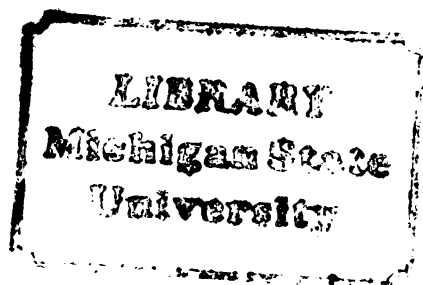


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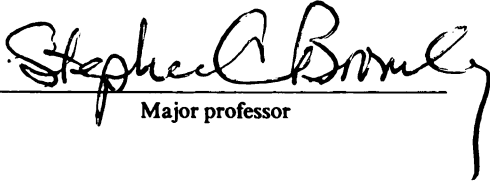
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AN EXAMINATION OF THE INTERACTION OF PROLACTIN WITH THE REGENERATING
LIMB OF THE NEWT NOTOPHTHALMUS VIRIDESCENS

By

Stephen Thomas Furlong

A DISSERTATION

Submitted to
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ABSTRACT

AN EXAMINATION OF THE INTERACTION OF PROLACTIN WITH THE REGENERATING LIMB OF THE NEWT NOTOPHTHALMUS VIRIDESCENS

By

Stephen Thomas Furlong

It is well-known that amphibian limb regeneration is influenced by a number of types of growth factors, including those originating in the pituitary. Past studies have indicated that limb regeneration is severely retarded in the absence of the pituitary and that regeneration in intact animals can be stimulated by injection of crude pituitary extracts or prolactin. It has not been determined, however, if levels of pituitary hormones fluctuate during the course of regeneration or in the manner in which these hormones exert their effect.

In this study, the relationship between one pituitary hormone, prolactin, and regenerating limb tissue was studied in detail. It was found that in the newt, pituitary hormone levels are within the range expected on the basis of studies of other amphibians. It was also found that during the course of regeneration there are no changes in the concentrations of any pituitary proteins in general, or of the protein identified as prolactin, in particular. Using

I-125 labeled ovine prolactin it was found that saturable binding sites are found in both unamputated and amputated newt limb tissue. These binding sites are specific for prolactin. During the course of regeneration there is increased binding at three days postamputation but no significant difference between regenerating and unamputated tissue at any other stage of regeneration. When I-125 prolactin is injected into animals unilaterally amputated, there is a marked accumulation within six hours in animals three days or more postamputation. In animals less than three days postamputation the accumulation effect is absent. The accumulation effect could also be demonstrated in unilaterally amputated animals which had also been either hypophysectomized or denervated.

Autoradiography of tissue sections from animals injected in vivo confirmed the results of accumulation studies. A marked concentration of grains was observed in regenerating limbs three days or more postamputation and grains were in particularly high concentration in the wound epithelium. Fine structural studies indicated morphological differences between wound epithelium and stump epidermis which may be related to the accumulation effect.

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KEY TO ABBREVIATIONS

BSA.....bovine serum albumin, fraction V

CPM.....counts per minute

GH.....growth hormone

ND-PAGE....nondissociating polyacrylamide gel electrophoresis

o-prl-15...lot number of prolactin received from National Institute
of Arthritis Metabolism and Digestive Diseases

PB.....phosphate buffer

PBM.....phosphate buffer to which was added .01 M magnesium chloride

P-S-8.....lot number of prolactin received from National Institute of
Health

prl.....prolactin

SDS.....sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Amphibian limb regeneration is a complex developmental system under the control of many interacting factors. These controlling factors are diverse in origin and include not only growth factors such as pituitary hormones, but also extracellular matrix molecules such as collagen and an important relationship between epithelium and underlying tissue. One of the most extensively studied of these is the "neurotrophic" factor (reviews, Singer, 1952, 1974; Thornton, 1968, 1970). As the name implies, this factor is thought to originate in the nerves associated with the limb. In the absence of nerve supply to an amputated limb, regeneration will not take place. In general, denervation causes a decline in DNA, RNA, and protein synthesis (Dresden, 1969; Lebowitz and Singer, 1970; Jabaily and Singer, 1977; Singer and Ilan, 1977). Baseline levels of these activities continue, however, indicating that there is not absolute neural control (Lebowitz and Singer, 1970).

In both in vivo (Singer, et al., 1976) and in vitro studies (Mescher and Loh, 1981; Carlone and Foret, 1979) a soluble protein fraction from homogenized newt brain may substitute for the neurotrophic factor with respect to stimulation of DNA, RNA, and protein synthesis. Exactly how the neurotrophic factor exerts its effect on the regenerating limb is unknown but one recent finding of possible significance in this regard is that innervation influences histone phosphorylation (Kelly and Singer, 1981). In limb regeneration this is

the first documented instance of a direct relationship between growth factor and genomic activation. Other recent work indicates that fibroblast growth factor (FGF) and the neurotrophic factor have similar effects in terms of stimulating protein and DNA synthesis (Carlone, et al., 1981; Mescher and Loh, 1981).

Another aspect of the control of limb regeneration is the epithelial/mesenchymal type relationship between the wound epithelium, which grows over the cut surface of the limb, and the underlying tissue (Thornton, 1968; Stocum, 1975). The function of the wound epithelium has variously been described as influencing aggregation of blastemal cells (Thornton, 1968), the mitotic cycle of blastemal cells (Globus, 1978; Tassava and Mescher, 1975) and the proximodistal progression of cartilage differentiation (Globus, et al., 1980). The mechanism by which the wound epithelium exerts its effect, however, is still unknown.

Changes in the distribution of extracellular matrix molecules and enzyme activity associated with these molecules are also known to accompany regeneration (Dresden and Gross, 1970; Grillo, et al., 1975; Johnson and Schmidt, 1974; Linsenmayer and Smith, 1975; Smith, et al., 1975; Toole and Gross, 1971) as well as changes in electrical activity of the limb (Borgens, et al., 1979, 1977; LaSalle, 1979) and permeability of the wound epithelium (LaSalle, 1980). It is obvious, then, that the regenerating limb is a very complicated system and the isolation of individual controlling factors a difficult task. Complex interactions exist between growth factors from diverse origins and concomitant changes in local tissue environment make separation of cause/effect relationships difficult. One final group of factors which influence limb regeneration is hormonal factors and it is this area that is the subject of this study.

Influence of the Pituitary on Limb Regeneration

Although hormones originating in a number of tissues have been shown to influence regeneration, those originating in the pituitary are probably the most well studied. Schotte's (1926) pioneering study showed that in the European newt, Triton, regeneration will not proceed at a normal rate in the absence of the pituitary. This observation was later confirmed in Notophthalmus viridescens (Richardson, 1945; Connelly, et al., 1968; Tassava, 1969a; Bromley and Thornton, 1974; Hall and Schotte, 1951). Schotte and Hall (1952) found that when hypophysectomy was delayed until three to 10 days after amputation, while no animals showed normal growth, the majority showed some growth by 24-43 days postamputation. In this experiment, of 155 animals examined 135 showed growth which was classified as abortive or delayed, while the remainder showed complete absence of growth. In this same experiment when hypophysectomy was delayed until 11-20 days postamputation, 17 of 83 animals regenerated normal limbs while the remainder were classified as delayed or abortive. Results from a similar type of experiment indicated that for animals hypophysectomized at 14 days postamputation the cross-sectional area of the limbs was 58.0% that of sham treated controls at 22 days postamputation (Tassava, 1969a). These results indicate decreased growth in limbs of hypophysectomized animals compared to controls. It is clear, however, that measurement of the amount of growth serves only as an indicator of the inhibition of limb regeneration caused by hypophysectomy for regeneration simply does not take place in the absence of the pituitary.

In addition to the stage of regeneration at which hypophysectomy is

performed the nutritional state of the animal also appears to influence the relationship between pituitary and regeneration. Tassava (1969a) reported that by feeding animals daily for two weeks prior to hypophysectomy survival and regenerative ability of animals was enhanced. In this experiment 12 of 16 "maximally fed" newts were alive 20 days post-hypophysectomy as opposed to one of 16 fasted for two weeks prior to hypophysectomy. In addition, in maximally fed animals amputated five days post-hypophysectomy, 16 of 16 showed signs of the early stages of regeneration by 26 days post-hypophysectomy. Another study on the relationship between pituitary nutrition and limb regeneration in adult Triturus pyrrhogaster (Sato and Inoue, 1973) indicated that limb regeneration is completed even in hypophysectomized animals, but is delayed about 15 days compared to controls. When hypophysectomized animals were injected every other day with a mixture of amino acids and glucose, growth of the regenerate was faster than hypophysectomized animals without such treatment. A delay in the early stages of regeneration was still evident, however.

In adult urodeles, then, the pituitary is clearly required for limb regeneration but the nutritional stage of the animal and stage of regeneration at which hypophysectomy is performed are important factors in this relationship. This is not the situation in larval urodeles for in these animals the pituitary is not required for successful limb regeneration (Schotte, 1961; Liversage, 1967; Tassava, et al., 1968). This point is generally underemphasized, for an understanding as to why the adult structures are dependent on pituitary influence but larval tissues are not may provide an important key to understanding control mechanisms in this aspect of limb regeneration.

Despite the fact that the pituitary has been shown to be necessary for successful regeneration in adults exactly which pituitary factors promote normal regrowth in the intact animal have not been conclusively shown. It is known that ectopic pituitaries are capable of sustaining essentially normal regeneration (Schotte and Tallon, 1960; Dent, 1967) as well as pituitaries which have been cultured for a period of time in the absence of the hypothalamus and subsequently implanted into hypophysectomized animals (Liversage and Liivamagi, 1971). The latter experiment allowed an examination of the importance of the pituitary at different stages of regeneration and indicated that the pituitary is important for initiation of regeneration as well as later differentiation. Pituitaries from larval urodeles are also capable of sustaining regenerative ability in adult animals (Tassava, 1969b). In the case of ectopic pituitaries, studies have indicated secretion of prolactin and TSH as determined by the induction of "water drives" behavior and maintenance of thyroid function in N. viridescens (Masur, 1962, 1969; Dent, 1966, 1967). It remains to be determined whether growth hormone or other factors are also released from amphibian pituitaries in the absence of the hypothalamus.

In intact animals, a number of hormone preparations have been shown to be effective in enhancing limb regeneration including impure pituitary extracts such as Antuitrin G and Phyone (Richardson, 1940, 1945) and prolactin (Schauble and Nentwig, 1974; Tassava and Kuenzli, 1979). Richardson (1945) reported growth of 4.4 mm in Antuitrin G treated animals vs. 4.0 mm for controls at ten weeks postamputation while Schauble and Nentwig (1974) report 0.69 cm of growth in prolactin treated vs. 0.48 cm growth in controls at 90 days postamputation (for

animals kept at 25 degrees C). Adrenocorticotrophic hormone, also an impure preparation, was reported to inhibit regeneration in intact animals (Schotte and Chamberlain, 1955), but other studies showed no effect (Bragdon and Dent, 1954). It is also interesting to note that treatment with thyroxine reportedly inhibits regeneration somewhat in intact animals (Richardson, 1945).

In hypophysectomized animals, several different hormones and combinations of hormones have been tried as replacement therapy with respect to sustaining normal limb regeneration. Injection of Antuitrin G permits limb regeneration to proceed (Richardson, 1940, 1945) but again the impure nature of these preparations prevented determination of the active factor(s). Adrenocorticotrophic hormone has been reported to be effective (Schotte and Chamberlain, 1955) but later studies indicated that this result could have been due to a contaminant present in the preparation (Tassava, 1969a). Frog anterior pituitary extract is also effective (Liversage and Scadding, 1969) as well as embryonic or adult chicken anterior pituitary extract (Liversage, 1974).

Initial studies indicated that the best results for promoting regeneration in hypophysectomized animals were obtained with either growth hormone by itself (Wilkerson, 1963) or by prolactin in combination with thyroxine (Connelly, et al., 1968; Tassava, 1969). As cross-contamination between pituitary hormone preparations was a major concern in these earlier studies, Bromley and Thornton (1974) re-examined the role of prolactin and growth hormone with more rigidly purified preparations. Their findings indicated that highly purified growth hormone was indeed capable of supporting limb regeneration as well as prolactin plus thyroxine. It is interesting in this regard that

while prolactin plus thyroxin was shown to restore regenerative ability to normal levels, growth hormone plus thyroxin significantly decreased the rate of regeneration. More recently Hessler and Landesman (1981a, b) have confirmed that injection of contamination levels of prolactin and TSH found in Wilkerson's growth hormone preparation were capable of sustaining a normal rate of regeneration. These same workers, however, have also confirmed that growth hormone, alone, is capable of supporting normal levels of regeneration. It has also been pointed out that while discontinuous administration of growth hormone is effective, continuous administration of prolactin/thyroxin is necessary (Landesman and Hessler, 1981). In this study a single injection of .029 I.U. of growth hormone permitted regeneration to the digitform stage in 40% of the animals. Three injections allowed regeneration to this stage in greater than 95% of the cases. By comparison no animals treated with prolactin/thyroxin on a similar regimen reached this advanced stage of regeneration. It is unlikely, though, that under physiological conditions a single large burst of any hormone would be responsible for stimulating regeneration.

