and MDH were found in adrenals but also in kidney and skin. Mouse adrenal showed the same characteristic zonation of SDH activity as reported by Levy et al. (1959). Since all cells which exhibit SDH activity are known to be synthesizing steroids (Levy et al., 1959) it can be concluded that the small adrenal islets of the adult newt do in fact synthesize steroid hormones. The exact steroids produced by the newt adrenal tissue remain to be identified. Hydrocortisone and aldosterone have been identified in Rana adrenal tissue (Carstensen et al., 1961; Macchi and Phillips, 1966) whereas only aldosterone was identified in Pleurodeles adrenal (Ferrerri et al., 1967). It is of interest that both cortisol and aldosterone have mineralo-corticoid effects in frogs (Ferrerri et al., 1967) as well as glucocorticoid effects (Bergerhoff and Hanke, 1967). These latter workers concluded that in frogs one cannot distinguish between glucocorticoids and mineralocorticoids.



The newt adrenal SDH activity did not change significantly after hypophysectomy. Adrenal lipid quantity likewise was no different after hypophysectomy nor after ACTH, prolactin or GH treatment of hypophysectomized newts. These results are in agreement with findings of Wurster and Miller(1960) that the urodele amphibian adrenal shows less dependence on the pituitary than does the adrenal of anuran amphibians. A pituitary-adrenal axis apparently exists in Rana(Piper and DeRoos, 1967; Van Kemenade and Van Dongen, 1967) but even in Rana the adrenal is to a large part independent of the pituitary(Bishop, et al., 1961; Hanke and Weber, 1965; Jurani, et al., 1967). It may be that in the adult newt, after hypophysectomy, the synthesis and/or secretion of one or more specific adrenal hormones is diminished but the SDH activity does not change to any appreciable extent, since the presence of this enzyme does "not necessarily signify continued secretory activity"(Levy et al., 1959). This would seem unlikely, however, since ACTH does not increase survival of hypophysectomized adult newts whereas growth hormone or prolactin + thyroxine are very effective in this regard. Also, corticosteroids have no survival value in hypophysectomized newts(Schotté and Bierman, 1956).

Although adrenal hormones may be essential for limb regeneration and survival of adult newts, the histochemical data presented above suggest that adrenal hormones are not limiting because the adrenal continues to exhibit enzyme

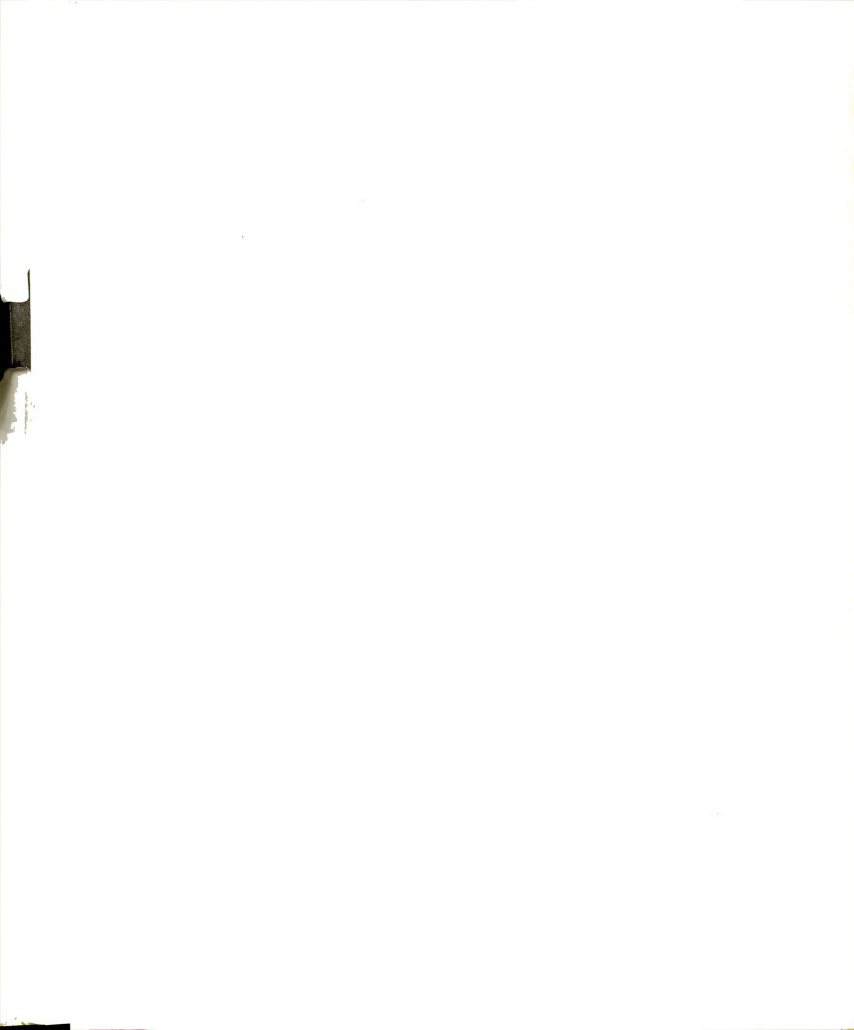
activity even two weeks after hypophysectomy, and because ACTH has no survival value in hypophysectomized newts. That the amount of ACTH administered did not cause an over stimulation (exhaustion) of the steroid synthesis capacity of the adrenal tissue is indicated by the fact that ACTH treatment of intact newts resulted in normal limb regeneration with no adverse effect on the health of the newts. On the other hand, it is likely that the dose of ACTH administered was sufficient since the MSH effect was pronounced (Geschwind, 1967) and comparable amounts of ACTH result in adrenal responses in frogs (Hanke and Weber, 1965).

The suggestion by Schotté (1961) that a "stress" mechanism is operative in initiating newt limb regeneration is not supported by the histochemical results of the present investigation. These results, therefore, support the finding of Liversage (1959) that newt limbs, at the time of amputation, can be isolated from the central nervous system by spinal cord ablation yet regeneration will occur normally. Neural stimulation of endocrine activities is not required (Liversage, 1959). In light of these findings, in addition to the demonstration that prolactin + thyroxine is an effective means (as is the ectopic pituitary) of enhancing survival and limb regeneration of hypophysectomized adult newts, the "stress" mechanism as proposed by Schotté (1961) should be abandoned. It is also unlikely that ACTH was the active hormone in the preparation (Armour) used by Schotté and



Chamberlain(1955) for 3 reasons: 1) the ACTH used by Schotté and Chamberlain(1955) contained 1.14 U ACTH/mg. of preparation (crude Armour ACTH, 1955 preparation) whereas it was not until 1962 that Armour produced purified ACTH (33 U/mg. of preparation)(Evans et al., 1966); the ACTH used in this investigation, on the other hand, was essentially pure ACTH (UpJohn Co., 1966 preparation); 2) the description of the response of the hypophysectomized newts to the crude ACTH given by Schotté and Chamberlain(1955) "newts became active, had good appetites, and slippery skin", closely resembles the response of the hypophysectomized newts in this investigation to prolactin + thyroxine or ectopic pituitary grafts. Hypophysectomized newts given pure ACTH (UpJohn) on the contrary, were sluggish, did not feed, and instead of having slippery skin, had coarse granular skin similar to that of saline treated hypophysectomized newts; 3) Schotté and Chamberlain(1955) reported that ACTH (1 U/newt/2 days) resulted in inhibition of limb regeneration in intact newts. This effect could also have been due to contaminating hormones. In this investigation, pure ACTH (1 U/newt/2 days) did not inhibit limb regeneration in intact newts.

It was clearly shown in Series II that amputated limbs of hypophysectomized newts will regenerate (although poorly) and survival is enhanced if the newt is in a particularly good nutritional state at the time of hypophysectomy.



Furthermore, small fragments of the pituitary, left behind due to partial hypophysectomies, do not significantly enhance survival. In the light of these findings, the data presented by Hall and Schotté¹(1951) can now be reinterpreted. The 28% of the hypophysectomized newts which did regenerate amputated limbs is a reasonable finding. Most of their newts died between day 10 and day 20 and since the normal regenerating limb does not show a distinct blastema until day 12, one would not expect to find very many blastema cells even at day 14 if regeneration is severely retarded. Hall and Schotté¹(1951) observed limb regeneration in 50% of the newts which survived to day 20 post-hypophysectomy. Although no exact counts of positive limb regeneration were made in this investigation on hypophysectomized newts treated with saline, thyroxine, or water, the limbs examined histologically of those newts surviving to day 20 or more post-hypophysectomy, exhibited positive regeneration in over 50% of the cases. Therefore, in the present investigation, as in the investigation of Schotté¹ and Hall(1952) regeneration could be correlated with survival. In the present investigation survival was correlated with the nutritional state of the newt at the time of hypophysectomy. The presence of regeneration could not, however, be correlated with the presence of pituitary remnants. The amputated limbs of hypophysectomized newts treated with thyroxine, saline, or water, when examined histologically after staining



for connective tissue with Masson's Trichrome (Merchant et al., 1964), exhibited some connective tissue, but did not possess a dermal pad. Schotté (1961) concluded that a dermal pad forms on non-regenerating limbs of hypophysectomized newts. Even if considerable connective tissue does form, it is not a block to regeneration since Wilkerson (1963) obtained good limb regeneration when growth hormone treatment of hypophysectomized adult newts was begun 14 days after amputation.

Since hypophysectomized adult newts may regenerate amputated limbs, why is the regeneration process delayed by hypophysectomy? Does only one phase of regeneration require pituitary hormone? The results of series III demonstrate that the normal growth rate of the blastema requires pituitary hormones. Whether the delay in differentiation is due to the lack of hormones or is indirectly due to the decreased rate of growth cannot be determined at this time. The work of Schotté and Hall (1952) also suggests that pituitary hormones influence blastemal growth and differentiation. Their data show that when hypophysectomy was delayed until 14 days after amputation, 79% of the limbs showed abortive or delayed regeneration. When hypophysectomy was delayed for from 7-13 days after amputation, regeneration was 100% abortive or delayed. It is surprising that Schotté and Hall (1952) concluded from this data that pituitary hormones were required only for the first 6 days of the regeneration

process. The suggestion of Hay(1956, 1966) that pituitary hormones are most important during the growth phase of regeneration is therefore very cogent. However, can one rule out an influence on dedifferentiation? There is no conclusive evidence to answer this question. However, when one considers that the blastema cells begin DNA synthesis (and therefore growth) as early as the 4th day after amputation(Hay and Fischman, 1961) and if cell proliferation is an essential part of blastema formation(Chalkley, 1954), then an influence solely on blastema growth could account for the observed abortive and delayed regeneration observed after hypophysectomy.

The effect of hypophysectomy on newt limb regeneration now appears to be very similar to the effect of hypophysectomy on newt lens regeneration. Stone and Steinitz(1953) found that when the lens was removed from newt eyes 5-8 days after hypophysectomy, a new lens regenerated but the process was considerably delayed and very often an abnormal lens was formed. The same result was observed after thyroidectomy. More important, when both the neural retina and the lens were removed from hypophysectomized newts, lens regeneration was even more retarded. These workers found that small pituitary fragments had no effect on the results. Some influence from the neural retina plays an important role in lens regeneration(Stone and Steinitz, 1953) and likewise, some neural influence (via the limb nerves) is required for limb regenera-

tion(Singer and Craven, 1946). Is it possible that the lens of an hypophysectomized newt regenerates even more slowly after neural retina removal because the primary effect of hypophysectomy is on neural tissue? Inoue(1958) has reported evidence which indirectly supports this idea. When growth hormone was administered to intact newts, limb skin proliferation increased but not in limbs which were denervated! Thus, a possibility which deserves future investigation is that the pituitary hormones' influence on blastemal growth is mediated via the limb nerves.

The results of Series IV demonstrate that prolactin in large doses (1.2 U/newt/2 days) enhances the survival of hypophysectomized adult newts. In addition, 100% of these surviving prolactin treated newts regenerated limbs. A prolactin-thyroxine combination was found to be even more effective in enhancing survival and also limb regeneration of hypophysectomized newts. This hormone combination resulted in survival of almost 100% of the hypophysectomized newts so treated, even when the hormones were not administered until 10 days post-hypophysectomy. This is a logical finding since an ectopic newt pituitary graft will also enhance survival(Dent, 1967) and limb regeneration(Schotté and Tallon, 1960) of hypophysectomized adult newts. The ectopic newt pituitary is known to produce prolactin(Chadwick, 1941) and thyroid stimulating hormone (TSH)(Dent, 1966). It would be of interest to determine the minimum effective dose of

both prolactin and thyroxine in enhancing survival and/or limb regeneration of hypophysectomized adult newts.

Thyroxine alone in the concentration used was ineffective in enhancing survival and limb regeneration and prolactin alone in the smaller dose (0.015 U/newt/2 days) was also ineffective. These findings strongly suggest that prolactin and thyroxine synergize in some as yet unknown way and that the combination of the two hormones is essential to normal health and limb regeneration of the adult newt. Hypophysectomized newts treated with thyroxine alone appear normal, and the stratum corneum of the epidermis does not build up, but these newts nevertheless do not survive any longer than saline-treated hypophysectomized newts. Thus, the suggestion by Dent(1966) that the cause of death of hypophysectomized newts is due to the build up of the epidermis, does not seem likely. Growth hormone was shown to be as effective as the prolactin-thyroxine combination in enhancing survival and limb regeneration of hypophysectomized newts, even at 1/10 the quantity used by Wilkerson(1963). The effectiveness of this smaller quantity of growth hormone (NIH) used in the present investigation may have been due to the fact that it contained 10 times as much prolactin per mg. of preparation as the growth hormone (NIH) used by Wilkerson. Thus, the newts given 0.03 mg. of growth hormone received 0.015 U prolactin/newt/2 days. It was pointed out by Berman et al. (1964) that this amount of prolactin, equivalent to the

prolactin contamination Wilkerson(1963) administered to newts, will elicit a growth response when injected into frog tadpoles. The crude growth hormone used by Wilkerson (1963) and also by Richardson(1945) also contained prolactin (Chadwick, 1940). It cannot be said with certainty whether the growth hormone used in this investigation contained enough TSH to activate the hypophysectomized newt thyroid. However, occasional molting or partial molting was observed during the treatment period suggesting some thyroid activation. Richardson(1945) and Wilkerson(1963) both suggested that some thyroid activity may be important to normal limb regeneration. Additional investigations should determine whether growth hormone, completely free of prolactin, would still be effective in supporting limb regeneration and survival of hypophysectomized newts. It was found recently (Tassava, unpublished) that 0.77 micrograms of growth hormone is not effective in influencing the water drive behavior of newts whereas the same amount of prolactin (0.77 micrograms) is effective. It is necessary to test whether this amount of GH + thyroxine will be as effective as the same amount of prolactin + thyroxine in enhancing survival of hypophysectomized newts.

The exact pathway by which prolactin and thyroxine influence survival and limb regeneration is unknown. Neither is it known whether the two effects are directly related. Inoue(1956) found a diminished mitotic proliferation in



epidermal cells of amputated limbs of hypophysectomized adult newts. Waterman(1965) and Niwelinski(1958) increased the rate of intact newt limb regeneration by prolactin treatment. Waterman(1965) also found that prolactin increased appetite and body weight of intact newts. Thyroxine will act directly on the skin of adult newts(Clark and Kaltenbach, 1961; Taban and Tassava, unpublished) and Grant and Cooper (1965) found that prolactin would maintain newt skin in organ culture but thyroxine alone was ineffective. Prolactin also acts on the skin of lizards by raising the frequency of sloughing(Maderson and Licht, 1967). Prolactin treatment will increase the molting frequency and the mitotic rate of red eft skin(Chadwick and Jackson, 1948). Whether prolactin and/or thyroxine may act directly (locally) on the blastema is presently being investigated(Tassava and Taban, in progress). Grant(1961) suggested that prolactin may induce the water drive of the red eft because of the role of this hormone in the water balance of the newt. This suggestion is important since it is known that prolactin does influence water balance in eels(Oliverreau and Ball, 1964), Fundulus(Ball and Ensor, 1967) and Tilapia(Dharmamba et al., 1967). In this investigation, survival of hypophysectomized newts was not enhanced by maintaining newts in NaCl solutions but this observation does not rule out a role of prolactin in water balance. Prolactin may also play a role in metabolism of fat, protein and carbohydrate.

Hypophysectomized Tilapia mossambica cannot form liver glycogen from amino acid precursors (Swallow and Fleming, 1967). Prolactin enhances food consumption and body weight gain in both newts (Waterman, 1965) and lizards (Licht, 1967). Prolactin treated lizards also show a significant weight increase of regenerating tails. Prolactin and thyroxine may be involved in normal metabolism in the adult newt and these hormones may be required for energy (glucose) production from protein. Thus, hypophysectomized newts which were previously fasted, survived only when given prolactin + thyroxine. However, hypophysectomized newts which were previously well fed survived significantly longer than fasted hypophysectomized newts. Newts in particularly good nutritional state at the time of hypophysectomy may contain food reserves, such as liver glycogen, which can be utilized for energy in the absence of hormones. Whether prolactin and thyroxine act on cell metabolism, which cells are acted upon (blastema cells?) and exactly how these hormones interact in enhancing survival and limb regeneration of adult newts will receive further attention in future investigations.

Schotte¹ (1961) reported that larval Ambystoma punctatum and larval newt pituitary grafts did not support survival and limb regeneration in hypophysectomized adult newts. It was therefore surprising to discover in this investigation that larval Ambystoma mexicanum (axolotl) pituitaries (2 pituitary grafts/newt) did enhance survival and limb

regeneration of hypophysectomized newts. If this effect is due to prolactin secretion it may be related to the fact that the axolotl is neotenuous. Prolactin has been identified in the pituitary of Necturus(Nicoll et al., 1966), another neotenuous amphibian. Prolactin is known to antagonize with the action of thyroxine at the level of the thyroid (Gona, 1967) and at the tissue level(Bern et al., 1968; Etkin, 1968) and the reason the axolotl does not normally undergo metamorphosis, as opposed to other urodeles, may be related to the presence of prolactin. Failure of Ambystoma mexicanum to undergo metamorphosis has been suggested as due to deficiency in its thyroid secretion, insensitivity of its tissues to thyroxine or lack of TSH secretion by the pituitary(Lynn and Wachowsky, 1951; Tassava et al., 1968). This investigation has demonstrated that thyroxine (or TSH), combined with prolactin, is essential to normal limb regeneration and survival of newts. Thus, it is tempting to speculate that the axolotl pituitary, ectopically transplanted to the newt, does, in fact, secrete TSH. This speculation is also supported by the fact that the epidermis of hypophysectomized newts with ectopic axolotl pituitary grafts does not build up as in hypophysectomized newts given only prolactin. It may be that in the axolotl no TSH releasing factor is present whereas grafted in the newt the axolotl pituitary responds to TSH releasing factor reaching the pituitary through the blood circulation.