Despite many studies aimed at determining which pituitary hormones are active in promoting limb regeneration little has been done with regard to how this interaction comes about. Vethamany-Globus and Liversage (1973a, b) have shown that hypophysectomy in the newt affects the histology of the pancreas and that experimentally induced diabetes interferes with both limb and tail regeneration. They have not shown in vivo, however, that replacement therapy with insulin can substitute for the influence of the pituitary. It has been suggested that one effect of prolactin/thyroxine or growth hormone may be in maintaining the

integrity of the relationship between wound epithelium and underlying tissue (Hessler and Landesman, 1981b; Landesman and Hessler, 1981). Even in these studies, however, a direct relationship between hormone and limb tissue was not established and it is conceivable that some other factor, such as somatomedin, may be acting as intermediary (Hessler and Landesman, 1981a). An examination of the effect of somatomedin on regeneration in intact and hypophysectomized animals may provide some interesting insights into hormonal control of regeneration. Until recently, however, somatomedin preparations have not been as readily available as those of pituitary hormones.

Another relationship which has been suggested in the past as important for regeneration is that between pituitary and adrenal (interrenal) tissue (Schotte, 1961). Schotte and Chamberlain (1955) reported that ACTH enhanced limb regeneration and survival in hypophysectomized animals but adrenal steroids injected alone reportedly do neither (Schotte and Bierman, 1956). Tassava (1969) has attributed the effect of the ACTH in the study by Schotte and Chamberlain to contaminants in their preparation. More purified preparations of ACTH are not effective in enhancing survival or limb regeneration (Tassava, 1969). It is interesting to note in this regard that lack of effect of purified ACTH does not necessarily rule out the existence of a "pituitary/adrenal axis." Prolactin, for example, has been shown to bind and be active in stimulating adrenal tissue (Solyam, et al., 1971; Edwards, et al., 1975; Marshall, et al., 1975).

To date, the only study directly linking changes in the affinity of regenerating limb tissue for any hormone is that of Bromley (1977). In this study labeled corticosterone, cortisol and estradiol preferentially

accumulated in the portion of the regenerating limb bearing the amputation site. Whether this change in affinity for these hormones is related to changes in specific receptors has not yet been determined. As noted above, the fact that ACTH alone does not appear to be able to substitute for the pituitary does not rule out stimulation of interrenal tissue by other hormones and subsequent elevation of corticosteroid levels. It is also known that in some amphibians corticosteroids can be produced in the absence of pituitary stimulation (Ball, 1981).

In Vitro Studies

Numerous studies have been done on the influence of neurotrophic and hormonal factors on explanted regenerates. In general it has been suggested that as with in vivo regenerates, both a source of neurotrophic factor(s) as well as hormonal factors are required to maintain the regenerate (Globus, 1978). Several growth factors such as epidermal growth factor and fibroblast growth factor have been shown to stimulate mitotic index, DNA synthesis and protein synthesis (Carlone, et al., 1981; Mescher and Loh, 1981). This has led to the suggestion that they may be similar to the neurotrophic factor.

With respect to hormonal factors, in a number of studies insulin alone appears to be as effective in stimulating growth of the regenerate as a number of combination of other hormones (Vethamany-Globus and Liversage, 1973a, b, c; and Vethamany-Globus, et al., 1978). It was reported, for example, that the presence of insulin in the culture medium resulted in 168% increase in C^{14} labeled amino acid incorporation into proteins as compared to controls (Vethamany-Globus, et al., 1978). With one exception, however, all of the in vitro studies have been on

later stages where the influence of the pituitary is known to be the least (Schotte and Hall, 1952). In the one study done on early stage regenerates, growth hormone was shown to result in better maintenance of the epidermis (Bromley and Angus, 1971).

In addition, as indicated above, no study has been done which indicates that replacement therapy with insulin can entirely replace the influence of the pituitary in vivo. In light of the fact that numerous growth factors are present in the serum used in these in vitro studies, the evidence that insulin is the sole hormone necessary for regeneration is not compelling. Although studies with cultured regenerates allow for more careful control of experimental conditions it seems that earlier stage regenerates must be further examined before the mode of action of the pituitary can be fully appreciated in cultured regenerates.

Properties of Amphibian Pituitary Hormones

Although it is generally accepted that pituitary hormones play a role in limb regeneration in the intact animal, no systematic study has been undertaken to determine if fluctuations occur in levels of pituitary hormones during the course of regeneration. By analogy, other developmental systems in which hormonal influences are prominent (i.e., mammary gland development, mammary cancer and amphibian metamorphosis) may show fluctuation in hormone levels, receptor levels or both (Kelly, et al., 1982; Clemons and Nicoll, 1977; White and Nicoll, 1979; Carr, et al., 1981; Meites, 1980). The fact that hormonal levels from animals with regenerating limbs have not been examined may be partially due to the difficulty in obtaining sufficient quantities of pure amphibian

hormone to create antisera for RIA. Although there is generally some cross-reaction between antisera to mammalian hormones and their amphibian counterparts, the amount of cross-reaction is variable and unpredictable (McKeown, 1973; Hayashida, et al., 1973; Hayashida, et al., 1975).

In replacement therapy studies, also, difficulty in obtaining sufficient quantities of pure amphibian hormones has led to the use of mammalian hormones of various levels of purity. Although these studies have served well to point out the major influences of pituitary hormones on limb regeneration, differences between the amphibian and mammalian hormones may have served to make the issue of which hormones are active even more confusing. Among mammalian hormones, there appears to be considerable overlap in structure and function among certain hormones. It is becoming increasingly apparent that prolactin and growth hormone belong to a family of molecules with similar structure and function (Ensor, 1978; Farmer, et al., 1977; Hayashida, et al., 1973). In general these molecules are relatively conservative, accounting for the fact that mammalian hormones show binding and biological activity in tissues from fish, amphibians and reptiles. It is interesting to note, however, that while mammalian prolactins and growth hormones are generally distinguishable by RIAs, when these RIAs are applied to amphibian hormones, cross-reactions are often evident between antisera to mammalian growth hormones and amphibian prolactins (Hayashida, et al., 1973; McKeown, 1973). In addition, even in mammalian systems, growth hormones and prolactins seem to bind to the same "lactogenic" receptors (Bhattacharya and Vonderhaar, 1981; Waters and Frieson, 1979).

Finally, it has been suggested that in some amphibians, prolactin is

the major "growth hormone" as indicated particularly by studies with ranid tadpoles (Ensor, 1978; Nicoll, 1978). It should not be inferred from the above discussion that prolactin and growth hormone are the same molecule for they are clearly distinguishable in nearly all vertebrates thus far examined. It should be noted, however, that prolactin and growth hormone exhibit a large degree of sequence homology and exhibit similar activity in a wide range of assays (Bentley, 1976).

In studies with non-dissociating polyacrylamide gel electrophoresis (Nicoll and Nichols, 1971), it was found that prolactin activity was associated with well-defined bands across a wide range of vertebrate species as determined by the pigeon crop sac test. In general prolactin migrates ahead of any other major protein band. Beyond that, however, there was a wide range of R_f values which did not appear to show any direct relationship to vertebrate class. Examination of tetrapod somatotropins also by non-dissociating PAGE revealed prolactins and somatotropins are electrophoretically separable molecules. In bioassays, however, both prolactins and somatotropin have somatotropic activity. Relative amounts of growth hormone and prolactin in the adenohypophysis also varies among vertebrate classes. While mammals have higher concentration of growth hormone than prolactin, birds and reptiles show no appreciable difference between the two, and amphibians are found to have relatively higher prolactin levels with lower concentration of growth hormone (Nicoll and Licht, 1971). It is interesting to note that in some urodele species no growth hormone activity was found at all. With respect to thyrotropic activity pituitaries across a wide group of vertebrates do not appear to have been examined as they were for prolactin and growth hormone. In A.

mexicanum, however, the pituitaries from sexually mature (neotenic) animals were examined by PAGE and a slow migrating peak was found to have thyrotropic activity (Schultheiss, 1980).

Classical staining procedures and immunocytochemistry have shown that in most amphibians prolactin is produced in acidophils (lactotrophs) in either the whole gland or the anterior two thirds while growth hormone is produced by acidophils (somatotrophs) in the dorsocaudal zone (Doerr-Schott, 1976 review). Release of amphibian pituitary hormones is somewhat under control of the hypothalamus but probably less so than in mammals (Ball, 1981). In Triturus cristatus prolactin release is under inhibitory control of the hypothalamus, probably dopaminergic in origin (Lodi and Mazzi, 1976) and this appears to be the general case for most amphibians (Ball, 1981). Thyrotropin releasing factor is abundantly contained in amphibian hypothalamus and forebrain such as in the newt (Grimm-Jorgensen and McKelvy, 1974) but a role for this molecule has been more clearly established in stimulating prolactin release than thyroid stimulating hormone (Hall and Chadwick, 1976, 1979; Clemons, et al., 1979). Secretion of GH has been shown to be independent of hypothalamic control in some urodeles (Holmes and Ball, 1974; Schultheiss, 1979) while dependent on a releasing factor in anurans (Holmes and Ball, 1974; Hall and Chadwick, 1979).

As described above, both growth hormone and prolactin in combination with thyroxine have been shown to be capable of supporting regeneration in hypophysectomized animals. It is not known, however, in what manner these hormones exert their influence in this system nor has this question been systematically addressed. In the following paragraphs is a brief review of the effects of these hormones which may be particularly pertinent to the present study.

Many studies have been done on the effects of somatotropins on amphibian tissues and in general these actions can be divided into several broad categories including: growth promoting activity, reproductive activity, osmoregulatory activity, integument effects, and metabolic actions (Ensor, 1978; Clarke and Bern, 1980, reviews). As cited above, there is some question as to whether amphibian prolactin or growth hormone has more growth promoting activity, particularly in larval stages (Ensor, 1978). In fact, Nicoll (1978) has suggested that prolactin may be more active than growth hormone in this regard in fetal and early postnatal mammals. Regardless of which hormone is more important it seems clear that both have some growth stimulating activity. Prolactin appears to stimulate growth of the regenerate in intact newts (Schauble and Nentwig, 1974) however, the manner in which the hormone was acting was not addressed. This is an interesting point because it indicates that not only are prolactin or growth hormone necessary for regeneration but also that the concentration of these hormones may be a limiting factor with respect to rate of growth. It should be noted, however, that purified growth hormone has not been examined in this manner in intact animals.

One interesting clue as to the nature of prolactin's influence on regeneration is through its effect on skin. It has been shown that prolactin stimulates mitotic activity in the epidermis in intact animals and this activity is depressed in hypophysectomized animals. This decrease in activity is not corrected by addition of thyroxine alone (Hoffman and Dent, 1973a). In this same study it was also shown that ergocryptine, an inhibitor of prolactin release, also lowers mitotic activity. Other studies in N. viridescens have shown that prolactin

influences other integumental structures such as nuptial pad development and tail fin height (Tassava and Kuenzli, 1979; Singhas and Dent, 1975; Dent, 1975) as well as influencing skin texture and mucous secretion (Dent, et al., 1973). As reported by Hessler and Landesman (1981b) one of the primary effects of both prolactin and growth hormone in limb regeneration appears to be on the wound epithelium, a structure which originates in the stump epidermis adjacent to the cut surface of the limb.

Another important effect of prolactin is in osmoregulation. Brown and Brown (1973, 1978) have reported an interaction with thyroxine in the newt (N. viridescens) which influences cutaneous secretion, hydromineral metabolism and molting. It has also been reported that prolactin depresses sodium transport across newt skin (Lodi, et al., 1978) and stimulates influx of sodium across isolated frog skin (Howard and Ensor, 1978). Furthermore, it has been suggested that many of the prolactin-associated events in amphibian metamorphosis are mediated by changes in the ionic environment (Clarke and Bern, 1980 review). The effect of prolactin on ion transport, then, is particularly interesting in light of the proposed sodium ion involvement in bioelectrical events in limb regeneration (Borgens, et al., 1977, 1979). It has also been suggested that many of the prolactin-associated events in amphibian metamorphosis are mediated by changes in the ionic environment (Clarke and Bern, 1980 review). It would not seem unreasonable, then, to expect that a relationship exists between hormonal and electrical events in developmental systems such as the regenerating limb. This relationship may come about by a hormone, such as prolactin, causing changes in ionic distribution which may, in turn, affect distribution of electrical charge between or among tissues.