SUMMARY

This investigation has demonstrated that newt limb regeneration does not depend upon activation of the adrenal gland by ACTH released after the stress of amputation; ACTH and consequently the adrenal hormones, are not the limiting factors for limb regeneration after hypophysectomy. Survival and limb regeneration after hypophysectomy depend upon the nutritional state of the newt at the time of hypophysectomy; remaining small pituitary fragments exert little or no effect. Hypophysectomy, performed after limb regeneration has progressed through the wound healing and dedifferentiation phases, causes a significant retardation in the growth of the blastema. Prolactin and thyroxine, administered together, effectively prolong the life of hypophysectomized adult newts, restore their appetite for food, and generally appear to duplicate the effect of an ectopic pituitary graft. Furthermore, hypophysectomized adult newts given prolactin + thyroxine regenerate amputated limbs in a typical fashion. Thyroxine alone, thyroxine + ACTH, ACTH, or saline were not effective in restoring the health of hypophysectomized adult newts and were not effective in restoring normal limb regeneration ability.

TABLE 1 The composition of the medium used for demonstrating
steroid dehydrogenase activity.

TABLE I

Constituent	Amount	Final Molarity
DHA ¹ (dehydroepiandrosterone)	2 mg.	ca. 1 mM
Propylene glycol	5 ml.	1 M
Nitro-BT ² soln., 1 mg./ml.	10 ml.	0.16 mM
Nicotinamide soln., 1.6 mg./ml.	7 ml.	4 mM
DPN Soln., 3 mg./ml.	8 ml.	0.54 mM
Phosphate buffer, 0.1 M., pH 7.1-7.4	40 ml.	0.057 M

- ¹ 2.0 mg. of DHA was put in a dry Columbia jar and the other constituents were then added. The propylene glycol in the medium facilitates dissolving the substrate, which is present in slight excess of its solubility.
- ² 2,2'-Di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride.

TABLE 2 The lipid in adrenal tissue¹ of intact and hormone treated² hypophysectomized adult newts estimated by sudan black staining^{3,4}.

TABLE 2

No. of Test	No. of Animals	Intact or Hypoxed	Treatment of Animal	Tissue	Time of Incubation	Estimated Lipid Quantity
1	3	Intact	-	Adrenal	12 hours	++
2	3	Hypoxed	ACTH	Adrenal	12 hours	++
3	3	Hypoxed	Prolactin	Adrenal	12 hours	++
4	3	Hypoxed	Growth Hormone	Adrenal	12 hours	++
5	3	Hypoxed	Saline	Adrenal	12 hours	++
6	3	Intact	-	Adrenal	1 hour	+++
7	3	Hypoxed	-	Adrenal	1 hour	+++
8	1	Intact	-	Mouse Adrenal (Control)	1 hour	++++++

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- 1 Tissues examined 2 weeks post-hypophysectomy.
- 2 Ten days of hormone treatment begun four days post-hypophysectomy.
- 3 Tests 1-5 incl. paraffin tissue sections.
- 4 Tests 6-8 incl. cryostat cut tissue sections.

TABLE 3 The activity¹ of steroid, malate and lactate
dehydrogenases in adrenal, kidney and skin of
normal and hypophysectomized adult newts².

TABLE 3

Histochemical Test ³	No. of Animals	Intact or Hypoxed	Tissue	Estimated Enzyme Activity
SDH	4	Intact	Newt Adrenal	+++
SDH	4	Hypoxed	Newt Adrenal	+++
SDH	1	Intact	Mouse Adrenal (Control)	+++++
SDH	4	Intact	Newt Kidney	0
SDH	4	Intact	Newt Skin	0
SDH	4	Hypoxed	Newt Kidney	0
SDH	4	Hypoxed	Newt Skin	0
MDH	2	Intact	Newt Skin Newt Kidney Newt Adrenal	+ ++ +++
LDH	2	Intact	Newt Skin Newt Kidney Newt Adrenal	+ ++ +++

¹ Enzyme activity was estimated in at least 3 adrenal islets of each newt.

² Tissues were examined 2 weeks post-hypophysectomy.

³ Tissues were incubated in SDH media for 4 hours and in MDH and LDH media for 1 hour.

TABLE 4 A comparison of the blastema areas of sham-operated
and hypophysectomized adult newts at 8 days
post-operation (22 days regeneration) and at
16 days post-operation (30 days regeneration).

TABLE 4

Mean Area of Blastema Sections 8 Days Post-operation (22 Days Regeneration)				Mean Area of Blastema Sections 16 Days Post-operation (30 Days Regeneration)			
Hypophysectomy			Sham*	Hypophysectomy			Sham
Limb	Mean Area of 5 Sections	Limb	Mean Area of 5 Sections	Limb	Mean Area of 5 Sections	Limb	Mean Area of 5 Sections
1	1.86	1	11.72	1	11.26	1	16.47
2	5.07	2	13.18	2	11.87	2	17.20
3	9.55	3	16.13	3	14.19	3	17.78
4	10.37	4	16.30	4	15.49	4	19.95
5	11.71	5	17.11	5	17.51	5	25.46
6	16.93	6	21.03	6	19.51	6	25.51
				7	20.26	7	27.20
				8		8	29.38
Mean = 9.25			Mean = 15.91	Mean = 15.22			Mean = 22.46

* The mean blastema areas (5 sections/limb) of the individual limbs of the sham-operated newts are significantly greater than the mean blastema areas (5 sections/limb) of the individual limbs of the hypophysectomized newts, both at 8 days post-operation and at 16 days post-operation (Mann Whitney U Test - 0.01 level of significance.)

Plate 1

- Fig. 1 A cross section of the kidneys of an intact adult newt showing the lipid containing islets of adrenal tissue. Tissues were fixed in formol-calcium, embedded in paraffin, and sectioned at 10 microns. A, adrenal tissue; K, kidney tubules; and VC, vena cava. Sudan Black Stain. 160X.
- Fig. 2 A cross section of the kidneys of a hypophysectomized adult newt showing the lipid containing islets of adrenal tissue. Tissues were fixed in formol-calcium 2 weeks post-hypophysectomy, embedded in paraffin, and sectioned at 10 microns. A, adrenal tissue; K, kidney tubules; and VC, vena cava. Sudan Black Stain. 160X.
- Fig. 3 A cross section of the kidneys of a hypophysectomized newt which was treated with ACTH (1 U/newt/2 days). Injections were begun 2 days post-hypophysectomy and continued until 14 days post-hypophysectomy at which time tissues were fixed in formol-calcium, embedded in paraffin and sectioned at 10 microns. A, adrenal tissue; K, kidney tubules; and VC, vena cava. Sudan Black Stain. 160X.
- Fig. 4 A cross section of the kidneys of an intact newt showing lipid droplets stained with Sudan Black. Tissues were frozen with solid CO₂-acetone and sectioned at 8 microns on a cryostat. L, lipid; K, kidney. 160X.
- Fig. 5 A cross section of the kidneys of a hypophysectomized newt showing lipid droplets stained with Sudan Black. Tissues were frozen with solid CO₂-acetone 2 weeks post-hypophysectomy. L, lipid; K, kidney. 160X.

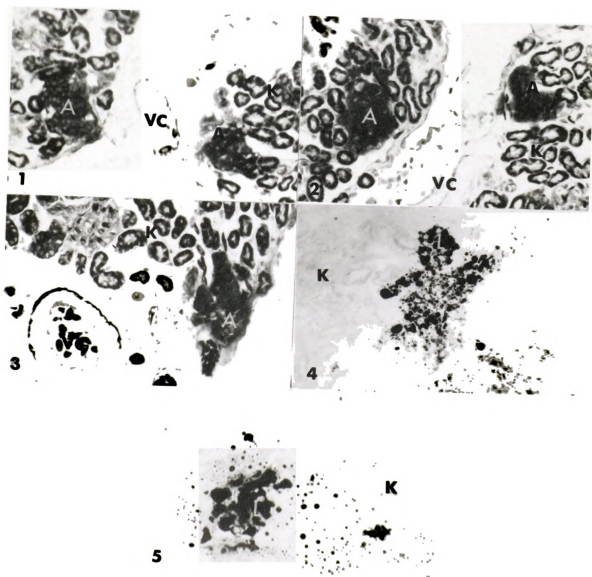


Plate 2

- Fig. 6 A cross section of a mouse adrenal gland cut at 8 microns on a cryostat and stained with Sudan Black. Note the characteristic zonation of the lipid with more intense lipid staining in the outer portion of the gland. 160X.
- Fig. 7 Formazan deposition in the adrenal tissue of an intact newt. Cryostat cut sections of frozen kidneys were incubated for 4 hours in a medium designed to demonstrate steroid dehydrogenase activity. The reaction product is formazan (F). Note lack of formazan deposition in kidney tissue (K). 160X.
- Fig. 8 Formazan deposition in the adrenal tissue of a newt hypophysectomized for 2 weeks prior to freezing of tissues. Cryostat cut sections were incubated for 4 hours in a medium designed to demonstrate steroid dehydrogenase activity. The reaction product, formazan (F), is present in equivalent amounts to that found in the adrenal tissue of intact newts. 160X.
- Fig. 9 Formazan deposition in the adrenal tissue of a mouse which was prepared in the same manner as newt tissue and incubated in the same medium and at the same time. Note the characteristic zonation in formazan deposition showing that steroid dehydrogenase activity is greater in the outer portion of the gland. 160X.

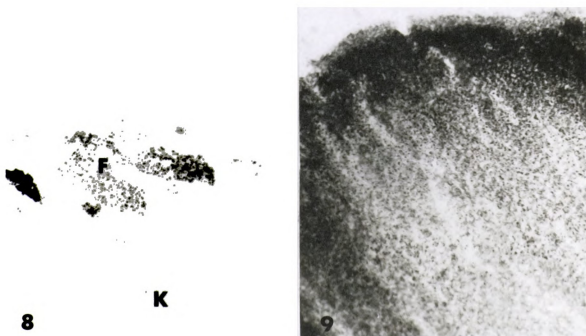
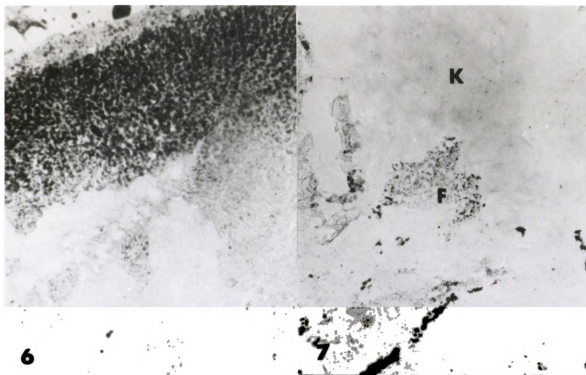


Plate 3

- Fig. 10 A longitudinal section through the regeneration blastema of a hypophysectomized newt which was fed for two weeks prior to hypophysectomy. This limb was amputated 5 days post-hypophysectomy and fixed 26 days post-hypophysectomy. Histological examination of the head of this newt demonstrated complete hypophysectomy. Hematoxylin-eosin. 100X.
- Fig. 11 A median sagittal section through the base of the infundibulum of a hypophysectomized newt which was fed for 2 weeks prior to hypophysectomy. At the time of fixation, 26 days post-hypophysectomy, the limbs of this newt showed positive regeneration. PAS stain. 100X.
- Fig. 12 A median sagittal section through the base of the infundibulum of a partially hypophysectomized newt which was fasted for 2 weeks prior to hypophysectomy. The small pituitary fragments (P) had little or no survival value since this newt survived for only 17 days post-hypophysectomy. PAS stain. 100X.
- Fig. 13 A longitudinal section through the regeneration blastema of a hypophysectomized newt which was fasted for 2 weeks prior to hypophysectomy. This limb was amputated only 2 days post-hypophysectomy and fixed 24 days post-hypophysectomy. Note the small size of the regenerate compared to that of Fig. 16. Masson's Trichrome. 100X.

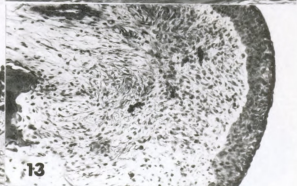
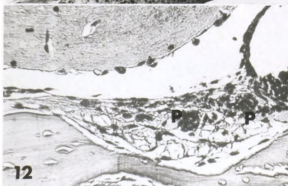
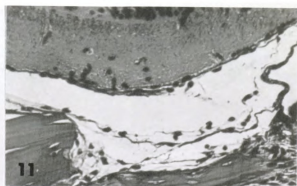
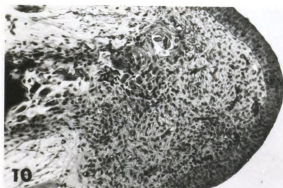


Plate 4

- Fig. 14 A longitudinal section of the regeneration blastema of an intact newt fixed 14 days after amputation. Dedifferentiation is essentially complete at this stage but the major growth of the blastema is beginning. Hematoxylin-eosin. 100X.
- Fig. 15 A longitudinal section of the regeneration blastema of a newt which was hypophysectomized 14 days after amputation of the limb. The limb was fixed 8 days post-hypophysectomy. Hematoxylin eosin. 100X. Note the smaller size of the blastema compared with that of Fig. 16.
- Fig. 16 A longitudinal section of the regeneration blastema of a newt which was sham-operated 14 days after amputation of the limb. The limb was fixed 8 days post-sham operation. Hematoxylin-eosin. 100X. Note the larger blastema size compared with that of Fig. 15.
- Fig. 17 A longitudinal section of the regeneration blastema of a newt which was hypophysectomized 14 days after amputation of the limb. The limb was fixed 16 days post-hypophysectomy. This blastema is smaller than that of Fig. 16 but larger than that of Fig. 15. Hematoxylin-eosin. 100X.

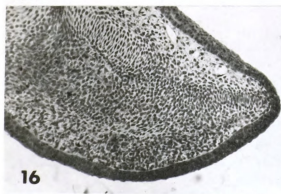
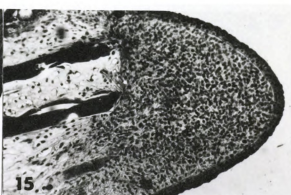
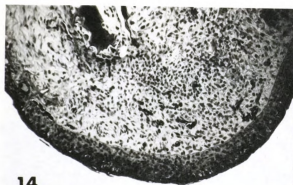


Plate 5

- Fig. 18 A longitudinal section of the differentiating regenerate of a hypophysectomized prolactin + thyroxine treated newt, 30 days post-amputation (35 days post-hypophysectomy). Note normal digit differentiation. This section is through the dorsal-ventral axis of the hand. Hematoxylin-eosin. 100X.
- Fig. 19 A longitudinal section of the differentiating regenerate of a hypophysectomized prolactin treated newt, 30 days post-amputation (35 days post-hypophysectomy). Note normal digit differentiation. Hematoxylin-eosin. 100X.
- Fig. 20 A longitudinal section of the limb of a hypophysectomized saline-treated newt, 15 days post-amputation. Compare with a normal regenerate of approximately the same age (Fig. 14). This limb exhibits retarded regeneration. Masson's Trichrome. 100X.

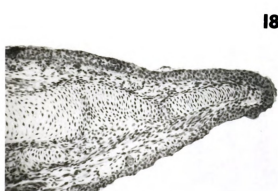


Fig. 21 A comparison of the percent survival of hypophysectomized adult newts treated with growth hormone (0.03 mg./newt/2 days), ACTH (1.0 U/newt/2 days), and 0.9% saline (0.1 cc/newt/2 days).

Injectons were begun 2 days after hypophysectomy. The percent survival is calculated by dividing the number of newts in a particular group observed to be surviving by the original number of newts in that group. Growth hormone-treated newts survived in significantly greater numbers (0.01 level of significance;

Chi Square Test).

Figure 21

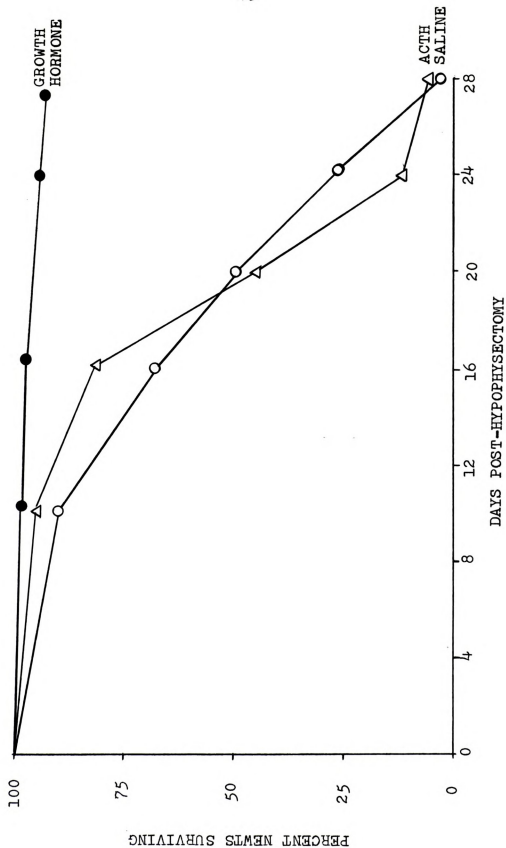


Fig. 22 A comparison of the percent survival of: 1) hypophysectomized newts fed for 2 weeks prior to hypophysectomy, 2) hypophysectomized newts fasted for 2 weeks prior to hypophysectomy, and 3) partially hypophysectomized newts fasted 2 weeks prior to partial hypophysectomy. ⁷⁴
Previously fed hypophysectomized newts survived in significant greater numbers (0.01 level of significance; Chi Square Test).