In addition to the effects listed above prolactin also appears to have general metabolic effects such as influences on lipid and carbohydrate metabolism and amino acid transport (DeVlaming, 1979). As growth hormone is also known to have general metabolic effects an important effect of these hormones with respect to limb regeneration is undoubtedly in this capacity. Cells depressed in essential metabolic capacities may be expected to be deficient in developmental function as well.

Receptor Studies

In the past it has generally been accepted that polypeptide hormones exert their influence through interaction with the plasma membrane (Cuatrecasas, 1974). More recent evidence from cultured mammary explants, however, suggests that in some cases internalization of hormones such as insulin and prolactin may mediate more subtle, long term effects, and undoubtedly has an influence on regulation of receptors (Djiane, et al., 1982). Regardless of the exact nature of the interaction between hormone and cell, however, receptor studies provide vital clues as to the involvement of hormones in various processes. For protein hormones, in nearly all cases receptor experiments are conducted using hormones labeled with Iodine-125. This is generally the method of choice because small amounts of hormone can be labeled to high specific activity, because labeling methods are generally gentle enough that hormones retain biological and immunological activity and finally because labeling methods are applicable to a wide range of hormones. In mammals a large number of studies have been done to examine binding of 125 I labeled hormones to various tissues. In amphibians the number of

studies is much more limited. In the species of interest in this study only prolactin receptors have been examined and then only in liver and kidney. Prolactin receptors have also been found in tail, gill, and kidney of other larval amphibians (Carr and Jaffe, 1981; Carr, et al., 1981; White and Nicoll, 1979) and liver, gut, bladder, and kidney of adult amphibians (White, 1981). In a study of the properties of the prolactin receptor, Carr and Jaffe (1981) found that the prolactin receptor complex was 114,000 daltons in liver and 103,000 in tail fin, both from Rana catesbiana. This compared with 170,000 daltons for the prolactin-receptor complex from rat liver. In addition, in this same study, charge differences between the receptor complex from liver and tail fin suggested that the receptor molecules differ from these two tissues. Despite numerous receptors studies on mammalian tissues, no examination of receptor binding for any protein hormone has been examined in limb regenerates. Receptor binding has been examined to some extent in regenerating mammalian liver (Pezzino, et al., 1981; Mourelle and Rubolcava, 1981; Leffert, et al., 1975) but these studies shed little light on the situation in the limb regenerate.

Purpose of Study

Although a number of studies in the past have pointed to the importance of pituitary hormones in regeneration several aspects of this interaction clearly need further examination. For example, it is not known if the trauma of amputation influences levels of pituitary hormones, either in the pituitary or in the circulation. Furthermore no study has examined the relationship between receptors for pituitary hormones and different stages of regeneration. It is, in fact

conceivable that pituitary hormones do not act directly on limb tissues, but rather through an intermediary such as somatomedin. Finally, it is not clear if pituitary hormones are equally necessary for all tissues of the regenerating limb or rather have a preferential effect on one tissue type. In this study, then, an attempt has been made to examine various aspects of pituitary involvement, particularly with respect to prolactin.

In general, the study can be broken down into three major parts:

- I. Examination of native pituitary hormones in the newt by polyacrylamide gel electrophoresis. These experiments will provide a means of answering several questions: 1) Are there any pituitary proteins, particularly prolactin or growth hormone, which change dramatically during the course of regeneration? 2) What, in fact, are the major newt pituitary proteins, as they have not previously been examined in detail? 3) Specifically, what is the concentration of newt prolactin in the pituitaries of intact animals and from those with regenerating limbs?

- II. Use of I¹²⁵ labeled ovine prolactin to examine interactions between this hormone and regenerating limb tissue. Using this labeled mammalian hormone it is possible to examine changes in receptor levels related to stage of regeneration and, in fact, to determine if receptors are present in limb tissue. Furthermore, use of this label with autoradiography of tissue sections permits localization of this hormone to particular cell and tissue types in the regenerating limb.

III. Fine structural examination of the wound epithelium. Previous studies have suggested that one structure in the regenerating limb which may be particularly influenced by the pituitary is the wound epithelium (Hessler and Landesman, 1981b; Tassava, 1969). Using a combination of scanning and transmission electron microscopy this structure was studied in detail from both intact and hypophysectomized animals. In addition, the regenerate of an anuran which regenerates only a cartilaginous spike rather than a complete limb was also studied in this manner. The intent of this work, then, was to attempt to correlate morphological changes in the wound epithelium with different stages of regeneration and under conditions in which successful regeneration is known to not take place.

By correlating the results from these different types of studies it is hoped that a clearer picture will emerge of exactly how pituitary hormones influence the regenerating limb.

Methods and Materials

Animals

Newts were obtained from Bill Lee's newt farm, Connecticut Valley Biological Supply or collected in Southern Ohio. Animals from different sources were kept segregated to minimize variations which may arise due to different lengths of time in the laboratory and other variables. During the entire course of study animals were routinely fed with beef or pork liver and kept in tap water at ambient room temperature.

Electrophoresis of Newt Pituitary Proteins

Non-dissociating disc gel electrophoresis was performed according to the method of Davis (1964). Pituitaries were removed as described by Bromley and Thornton, (1974), homogenized in 100 μ l of stacking gel, buffered and layered onto the stacking gel. Gels were 1.0 cm i.d., the resolving (7.5% acrylamide) gel 5.0 cm long and the stacking gel (3% acrylamide) 0.5 cm. Gels were run at 1.5 ma/tube until the tracking dye (bromphenol blue) reached the resolving gel and subsequently run at 2.5 ma/tube. Gels were removed from tubes by rimming with a syringe needle and stained in 0.25% Coomassie Blue R-250 (Bio-Rad) in methanol/acetic acid/H₂O (5:1:5) overnight or buffalo black. Destaining was done in methanol/acetic acid/H₂O (1.5:1.0:17.5) in a diffusion destainer. Destained gels were stored in 7% acetic acid until photography or densitometry.

SDS Gel Electrophoresis

SDS electrophoresis was run according to Maizel (1971). Gels were cast in 4 mm i.d. glass tubes 135 mm in length. The resolving gel was 10% acrylamide and 9.5 cm long, the stacking gel 3% and 2.0 cm long. Pituitaries were homogenized in 100 l sample buffer (10% mercaptoethanol .002% bromphenol blue, 10% glycerol, 1% SDS, .062 M Tris pH 6.7) immersed in a boiling water bath for two minutes, layered onto stacking gel and covered with 10% glycerol. Gels were run for approximately 3.5 hours (until tracking dye was approximately 1.0 cm from bottom of the tubes) at 100 V with a Heathkit constant voltage power supply. Following electrophoresis, gels were stained in 0.25% Coomassie Blue R-250 in methanol/acetic acid/H₂O (5:1:5) for one hour and diffusion destained for approximately 36 hours in Methanol/acetic acid/H₂O (1.5:1.0:17.5). Destained gels were subsequently scanned on a Beckman Model 24 spectrophotometer equipped with a gel scanner at 620 nm. Areas under the major peaks were integrated using a planimeter, by calculating 1/2 bh or by cutting out and weighing the peaks. These methods were found to give comparable results. Peaks were expressed as arbitrary density units/g body weight.

When slab gel electrophoresis was used, acrylamide concentrations, etc., were the same as used in tube gels (Maizel, 1971). Gels were cast 0.8 mm thick and the gel dimensions were 11 x 20 cm. Gels were run with a Heathkit power supply at 50 V until the tracking dye was into the resolving gel and subsequently at 100 V. Gels were stained and destained by the method of Fairbanks, et al. (1971) using .035% Coomassie Blue in 25% isopropanol and acetic acid. Gels were dried

using a Bio-Rad slab gel drier for one hour. Molecular weight standards were lysozyme (MW = 14,300), β -lactoglobulin (MW = 18,400), trypsinogen (MW = 24,000), pepsin (MW = 34,700), egg albumin (MW = 66,000) all from Sigma Chemical Co.

Preparation of I-125 Labeled Prolactin

Two methods of protein iodination were compared: Chloramine T (Aubert, et al., 1974) and lactoperoxidase (Sakai and Banerjee, 1979). I¹²⁵-labeled prolactin was prepared by each method as follows:

Chloramine T: added in order 1) 21 l phosphate buffer (0.05 M pH 7.2); 2) 5 μ l prolactin (1 mg/ml NIH p-S-8); 3) 10 μ l I¹²⁵ (0.1 μ Ci/ 1 Amersham; 4) 14 l chloramine T (5 mg/ml Sigma). Thirty seconds after addition of Chloramine T the reaction was stopped by the addition of 20 l Na metabisulfate.

Lactoperoxidase: added in order 1) 10 l I¹²⁵ (0.1 mCi/ 1 Amersham); 2) 25 l 0.5 M phosphate buffer (pH 6.9); 3) 10 l prolactin (0.5 mg/ml NIH p-S-8 or NIAMDD 0-prl-15 in 0.05 M NaHCO₃); 4) 5 l lactoperoxidase (1.0 mg/ml in 0.05 M phosphate buffer pH 7.3 A₄₁₂/A₂₈₀:minimum 0.9 (Sigma). The reaction was sustained by adding 10 l H₂O₂ (1:10,000) at 1, 3, 6 minutes. After 10 minutes the reaction mixture was diluted by addition of 500 l .05 M phosphate buffer pH 7.3.

In later experiments to ensure that I¹²⁵ was covalently bound to protein the lactoperoxidase procedure was modified such that amount of lactoperoxidase added was increased to 25 g and the concentration of H₂O₂ to 1:1000. When compared in binding studies the two lactoperoxidase prepared preparations behaved identically. The modified procedure, however, resulted in labeled prolactin completely free from non-covalently bound I¹²⁵ (as determined by SDS gel electrophoresis).

Separation of Labeled Protein From Iodine

Initially labeled protein was separated from free iodine by first running over sephadex G-25 (Fig. 1) and the labeled protein was subsequently run over sephadex G-100 (fine) with 1% BSA as eluting buffer. Fractions were monitored by 5 1 aliquots in 1.0N NaOH. In subsequent studies it was found that essentially the same results were obtained in binding studies by a single separation on Sephacryl S-200 with 1% BSA/.05M PB as eluting buffer (fig. 2). In general, lactoperoxidase catalyzed iodination followed by separation of iodine from labeled protein on Sephacryl S-200 emerged as the simplest and most effective technique. Using this method approximately 90% of the label could be precipitated with TCA as described by Greenwood, et al., 1963. The labeled product was also immunologically active and could be immunoprecipitated with rabbit antiovine antiserum.

In Vitro Receptor Studies

In vitro receptor studies were performed either on the low speed (600 xg) pellet of tissue homogenized in .05 M Tris (McNeilly, et al., 1980) or on a crude homogenate homogenized in .05 M PBM (Catt, et al., 1979; Barkey, et al., 1979). All homogenization was done in a glass/glass homogenizer. Figure 4 shows the areas from which tissue was harvested for various experiments.

In experiments on different stages of regeneration, for each data point 8 limb segments from 4 bilaterally amputated animals were homogenized with 100 strokes of the glass/glass homogenizer in 0.9 mls .05 M PBM. 100µls of homogenate was added to each of 6 tubes as

Figure 1

I¹²⁵ labeled prolactin was prepared by lactoperoxidase catalyzed iodination of o-prl-15 (NIAMDD). For separation of labeled hormone from free I¹²⁵ the preparation was run over a Sephacryl S-200 column eluted with .05 M phosphate buffer, 1% BSA, pH 7.3.