Figure 22

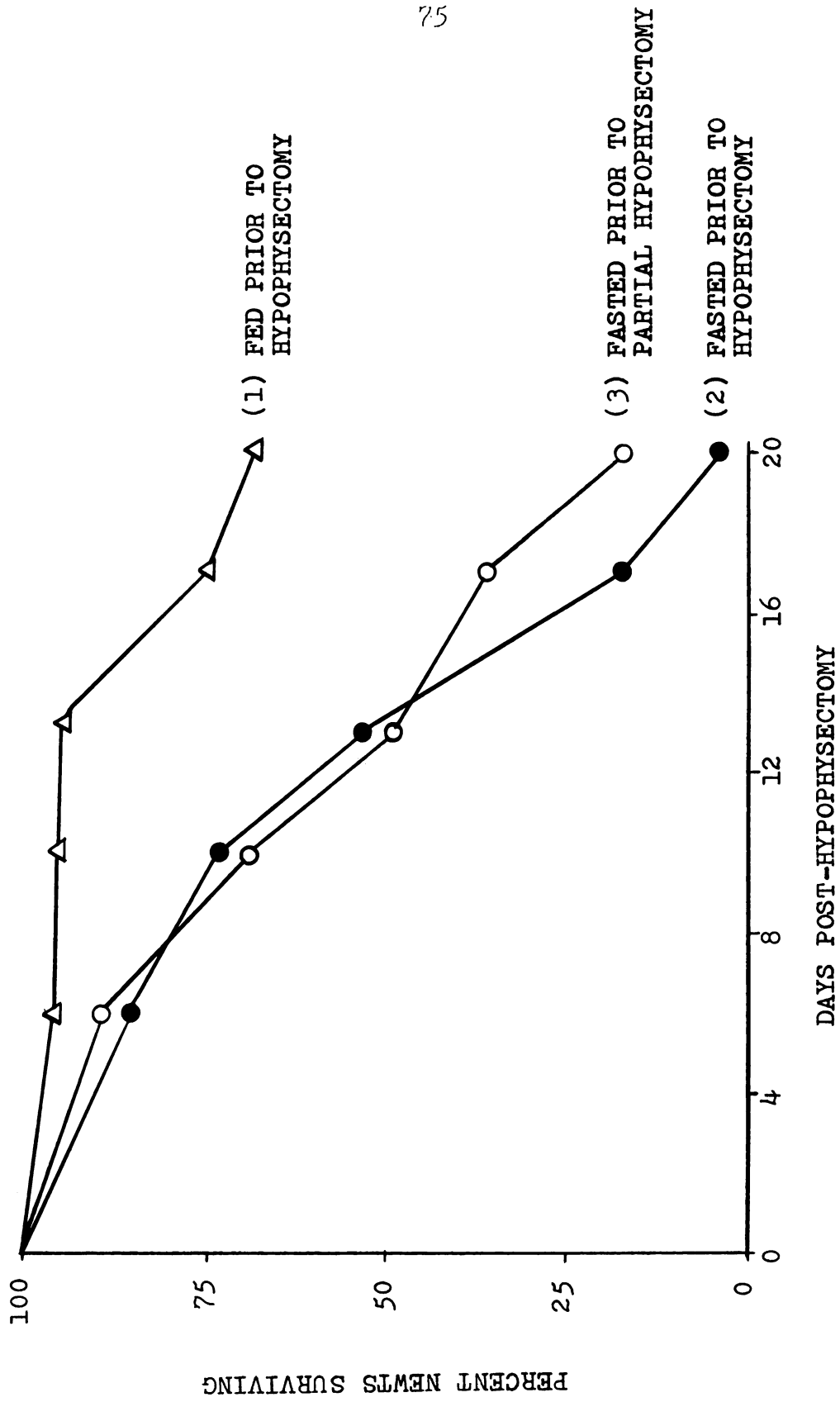


Fig. 22 A comparison of the percent survival of: 1) hypophysectomized newts fed for 2 weeks prior to hypophysectomy, 2) hypophysectomized newts fasted for 2 weeks prior to hypophysectomy, and 3) partially hypophysectomized newts fasted 2 weeks prior to partial hypophysectomy. ²⁴
Previously fed hypophysectomized newts survived in significant greater numbers (0.01 level of significance; Chi Square Test).

Figure 22

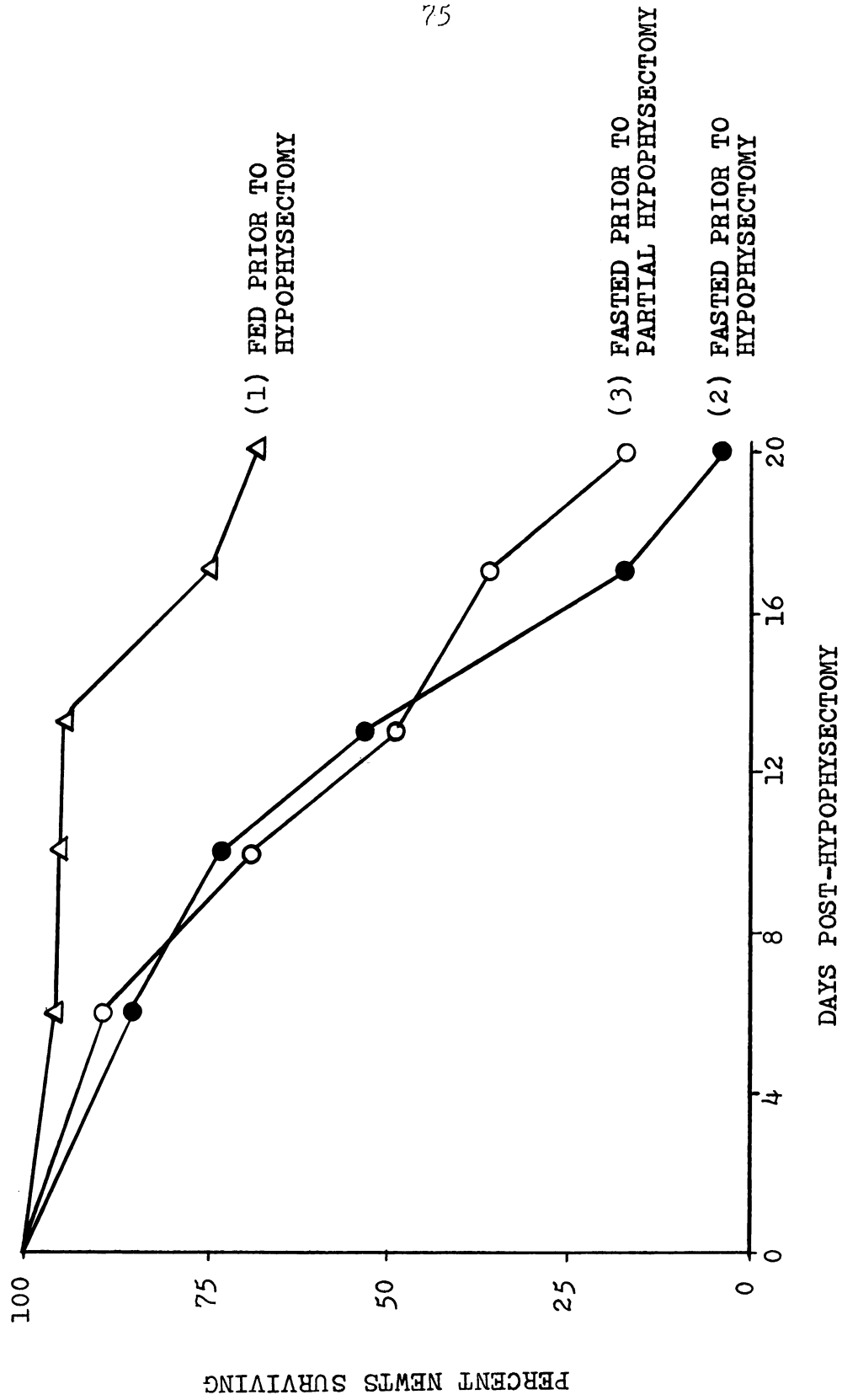
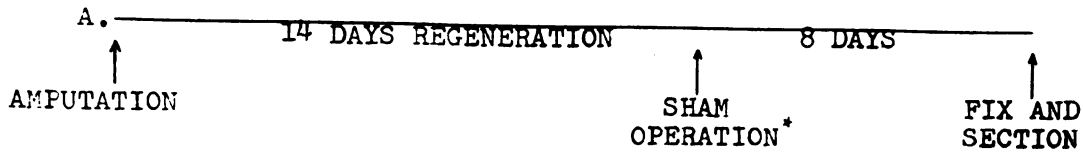
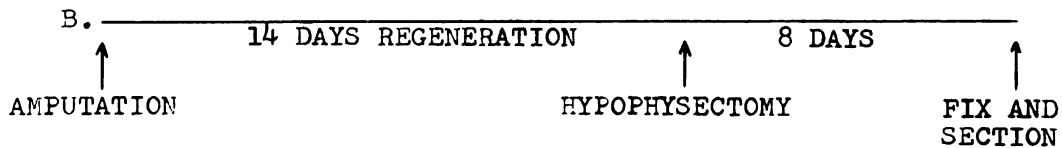
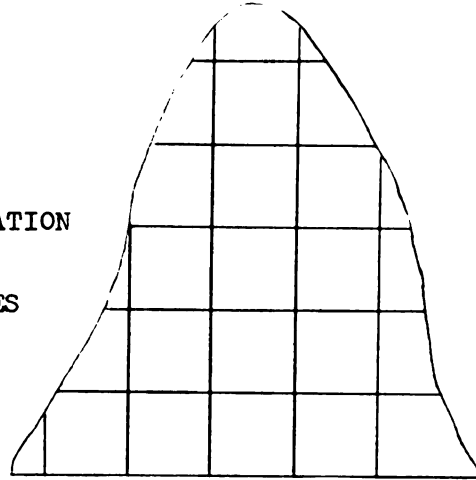


Fig. 23 The influence of hypophysectomy on newt limb blastema area. Fasted newts were hypophysectomized or sham-operated 14 days after amputation and 8 days after hypophysectomy or sham operation the limbs were fixed, sectioned and stained. A and B illustrate schematically the methods used. In A and B the section areas were obtained by projecting the blastema image onto graph paper from a constant height and tracing the blastema outline. The mean blastema areas of the sham-operated newts were significantly larger (0.01 level of significance; Mann-Whitney U Test).

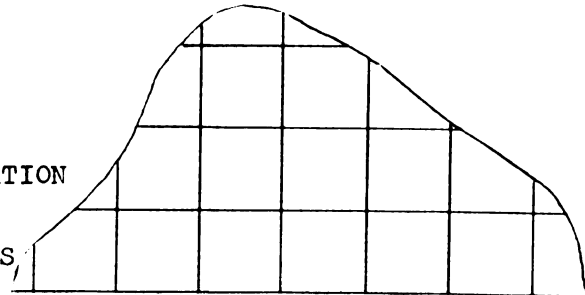
Figure 23



A. SAMPLE SECTION
 AREA = 16.11 SQUARES
 22 DAYS TOTAL REGENERATION
 MEAN OF GROUP = 15.91
 (6 NEWTS) SQUARES



B. SAMPLE SECTION
 AREA = 9.69 SQUARES
 22 DAYS TOTAL REGENERATION
 MEAN OF GROUP = 9.25
 (6 NEWTS) SQUARES



* SHAM-OPERATED NEWT BLASTEMAS EXHIBIT SIGNIFICANTLY LARGER AREAS (0.01 LEVEL, MANN-WHITNEY U TEST).

Fig. 24 A comparison of the mean blastema areas of newts hypophysectomized or sham-operated at 14 days after limb amputation and fixed 8 days post-operation or at 16 days post-operation. The blastema areas of Fig. 23 are here represented in bar graph form. The area of each square is equivalent to that of Fig. 23. The mean blastema areas of the sham-operated newts are significantly larger than those of the hypophysectomized newts both at 8 days post-operation and at 16 days post-operation (0.01 level of significance; Mann-Whitney U Test).

FIGURE 24

MEAN AREA OF BLASTEMAS 16 DAYS POST-SHAM	22.46 SQUARES	MEAN AREA FOR 8 LIMBS =
MEAN AREA OF BLASTEMAS 16 DAYS POST-HYPOX	15.22 SQUARES	MEAN AREA FOR 7 LIMBS =
MEAN AREA OF BLASTEMAS 8 DAYS POST-SHAM	15.91 SQUARES	MEAN AREA FOR 6 LIMBS =
MEAN AREA OF BLASTEMAS 8 DAYS POST-HYPOX	9.25 SQUARES	MEAN AREA FOR 6 LIMBS =

Fig. 25 A comparison of the percent survival of hypophysectomized newts treated with prolactin ($0.015 \text{ U/newt/2 days}$) + thyroxine (1×10^{-7} conc. in aquarium water), prolactin alone ($1.2 \text{ U/newt/2 days}$), thyroxine alone (1×10^{-7} conc.), and 0.9% saline ($0.1 \text{ cc/newt/2 days}$). Injections were begun 5 days after hypophysectomy. Prolactin + thyroxine, and prolactin significantly increased survival (0.01 level of significance; Chi Square Test).

Figure 25

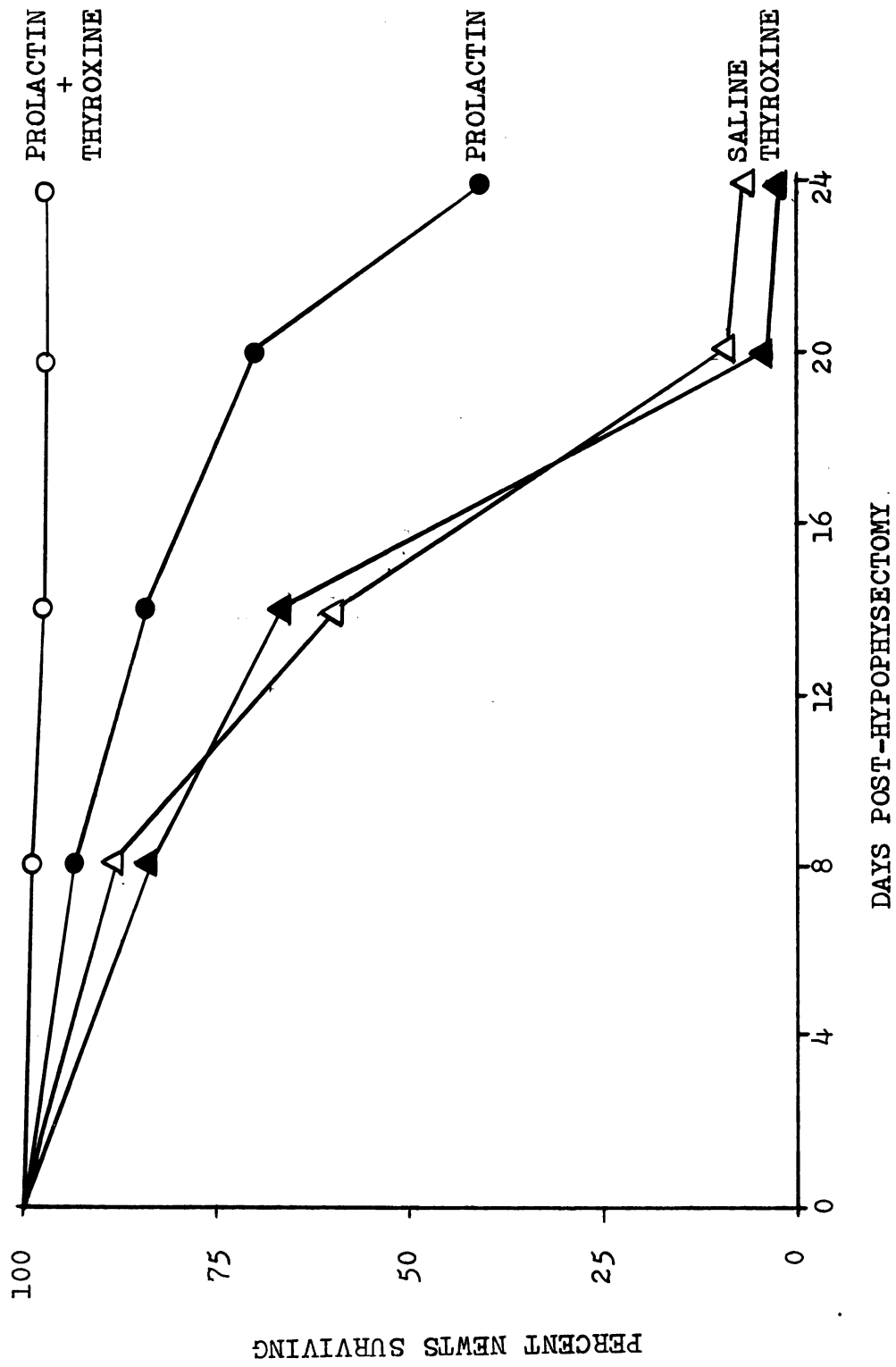


Fig. 26 A comparison of the percent survival of hypophysectomized newts receiving no treatment, prolactin (0.015 U/newt/2 days) + thyroxine (1×10^{-7} conc.), ACTH (1 U/newt/2 days), water (0.1 cc/newt/2 days). Injections were begun 10 days after hypophysectomy.