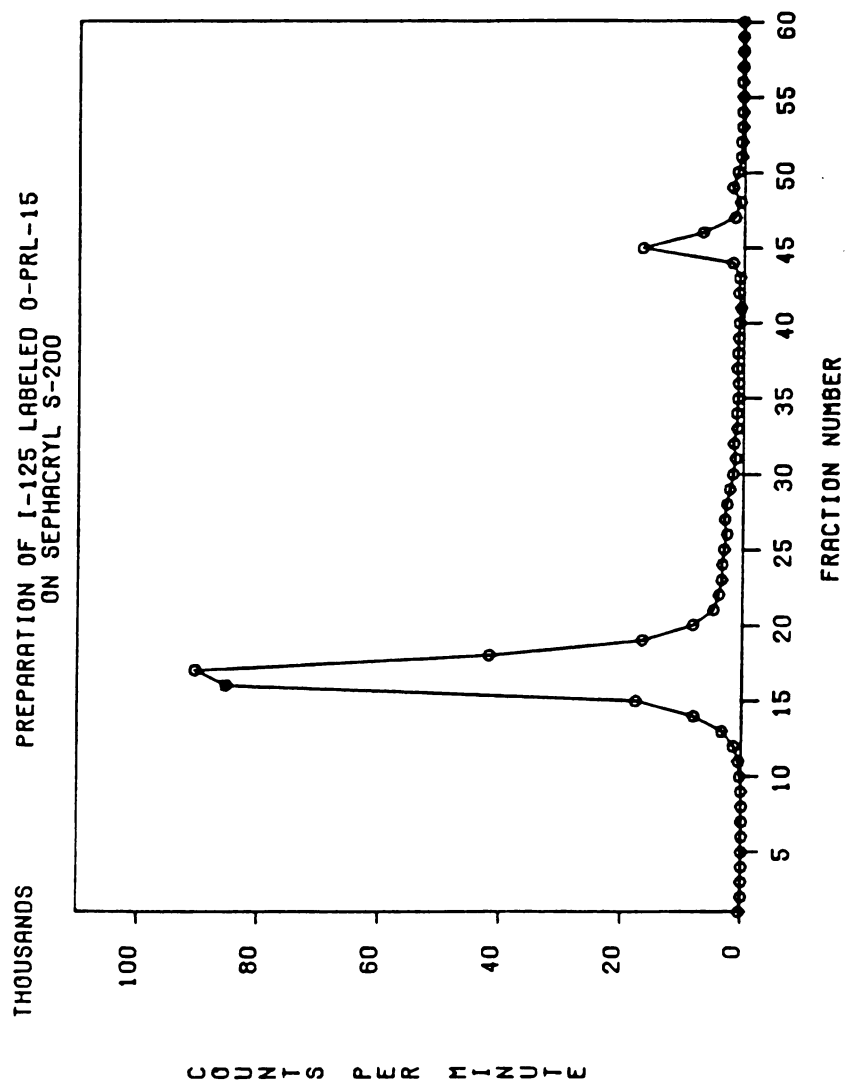
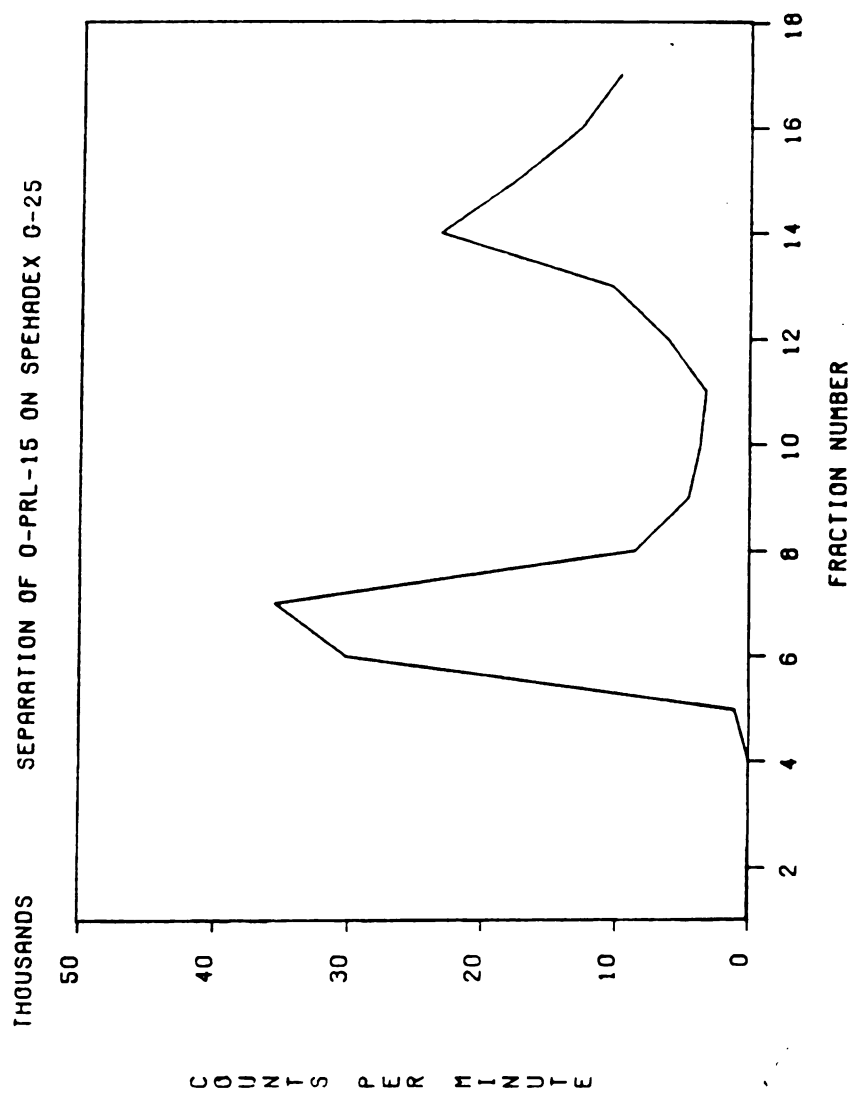




Figure 2

Separation of labeled prolactin from free iodine on Sephadex G-25. Labelled material was eluted from the column with .05 M phosphate buffer, 1% BSA, pH 7.3.



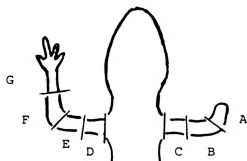


Figure 3 Line diagram of anterior portion of a newt illustrating location of segments sampled for accumulation experiments, dorsal view.

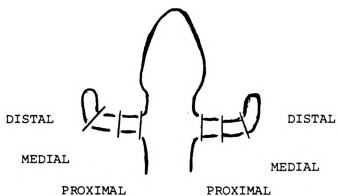


Figure 4 Line diagram of anterior portion of a newt illustrating location of segments sampled for in vitro binding experiments, dorsal view.

described below and 2, 25, or 50 μ l aliquots were saved for protein determination. The remainder was lost during homogenization or discarded.

In general, assays were performed as follows:

to each of six, 10 x 75 mm glass culture tubes was added:

1. 100 μ l (crude homogenate) or 100 g (600 xg pellet) newt limb tissue,
2. 25,000 counts I^{125} prolactin in 20 μ l prepared as described above,
3. 10 μ l .05 M NaHCO₃ or 10 l .05 M NaHCO₃ containing 5 g of ovine prolactin (3 each), and
4. 70 μ l PBM containing 2.5% BSA Fraction V.

Tubes were placed in test tube racks fastened to an orbital shaker and incubated 20 hours at 150 orbits per minute at room temperature. At the end of the incubation period 2.5 mls of cold PBM was added to each tube and bound prolactin separated from free by centrifugation at 5000 xg (crude homogenate) or 1500 xg (low speed pellet). Pellets were resuspended in 0.1N NaOH and counted. Specific binding could be determined by subtracting counts bound to the pellet in the presence of excess prolactin to that bound to the pellet incubated without excess.

Accumulation Studies

Animals were amputated, right forelimb, midway between elbow and wrist and allowed to regenerate for various periods of time. After the appropriate interval each animal was injected interperitoneally with 5 Ci of I^{125} labeled prolactin diluted to a volume of 0.1 ml in 0.9% NaCl in a tubercullin syringe with a #30 gauge needle. Label was routinely allowed to circulate for six hours as initial studies showed maximum

accumulation effect at that interval. At the end of the pulse interval, both amputated limbs were cut into segments as shown in Fig. 3. Each segment was placed in an omnivial with 0.5 mls 1.0 N NaOH and incubated at 53°C until solubilized. Samples were then counted in a Beckman Autogamma gamma counter for 1 minute counts. Following counting 1.0 ml 25% TCA was added to each vial and protein precipitated for several hours at 4°C followed by centrifugation at 2000 xg in a Sorvall RCB-2 centrifuge with #SS-34 rotor. The supernatant was decanted and precipitated protein quantitated by the modified Bradford method (Reed and Northcote, 1981). To ensure that samples were treated the same, all segments from an individual animals were precipitated, centrifuged and evaluated for protein simultaneously. The relative accumulation of label in each segment was expressed as in Bromley (1977).

Autoradiography of Tissue Sections

For autoradiography from tissue sections of animals injected in vivo animals were injected i.p. with 5 Ci of freshly prepared I^{125} prolactin with a tuberculin syringe in a volume of 0.1 ml. At the end of six hours, tissue was removed and fixed overnight in Bouin's fixative. Fixation was followed by dehydration in ethanol (25, 50, 75, 95, 95, 100) clearing in xylene and infiltration with paraffin. Sections were cut at 10 μ on an AO rotary microtone and mounted with Mayer's albumin. After allowing the slides to dry at least 24 hours sections were deparaffinized in 3 changes of xylene followed by two changes of 100% ethanol and air drying. Slides were coated with undiluted Kodak NTB-3 emulsion placed in black boxes containing dessicant and incubated at -20°C for 3 - 6 weeks. Following incubation

slides were developed with Dektol 1:1, air dried and following rehydration lightly stained with Mayer's hematoxylin and eosin dehydrated and coverslipped with Permount. All histological procedures were taken from Humason (1979).

To determine specific binding to tissue sections limbs were allowed to regenerate various periods of time then frozen in aluminum foil immersed in petroleum ether/dry ice. Tissue was mounted onto stubs with Tissue Tek and cut at 20 μ in a Lipshaw cryostat set in the normal temperature range (-25° to -20°C). Tissue not cut immediately was stored in the cryostat.

Tissue sections were thaw-mounted onto glass slides "subbed" with 0.1% gelatin/.01% chromium potassium sulfate. Slides were stored in plastic slide boxes at -20°C until binding studies were performed.

For binding studies a solid line was drawn across the slides 2.0 cm from the end with a wax pencil. This effectively held solutions on the end of the slide containing sections. In this manner slides were treated with the following solutions:

1. 0.8 mls 0.5 M PBM - one half hour,
2. 0.8 mls .05 M PBM containing 80,000, 160,000, or 320,000 counts of I¹²⁵ prolactin with or without an excess of unlabeled prolactin (5 g) - three hours,
3. 0.8 mls .05 M PBM - 3 brief rinses,
4. air dried overnight,
5. dipped in undiluted NTB-3 emulsion and incubate 2-3 weeks at -20°C with dessicant,

6. developed with Dektol 1:1.

For whole body autoradiography animals were injected i.p. with 5 μ Ci I¹²⁵ prolactin or 5 μ Ci I¹²⁵ alone and frozen as described above. Before cutting individual sections, a piece of tape was placed on the block face and the resulting 20 sections remained attached to the tape. Following freeze-drying of the tissue, sections were mounted with epoxy on glass slides. Sections were then lightly dusted with talcum powder, apposed to autoradiographic film (LKB ultrafilm) and stored in a light-tight box for 2 - 3 weeks. Following incubation film was developed with Microdol-x and printed by usual darkroom techniques.

RESULTS

Characterization of Newt Pituitary Proteins

In nondissociating PAGE for newt pituitary proteins the pattern is similar to that described previously for other amphibians (Nicolli and Nichols, 1971). One band, putative newt prolactin, with an R_f of 0.777 comprises 18-19% of total proteins migrating on the gel. This band can be seen as fairly well separated from the rest of the pituitary proteins both by densitometry (Fig. 5) and by visual examination. Other peaks, not well separated can be seen at R_f 0.638, 0.538, 0.338, and 0.020.. When ovine prolactin is run in a similar manner (Fig. 6) two peaks were found - a major peak with R_f of 0.507 and a minor peak with R_f of 0.569. Using the ovine prolactin as a standard it was estimated that the newt peak at R_f 0.777 comprised 0.16 g/g body weight of newts.

Using SDS PAGE the pattern is considerably different from that seen with nondissociating gels. Characteristically gels exhibit a pattern of three prominent relatively low molecular weight bands with 16-20 less prominent higher molecular weight proteins (fig. 7). The three most prominent peaks (designated I, II, III) had R_f s in 4.0x135.0 mm tube gels at 0.933, 0.880, and 0.765 respectively (average from 24 gels). By comparison, samples of bovine growth hormone (mw 18,000) and ovine prolactin (mw 23,000), had R_f s of 0.838 (GH), 0.79 (prl). Band II contained 23-26% of the total pituitary proteins and on estimated molecular weight of 17,000 (16,745). When newt pituitaries are incubated for 12 - 24 hours in Kreb's Ringer's bicarbonate the greater

relative amount of band II compared with any other band is striking. Based on molecular weight, relative abundance and the fact that release of this protein is increased relative to other proteins in the absence of hypothalamic inhibition (Ball, 1981) leads to the suggestion that this band represents newt prolactin.

Figures 8 - 10 show the relative amounts of protein present from 7-21 days postamputation. In the pituitary although the initial series of studies indicated a significant difference between levels of protein III compared with unamputated in male animals and a slight but not statistically significant difference in females, later studies indicated that that this difference could not be duplicated. No difference was detected in pituitary levels of band I or band II from 0 - 21 days postamputation. There was also no consistently large difference in bands II or band III throughout the course of study. In band I, however, a consistent 2-2.5 fold increase in males was found as compared to females.