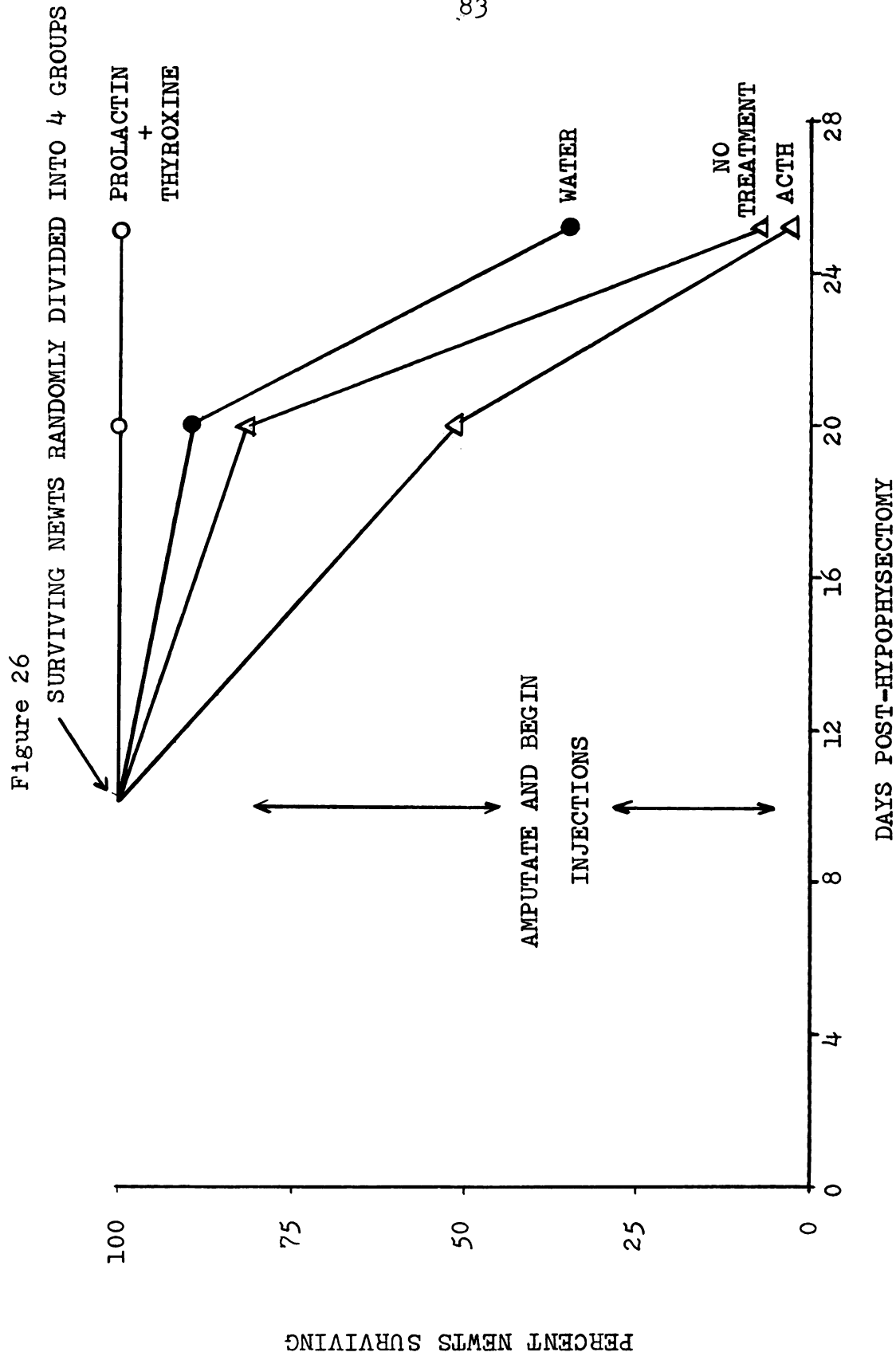


Fig. 27 A comparison of the percent survival of hypophysectomized newts

given growth hormone (0.03 mg/newt/2 days), prolactin (0.015 U/newt/2 days) + thyroxine (1×10^{-7} conc.), prolactin alone (0.015 U/newt/2 days), ACTH (1 U/newt/2 days), 0.9% saline (0.1 cc/newt/2 days) and ectopic, Ambystoma mexicanum pituitary grafts (2 pituitaries/newt).

Injections were begun and pituitaries were grafted 5 days after hypophysectomy. Prolactin + thyroxine, growth hormone, and Ambystoma mexicanum pituitary grafts significantly increased survival (0.01 level of significance; Chi Square Test).

Figure 27

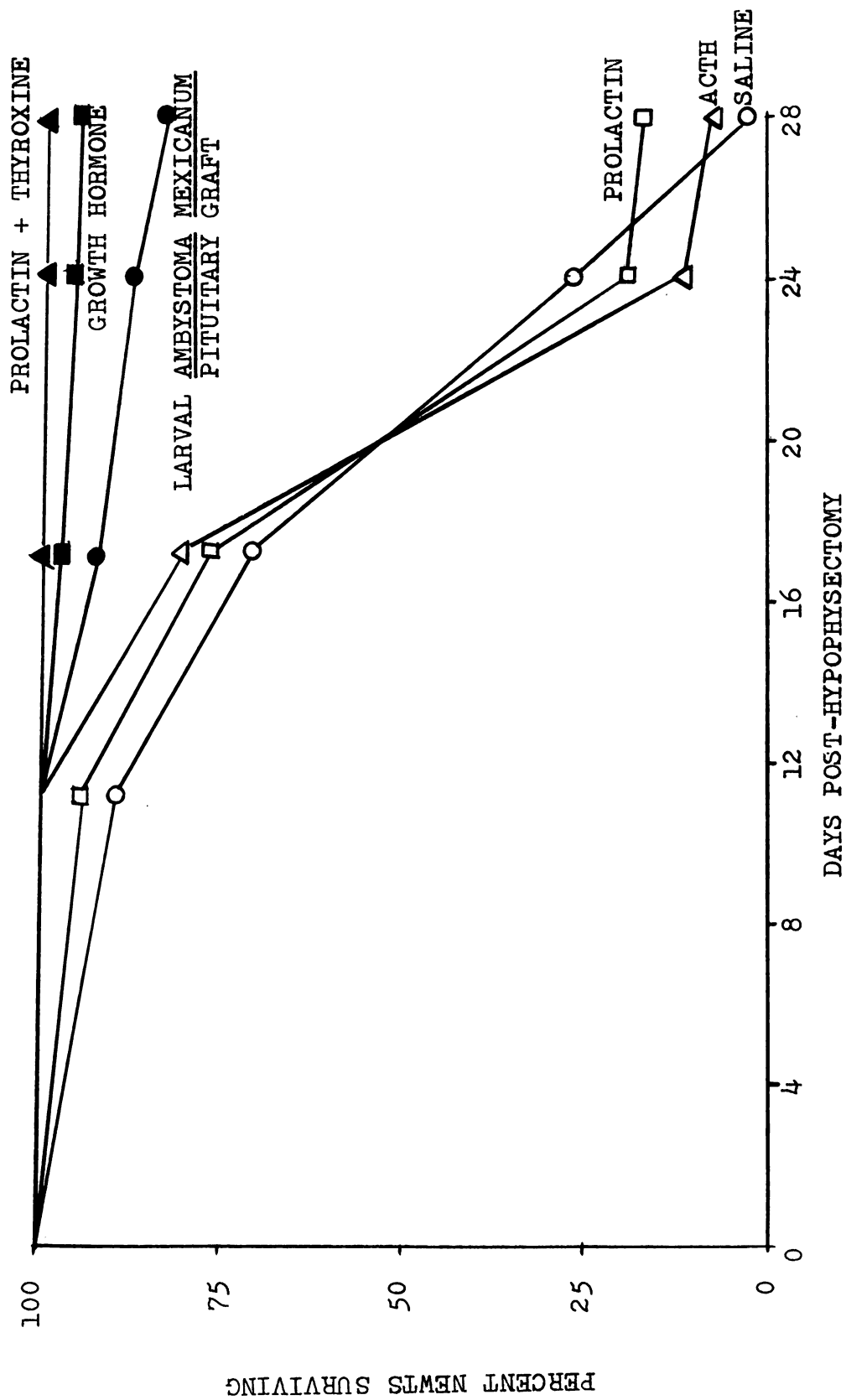


Fig. 28 A comparison of the percent survival of hypophysectomized newts treated with prolactin ($0.015 \text{ U/newt/2 days}$) + thyroxine ($1 \times 10^{-7} \text{ conc.}$), thyroxine alone ($1 \times 10^{-7} \text{ conc.}$), and 0.9% saline ($0.1 \text{ cc/newt/2 days}$). Injections were begun 10 days after hypophysectomy. Prolactin + thyroxine significantly increased survival ($0.01 \text{ level of significance; Chi Square Test}$).

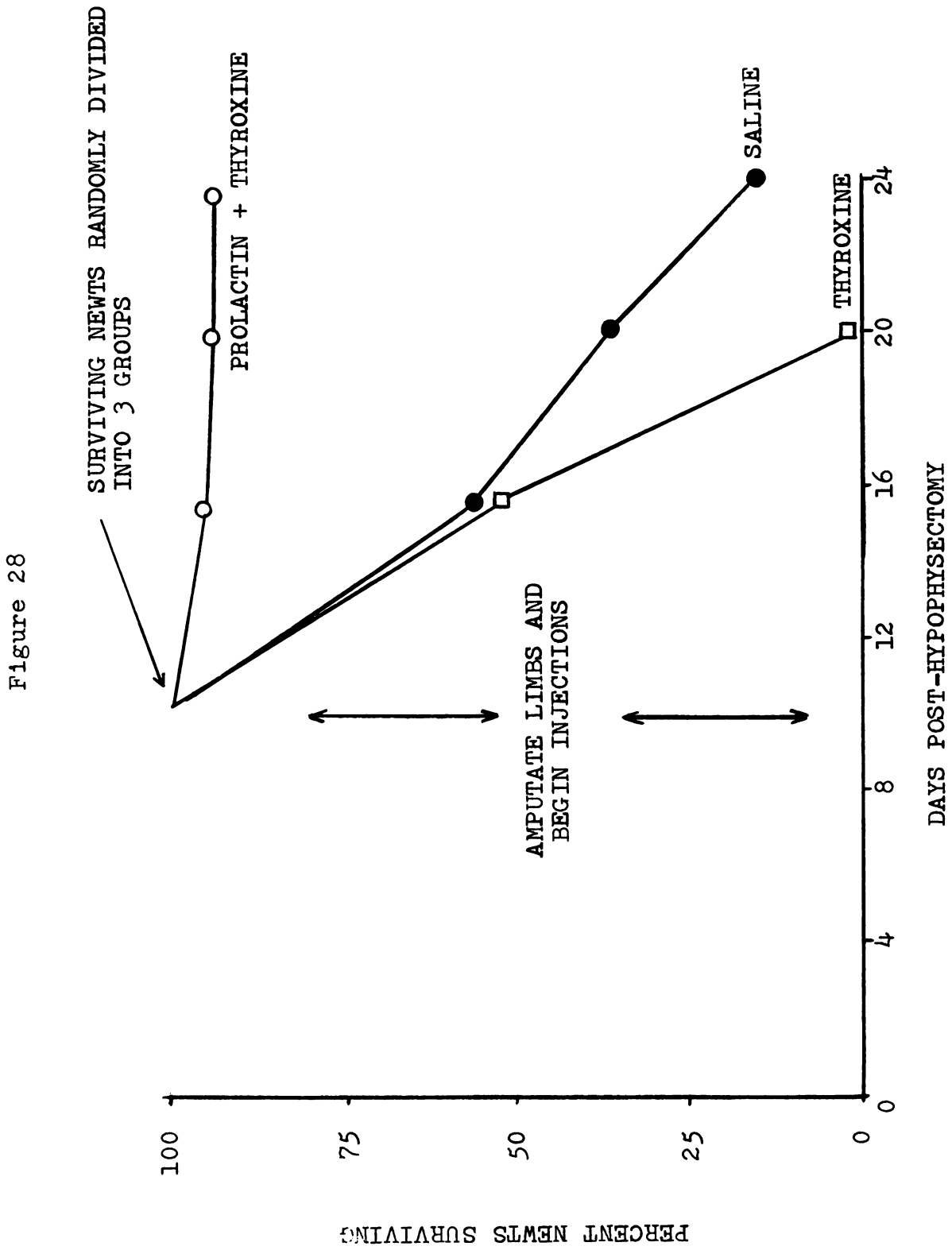
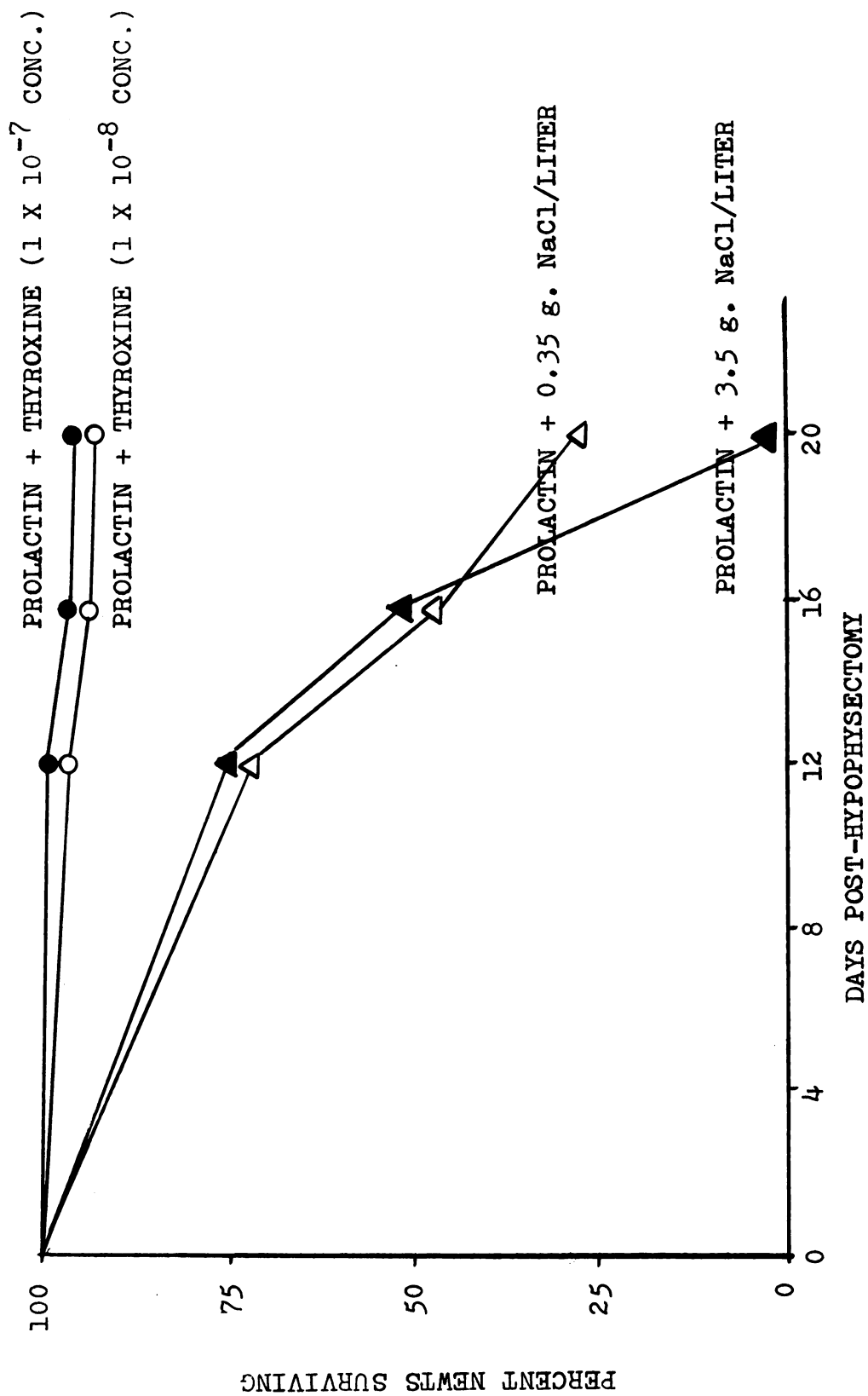


Fig. 29 A comparison of the percent survival of hypophysectomized newts

treated with prolactin ($0.015 \text{ U/newt/2 days}$) + thyroxine (1×10^{-7} conc.), prolactin ($0.015 \text{ U/newt/2 days}$) + thyroxine (1×10^{-8} conc.), prolactin ($0.015 \text{ U/newt/2 days}$) and maintenance in aerated water containing $0.35 \text{ grams NaCl/liter}$, and prolactin ($0.015 \text{ U/newt/2 days}$) and maintenance in aerated water containing $3.5 \text{ grams NaCl/liter}$.

Treatment was begun 1 day after hypophysectomy. Prolactin + thyroxine of either conc. significantly increased survival (0.01 level of significance; Chi Square Test).

Figure 29

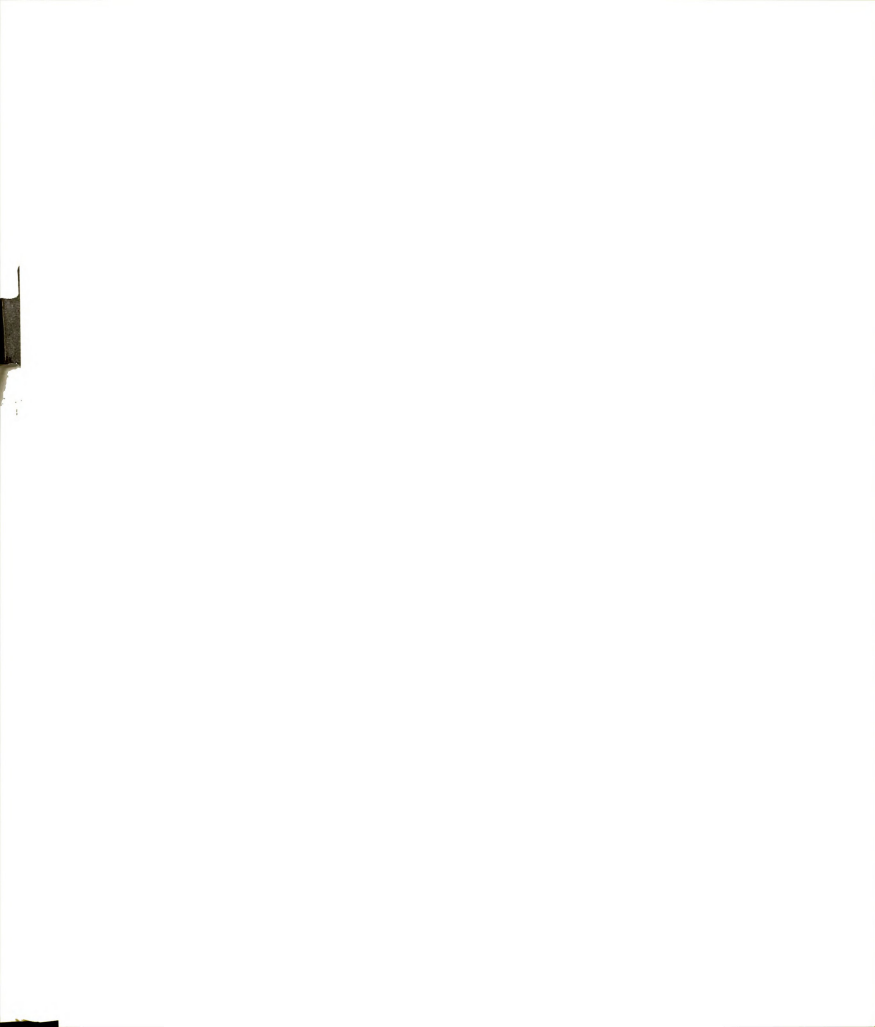


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