In general, then, it appears that there are no clearly discernible quantitative or qualitative differences in pituitary proteins from animals with regenerating limbs. Data derived from these experiments indicate that SDS gel electrophoresis is superior in resolution and sensitivity for detecting newt pituitary proteins.

In Vitro Binding Assays

Binding sites for I^{125} prolactin were detected in both low speed pellet and crude homogenate of unamputated newt limb tissue (Figs. 12 and 13). The labeled prolactin could be displaced by an excess of unlabeled prolactin but not by equivalent amounts of insulin, thyroid

stimulating hormone (TSH) or thyroxine (Fig. 14). The labeled hormone was somewhat displaceable by growth hormone but not to the same degree as prolactin. In experiments conducted in this study, specific binding (defined as CPM/ g protein bound in the absence of unlabeled prolactin less CPM/ g bound in the presence of excess unlabeled prolactin) averaged 35.0% and ranged from 2.5% to 72.1%. These values were determined after appropriate corrections for glassware blanks. Figure 11 shows that the amount of label found is proportional to amount of tissue present (correlation coefficient = .947). This indicates that radioactivity detected is the result of binding to limb tissue and is not artifactual. A further indication of the nature of prolactin binding to this tissue is provided by figures 10 and 11. These experiments indicate that binding of labeled prolactin can be significantly displaced by as little as 10 ng of unlabeled hormone and offer further evidence that specific prolactin binding sites are present in newt limb tissue.

With respect to binding of prolactin to regenerating limbs, one point to emphasize is that both unamputated and regenerating limbs from all stages examined demonstrated prolactin binding. Several differences were noted, however, in both specific and non specific binding during the course of regeneration. (See Table 1 and Figs. 15 - 17). Figure 4 shows the locations from which tissues were sampled.

In the distal segment, containing the amputation site, specific binding is higher at three days postamputation than in tissue from unamputated animals or tissue from any other stage of regeneration. This result is evident in both series of experiments in which the three day postamputation stage was included (series B and C; Table 1) and is

statistically significant when using Tukey's test for multiple means ($P < .05$). This increase is also evident when data from all three series is compiled (Fig. 15). When examining non-specific binding from all three series a general decrease is noted in the distal segment from 0 - 21 days postamputation (Fig. 16). It should be noted, however, that this change does not represent a change in prolactin binding sites but is due to some other factor.

In contrast to the distal segment, specific prolactin binding in the medial segment is higher in tissue from unamputated than amputated limbs ($P < .05$). A steady progressive decline in specific binding is seen from 7 - 21 days postamputation, at which point the specific binding for distal, medial and proximal segments is roughly equal. An increase in binding at three days postamputation is seen but of less magnitude than the increase detected in the distal segment (Fig. 15). In general, then, the most salient aspects of the in vitro binding studies appear to be that: 1) there are, indeed, specific prolactin binding sites both in unamputated and regenerating limb tissue; 2) specific binding increases at three days postamputation but later stage regenerates show no difference compared to unamputated controls; 3) non-specific binding, by definition not attributable to specific prolactin binding sites, decreases in the distal segment during the course of regeneration but shows no consistent decrease in medial or proximal segments.

Accumulation of I-125 Labeled Prolactin and β -lactoglobulin

When I^{125} prolactin was injected i.p. and segments cut and processed as shown in Figure 3, label is found to preferentially accumulate in the segment bearing the amputation site. Using animals

seven days postamputation this effect was most pronounced when label was allowed to circulate for six hours. This effect was also present using a labeling interval of three hours but diminished when a twelve hour interval was used (Table II).

Using the six hour labeling interval no consistent accumulation effect is present in unamputated tissue or in animals of three, six, 12, or 24 hours postamputation (Table III). By three days postamputation accumulation is evident in the segment bearing the amputation surface as shown in Table III, and this effect continues up to 42 days postamputation. In general the magnitude of the effect does not appear to be strictly correlated with the stage of regeneration and appears to range between 1.2 to 1.4 from three to 42 days postamputation.

Accumulation of I^{125} labeled prolactin is also evident in animals with denervated limbs (Table IV) as well as those which have been hypophysectomized (Table V). Denervated, amputated limbs actually appeared to have slightly higher relative accumulation than corresponding segments from either sham denervated or unoperated controls. At ten days postamputation, however, the two most distal segments appeared lower than corresponding segments from unoperated controls. Table V shows that the accumulation effect is essentially the same in hypophysectomized animals at seven days postamputation as in sham hypophysectomized controls.

In order to determine whether the accumulation effect would be evident with proteins other than prolactin, β -lactoglobulin, a protein with no known relationship to regeneration, was used. Table VI shows that a marked accumulation effect was seen at 3, 7, and 14 days postamputation. To further examine the nature of the accumulation effect, I^{125} alone was injected with results as shown in Table VII.

It appears from these studies then, that a marked accumulation effect of I^{125} prolactin is detected in all stages subsequent to three days postamputation. A similar effect can be seen with I^{125} lactoglobulin but not with I^{125} alone. It seems likely that accumulation as described here is not due solely to changes in specific prolactin binding but other less specific factors as well.

Tissue Localization of I^{125} Labeled Prolactin

The most striking result from autoradiographic localization both in vivo and in vitro is the difference in grain distribution between epithelial structures and underlying tissue. In over 200 autoradiograms examined the intensity of the labeling in intact muscle, blastemal cells and other internal structures never exceeded that seen in the integument and particularly the wound epithelium. In fact, these experiments generally indicate that the affinity of the labeled prolactin is much greater for the integument than underlying structures. One of the areas to most consistently show intense grain distribution was the outer layer of cells in the wound epithelium (Fig. 21). This area contained large numbers of grains both in animals injected in vivo and in sections treated with I^{125} prolactin in vitro. The inner layers of the wound epithelium were also consistently labeled heavily but the intensity is less than that of the outer layer. Figures 27 and 28 illustrate that while some of the binding in the wound epithelium is due to specific prolactin binding, non-specific binding also plays a significant role.

In addition to autoradiography of I^{125} prolactin, autoradiography was also done on tissue sections from five animals seven days postamputation injected with 5.0 Ci I^{125} alone. None of the animals in

this group showed the grain distribution characteristic of those injected with I^{125} prolactin.

With respect to grain distribution and stage of regeneration, in all stages examined (3 - 21 days postamputation) grains were more numerous over the wound epithelium than any other structure. Other than this, however, no clear relationship emerged between stage of regeneration and tissue localization of I^{125} prolactin. In the only examples of areas of intense grain distribution in underlying tissues, several instances were observed in which increased affinity for prolactin was noted accompanying muscle breakdown (Fig. 24). This phenomenon was restricted to 3 and 7 days postamputation. As noted above, blastemal cells in later stages were rarely seen with more than a sparse distribution of associated grains.

Results from whole body autoradiograms appear to tie together much of the work discussed in the previous paragraphs (Fig. 30). Several points become immediately obvious from these preparations: 1) with respect to regenerating limb tissue I^{125} prolactin does have a preferential affinity for integument; 2) this effect could not be duplicated by injection of I^{125} alone; 3) I^{125} prolactin is found not only at the distal end of the limb but also for a considerable distance proximal to the amputation surface; 4) consistent with the results from accumulation studies prolactin localization is more intense in the regenerating limb than in the contralateral, unamputated limb.

In general, then, it appears that changes come about in both the wound epithelium and stump epidermis following amputation which result in increased affinity for prolactin. Specific prolactin receptor sites are found in these tissues but it also appears that non-specific affinity for prolactin may also increase.

Figure 5

Typical gel scan from nondissociating disc gel electrophoresis. Tubes were 6.5 x 1.0 cm, 7.5% resolving gel, 3% stacking gel run at 100 V. This gel represents the equivalent of 7 pituitaries layered on top of the gel which were stained following electrophoresis with .25% Coomassie Blue R-250 in Methanol: Acetic Acid: dH₂O (5:1:5). Gels were scanned on a gel scanning attachment of a Beckman Model 25 spectrophotometer at 620 nm (slit 0.2 nm, span 1A, scan 0.5 cm/min. chart speed 0.5 inches/min.).

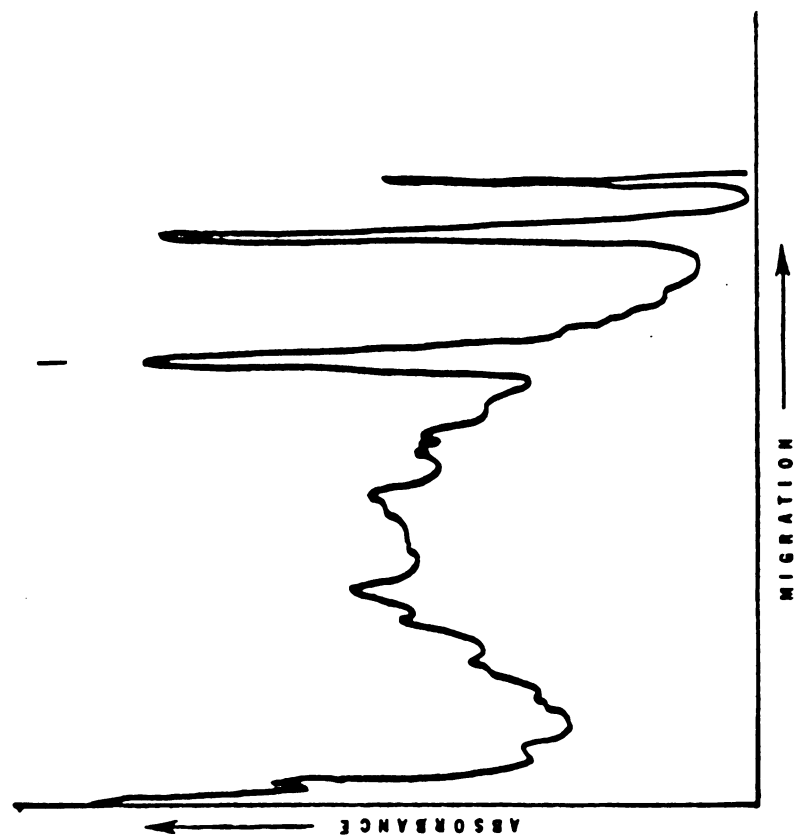


Figure 6

Nondissociating disc gel electrophoresis of ovine prolactin run as described in Figure 3. The minor peak represents a deamidated form of prolactin (NIH-p-s-8).

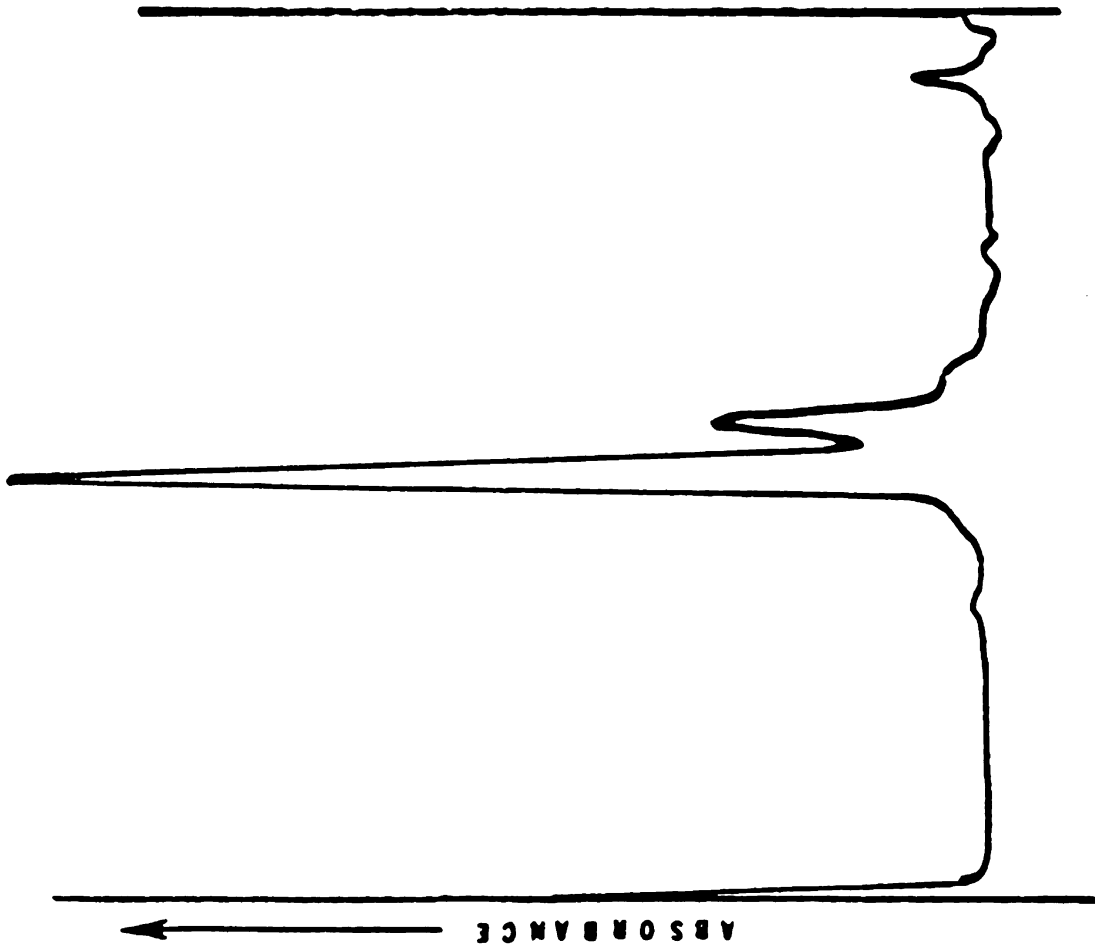
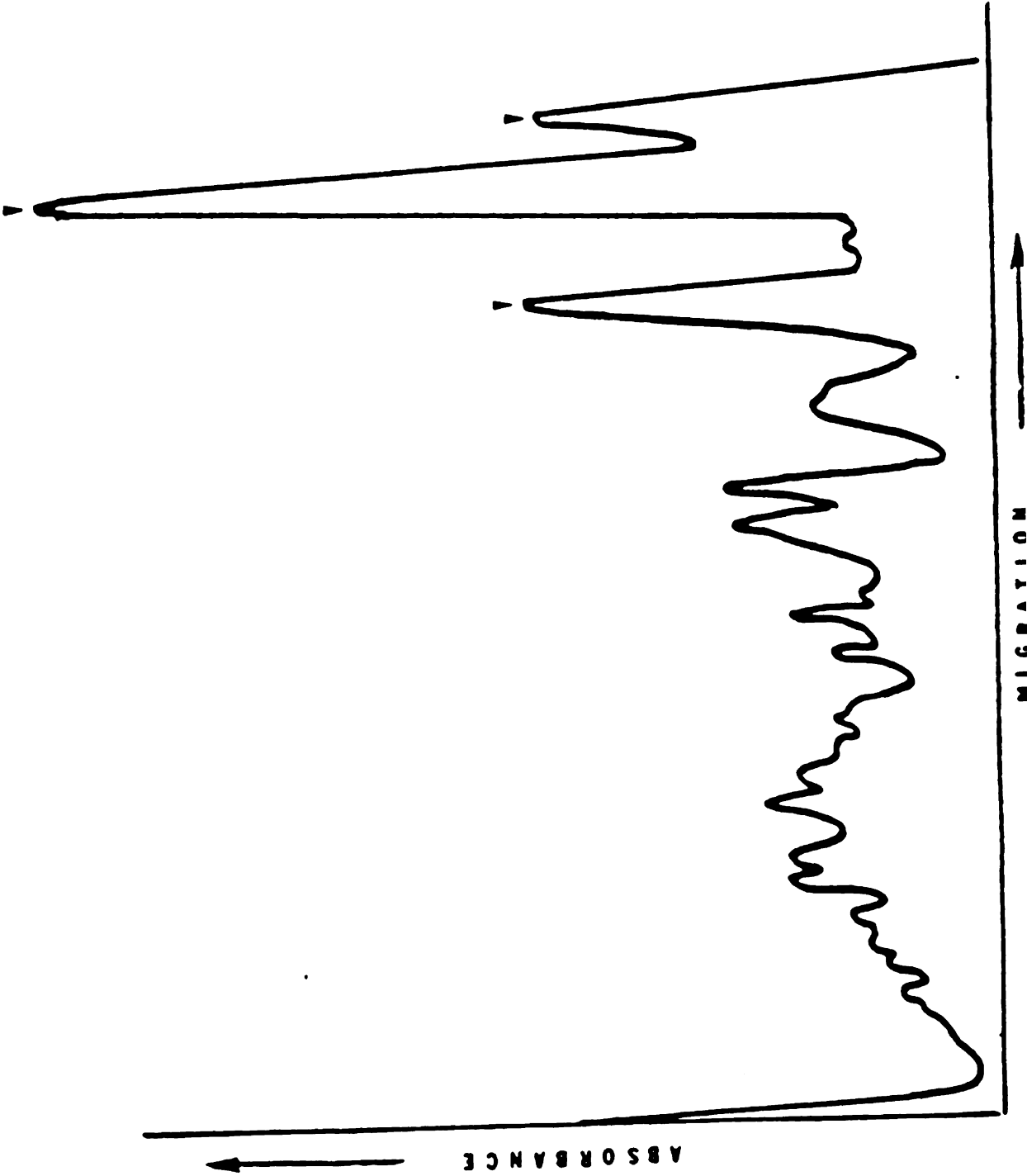


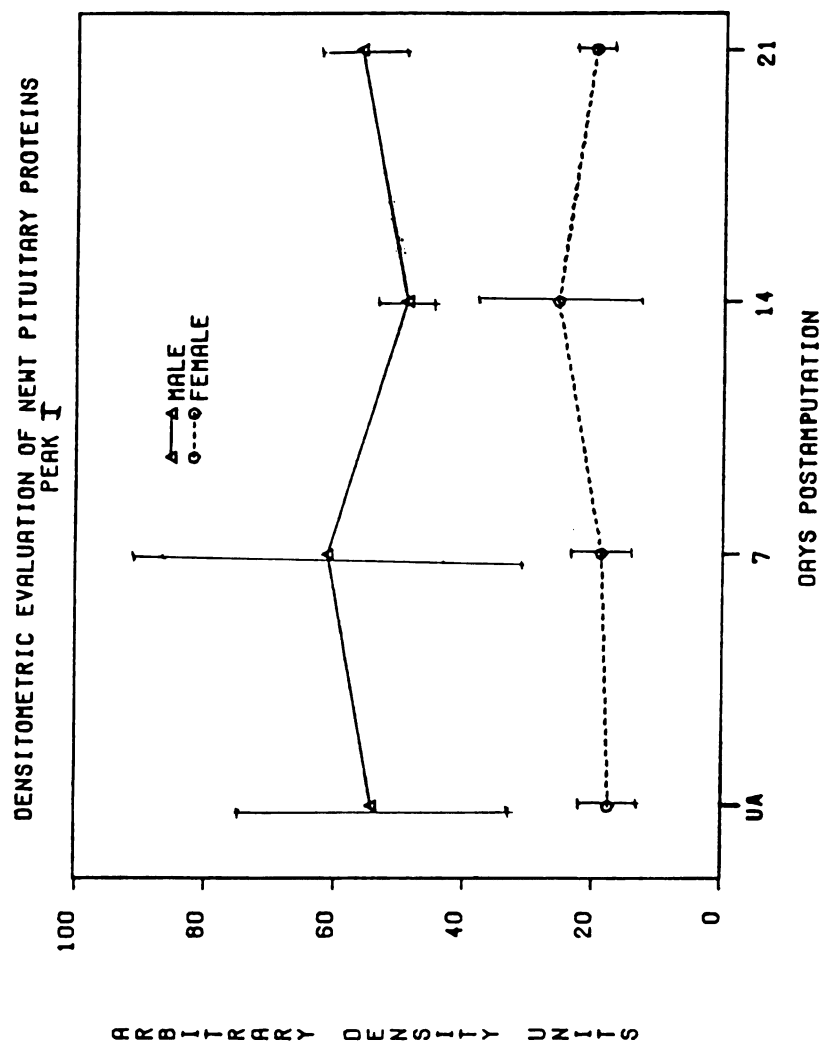
Figure 7

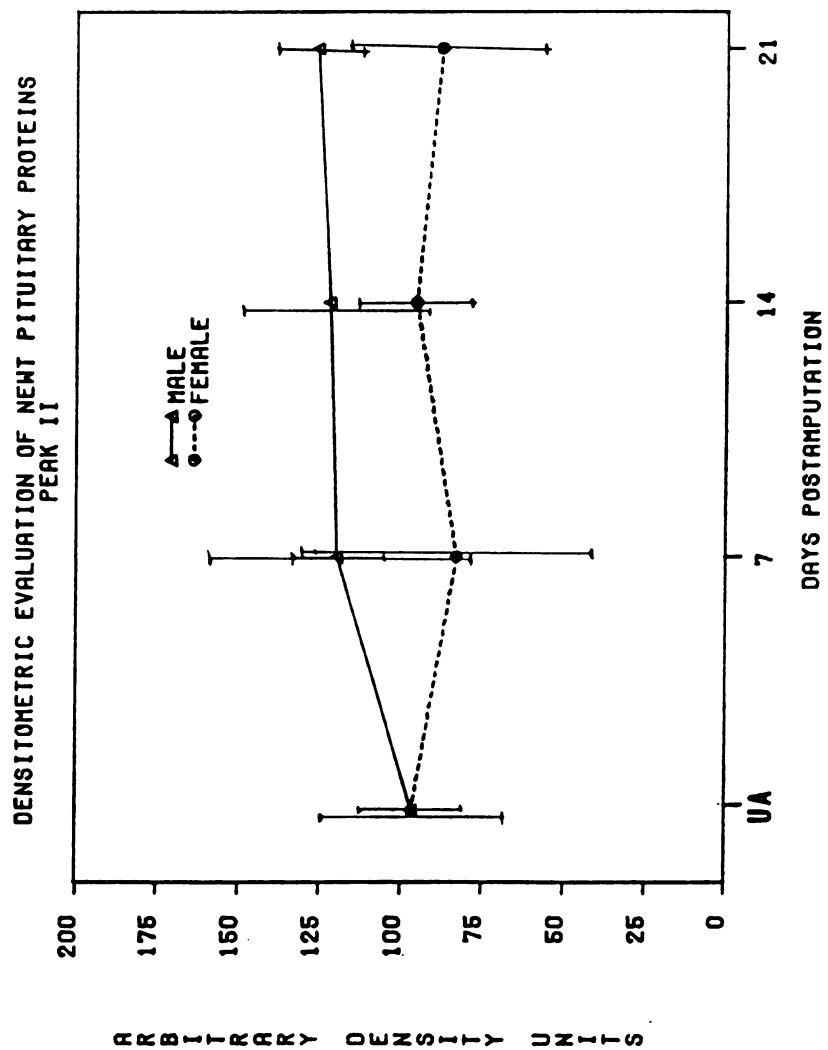
Typical gel scan from SDS disc gel electrophoresis. Tubes were 135 mm x 4.0 mm (i.d.), 10% resolving gel, 3% stacking gel run at 100 V. This gel represents three pituitaries layered on top of the gel which was stained following electrophoresis with .25% Coomassie Blue R-250 in Methanol: Acetic Acid: dH₂O (5:1:5). Gels were scanned on a gel scanning attachment to a Beckman Model 25 spectrophotometer at 620 nm (slit 0.1 mm, span 2A, scan 2 cm/min., chart speed 2 in/min.).



Figures 8 - 10

Densitometric evaluation of major protein peaks by SDS PAGE. Each point is the average of three peaks, integrated by planimetry, corresponding to identical locations on three separate gels. Each gel represents three pooled pituitaries run as shown in Figure 7. Points are expressed as Arbitrary Density Units per gram body weight of animals. Peak II is thought to represent newt prolactin.







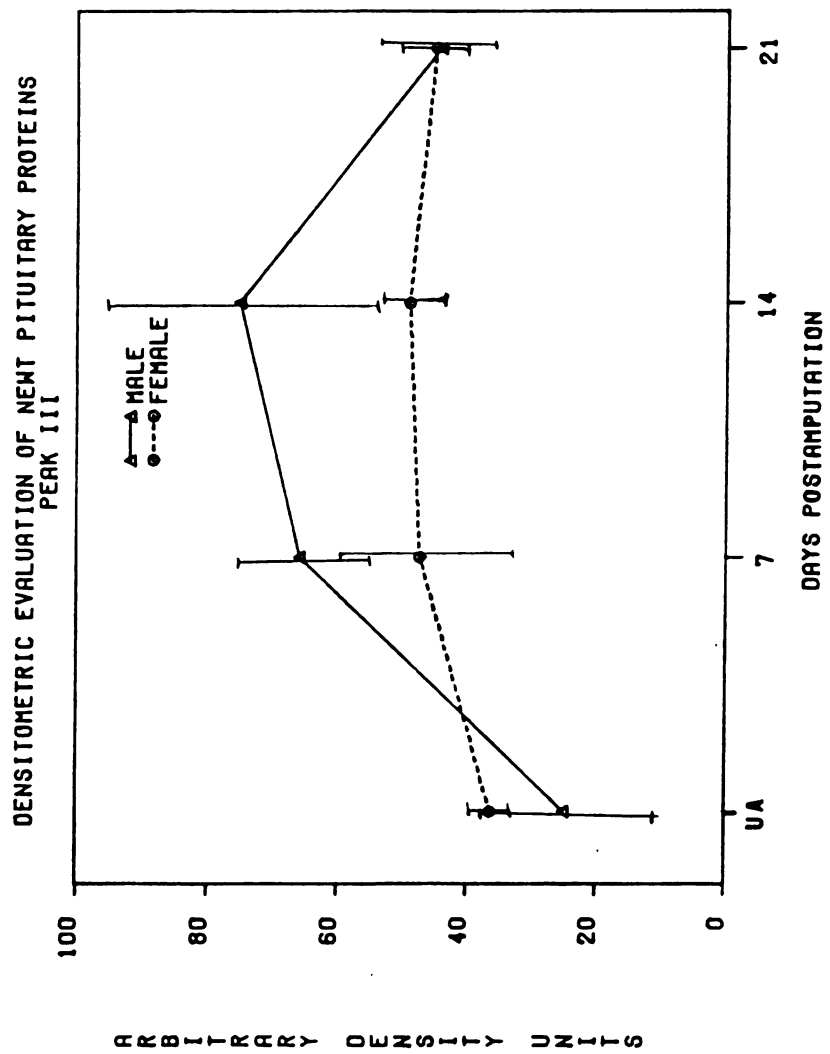


Figure 11

Binding of I^{125} labeled prolactin to crude homogenate of unamputated tissue (effect of protein concentration on binding). Tissue was homogenized in .05 M phosphate buffer pH 7.4, .01 M $MgCl_2$ (PBM) in a glass/glass homogenizer. 0.1 ml aliquots were added to 10 x 75 mm glass culture tubes to which were also added 25,000 counts I^{125} prolactin and PBM containing BSA to yield a final concentration of 1% BSA. The final volume of the incubation mixture was 200 μ l and incubation was carried out for 20 hours on an orbital shaker at 150 rpm. At the end of the incubation period 2.5 mls of cold PBM/1% BSA was added to each tube and centrifuged at 5000 xg in an SS-34 rotor and Sorvall RCB-2 centrifuge. The supernatant was decanted and pelleted, resuspended in 0.1 N NaOH, transferred to plastic gamma vials and counted in a Beckman gamma counter. Each point represents the average of three separate determinations.

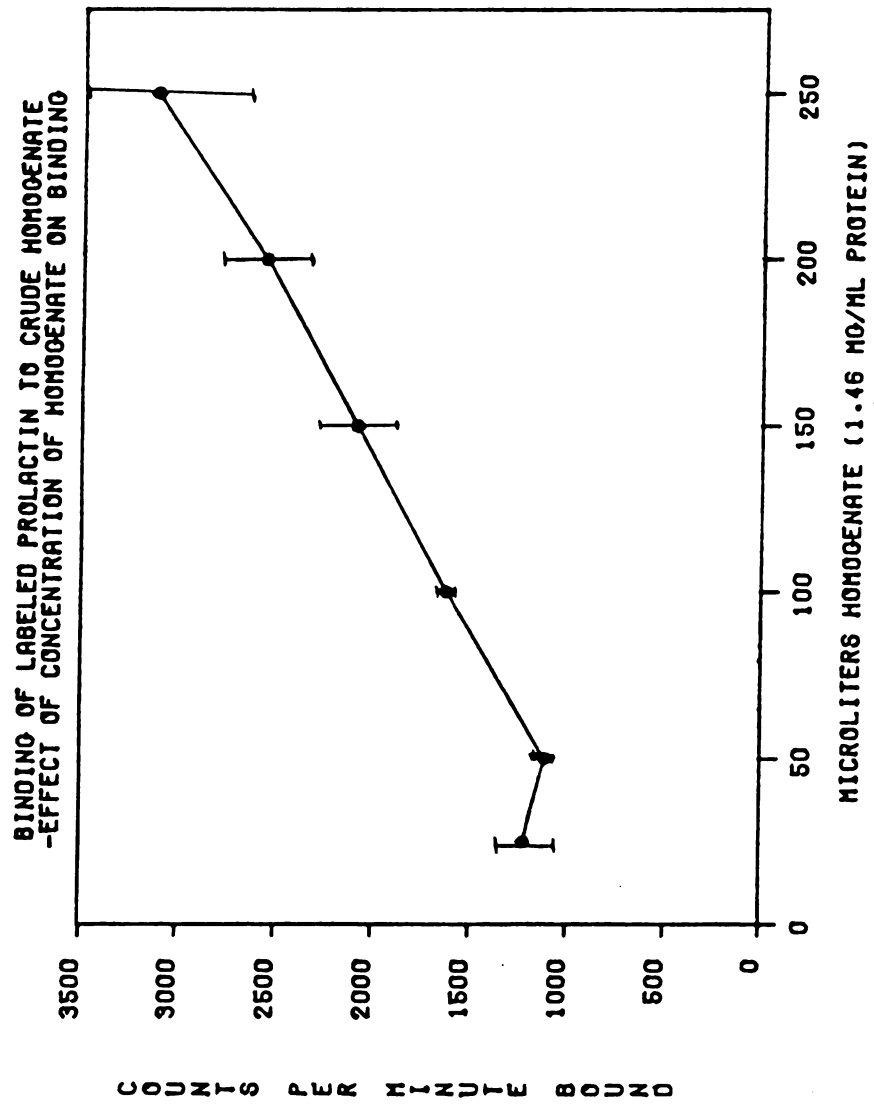




Figure 12

Binding of I^{125} labeled prolactin to crude homogenate of unamputated tissue (effect of increasing amount of unlabeled hormone on binding). Assay run as in Figure 11 with the exception that ten microliters of PBM or PBM containing O-prl-15 were added to each tube (containing a fixed amount of homogenate) to give the appropriate concentration of hormone.

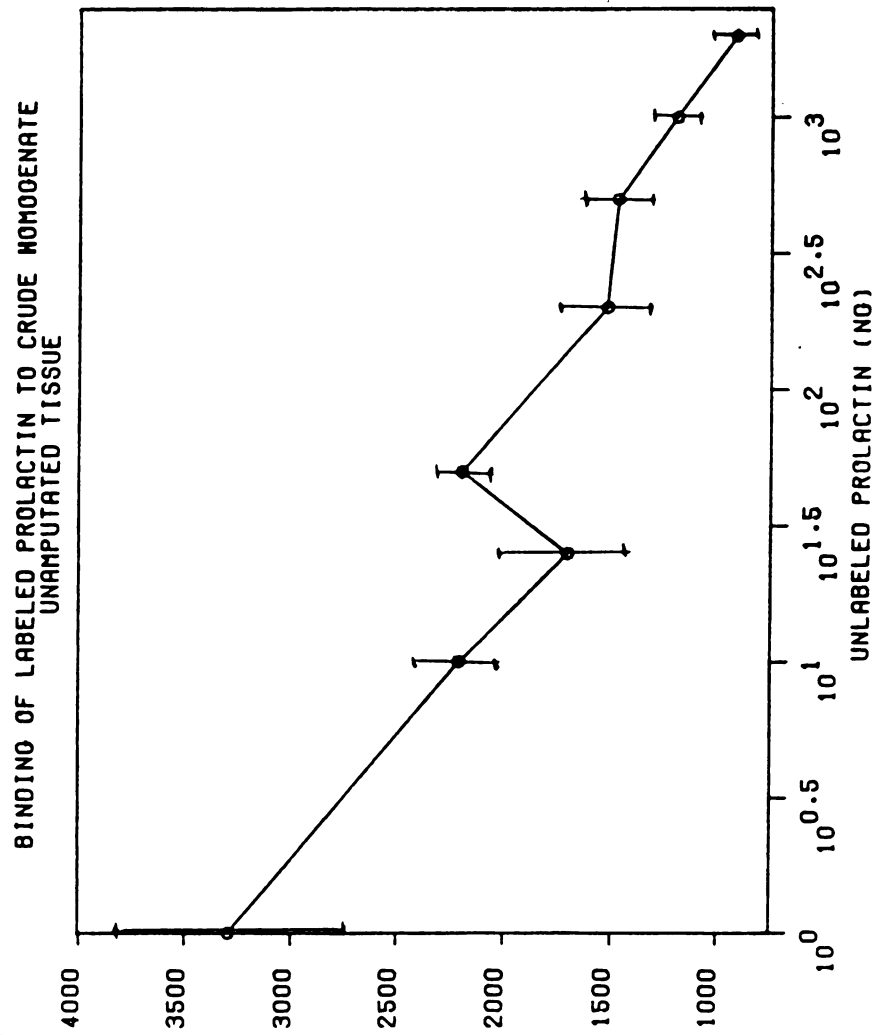




Figure 13

Binding of labeled prolactin to 600 xg pellet of tissue from regenerating limb seven days postamputation (effect of increasing amount of unlabeled hormone on binding). Distal segments from regenerating limbs seven days postamputation were homogenized in .05 M Tris buffer in a glass/glass homogenizer and centrifuged at 600 xg for one hour. The supernatant was decanted, the pellet resuspended in .05 M Tris and a protein determination was performed using the Coomassie Blue G dye binding assay. An amount corresponding to 100 g was added to each tube as well as 50,000 counts of 125 I prolactin and varying amounts of unlabeled prolactin. Bound hormone was separated from free by centrifugation at 1500 xg.

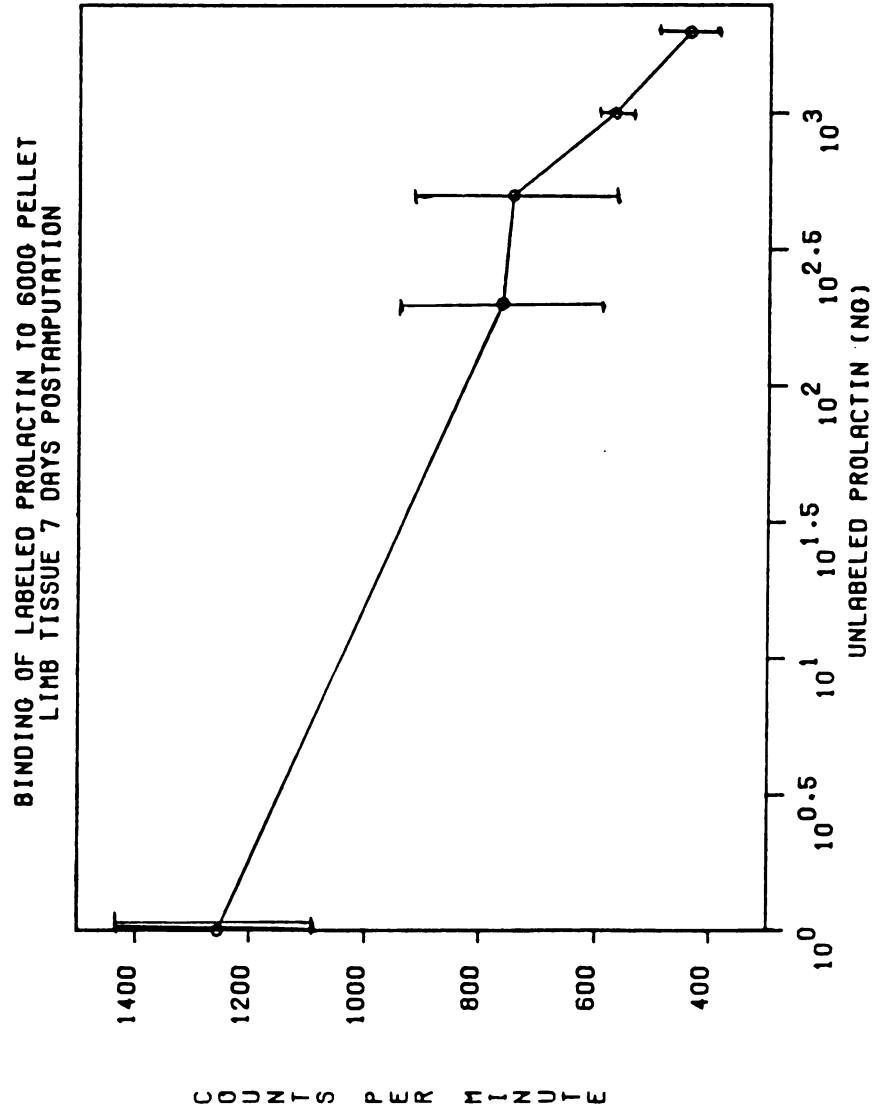




Figure 14

Displacement of I^{125} labeled prolactin by other hormones. This assay was performed on a crude homogenate essentially as described in Figure 11 with the exception that rather than adding varying concentrations of prolactin, 5 g of the indicated hormone was added to each tube. Triplicate determinations were performed for each hormone. Prl = prolactin; GH = growth hormone; TSH = thyroid stimulating hormone; INS = insulin; THY = thyroxine.

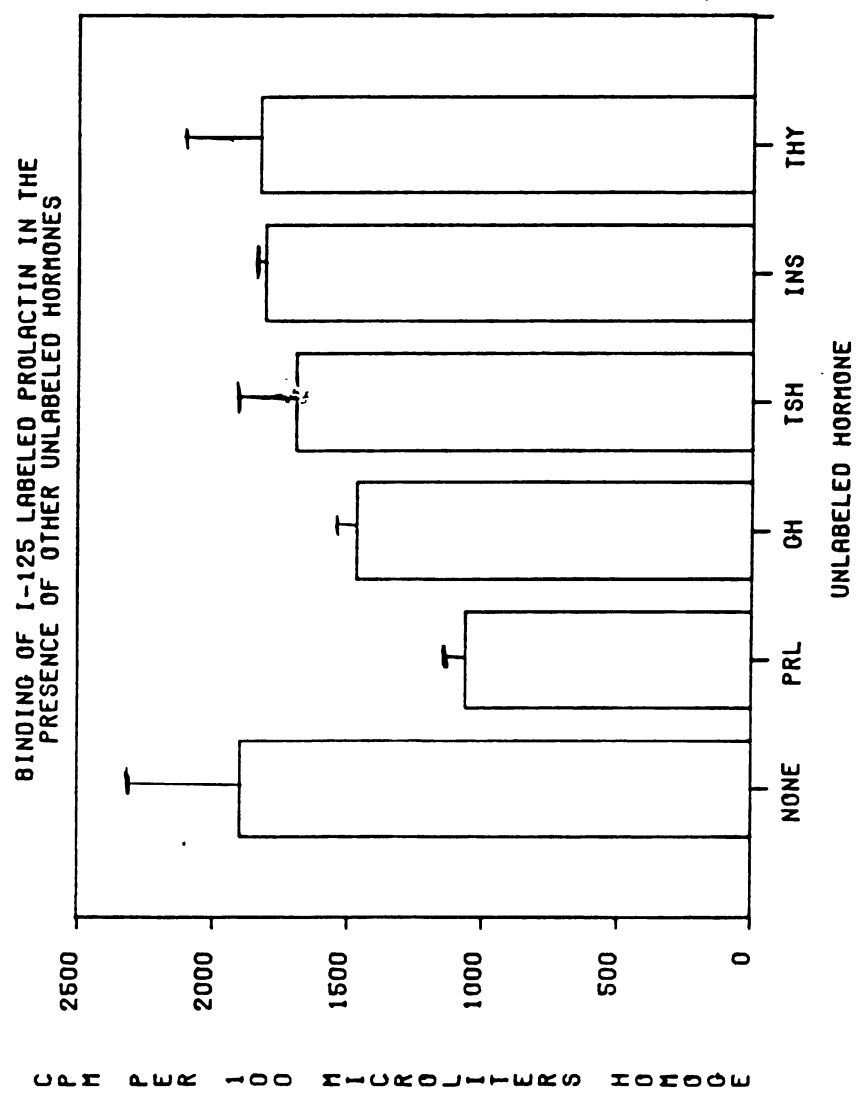


Table 1

This table represents a summary of all prolactin binding experiments to crude homogenates. All experiments were performed as described in Figure 11. In animals with amputated limbs the distal segment contains the amputation site. Binding to medial and proximal segments, not containing amputation sites, was done for comparison between regenerating and non-regenerating areas of the limb. Experiments were performed in duplicate on three separate occasions and individual determinations were performed in triplicate. Within each series of experiments the upper number represents the specific binding plus/minus the standard error while the lower number represents non-specific binding plus/minus the standard error.

Table I
Summary of In Vitro Binding Experiments

Distal Segments (counts/microgram)						
Series	UA	1PA	3PA	7PA	14PA	21PA
A1	10.8+10.84			26.1+1.10	17.5+7.48	19.9+1.89
	25.0+3.70			23.6+4.27	14.47+1.39	20.0+4.2
A2	17.2+3.70			17.3+0.60	4.9+2.58	11.0+1.88
	28.5+1.22			11.2+2.49	6.1+0.33	9.1+1.23
B1			33.9+13.77	12.8+3.08	14.0+3.42	12.9+3.04
			13.48+7.30	20.2+4.78	21.8+0.90	16.0+0.95
B2			31.5+9.46	16.2+2.16	14.5+0.64	
			35.1+5.92	19.7+3.43	14.14+3.51	
C1	11.9+4.64	12.1+11.43	20.7+7.47	2.51+6.33		
	48.7+1.86	49.3+1.00	48.1+9.13	34.5+4.44		
C2	13.05+2.15	15.1+3.75	26.1+6.95	7.27+3.38		
	55.4+1.35	46.4+3.41	53.1+19.9	48.8+3.62		
TOTAL	13.2+6.2	13.6+7.78	28.2+10.1	13.6+8.2	12.72+6.16	14.60+5.62
	39.4+13.6	47.8+2.76	32.4+16.8	26.3+12.92	14.1+6.05	15.0+5.30



Table I (cont'd)
Summary of In Vitro Binding Experiments

Medial Segments (counts/microgram)					
Series	UA	3PA	7PA	14PA	21PA
A1	34.0+4.8		12.84+4.30	26.3+14.60	36.2+4.06
	43.53+5.06		60.9+5.83	46.1+1.67	44.4+11.5
A2	21.3+1.14		32.3+21.3	5.4+11.28	1.6+20.58
	31.2+10.25		52.2+15.8	14.9+2.78	55.7+11.41
B1		20.9+13.02	42.0+13.61	11.12+7.20	25.59+8.41
		56.3+5.02	35.2+6.63	29.91+3.72	44.3+8.33
B2		37.8+7.81	18.9+8.82	16.8+4.56	
		32.8+6.74	23.4+8.14	25.7+3.87	
TOTAL	27.6+10.5	26.8+11.61	26.5+16.6	14.91+11.8	16.7+18.71
	38.3+9.2	39.6+12.98	42.9+17.4	29.2+12.01	48.2+10.73

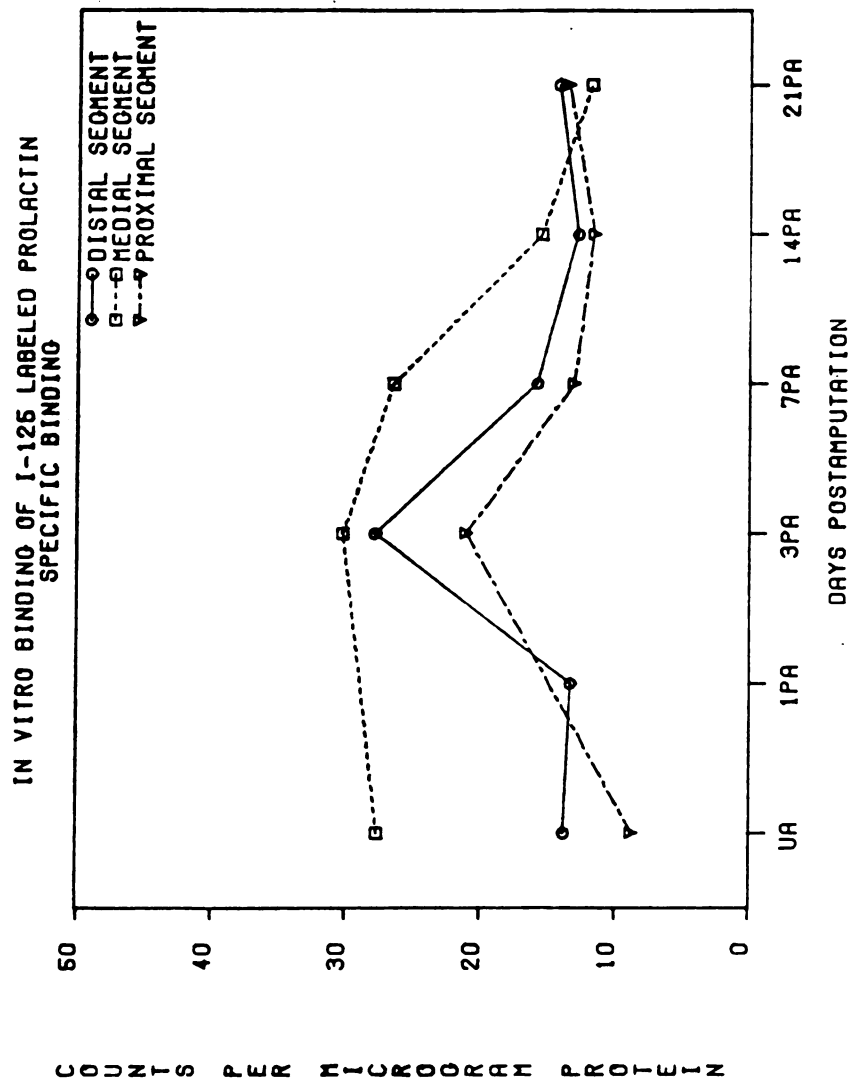


Table I (cont'd)
Summary of In Vitro Binding Experiments

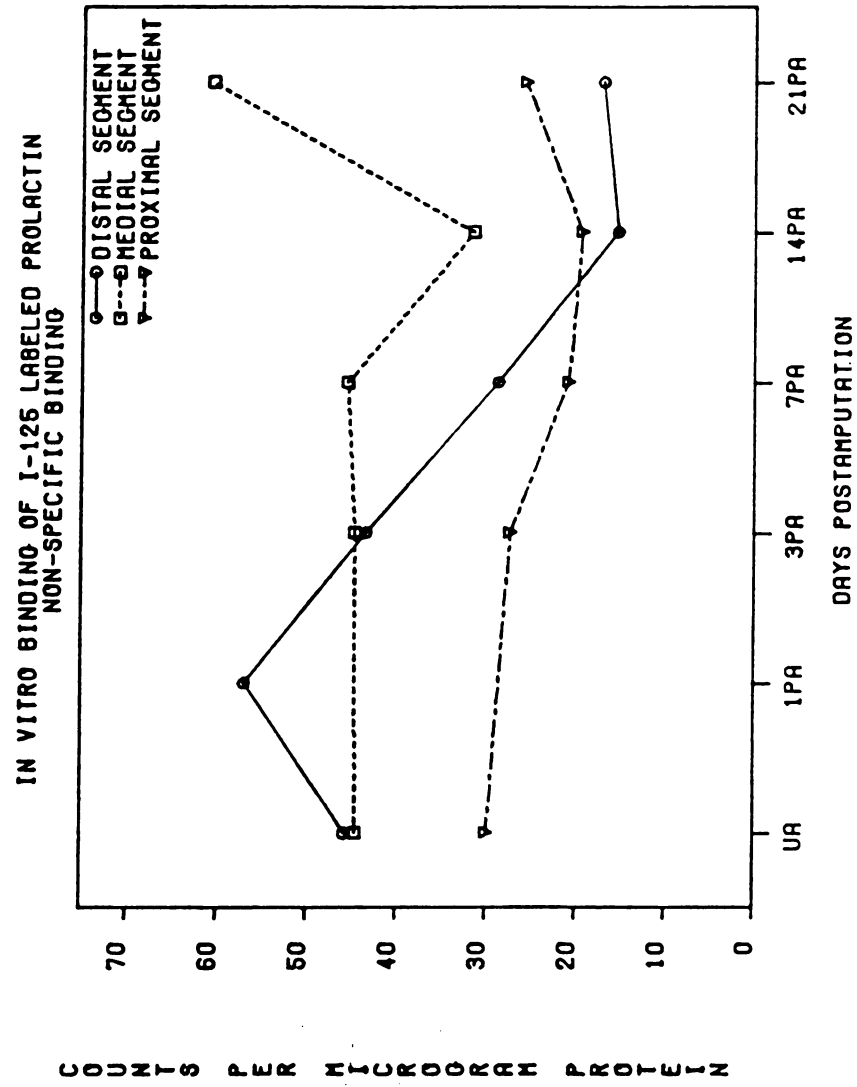
Proximal Segments (counts/microgram)					
Series	UA	3PA	7PA	14PA	21PA
A1	14.2+4.56		24.7+7.92	14.3+9.32	12.7+1.27
	25.0+2.77		14.15+3.51	25.4+3.88	27.9+0.68
A2	3.1+6.58		6.6+9.18	12.2+1.25	16.0+2.04
	22.94+1.96		27.4+1.91	9.53+7.86	23.5+1.18
B1		21.7+6.47	5.06+6.81	0.88+8.14	11.93+2.38
		27.5+4.42	15.2+2.03	19.29+7.13	20.7+1.92
B2		20.3+6.94	11.9+3.14	19.6+10.50	
		26.8+3.07	19.9+2.40	14.15+4.35	
TOTAL	8.6+7.96	21.0+6.05	12.8+10.6	11.1+10.53	13.5+2.66
	24.0+2.43	27.2+3.43	19.2+5.89	17.8+6.66	24.04+3.36

Figures 15 - 17

Graphic representation of results expressed in Table 1. These graphs represent data compiled from three separate series of experiments.







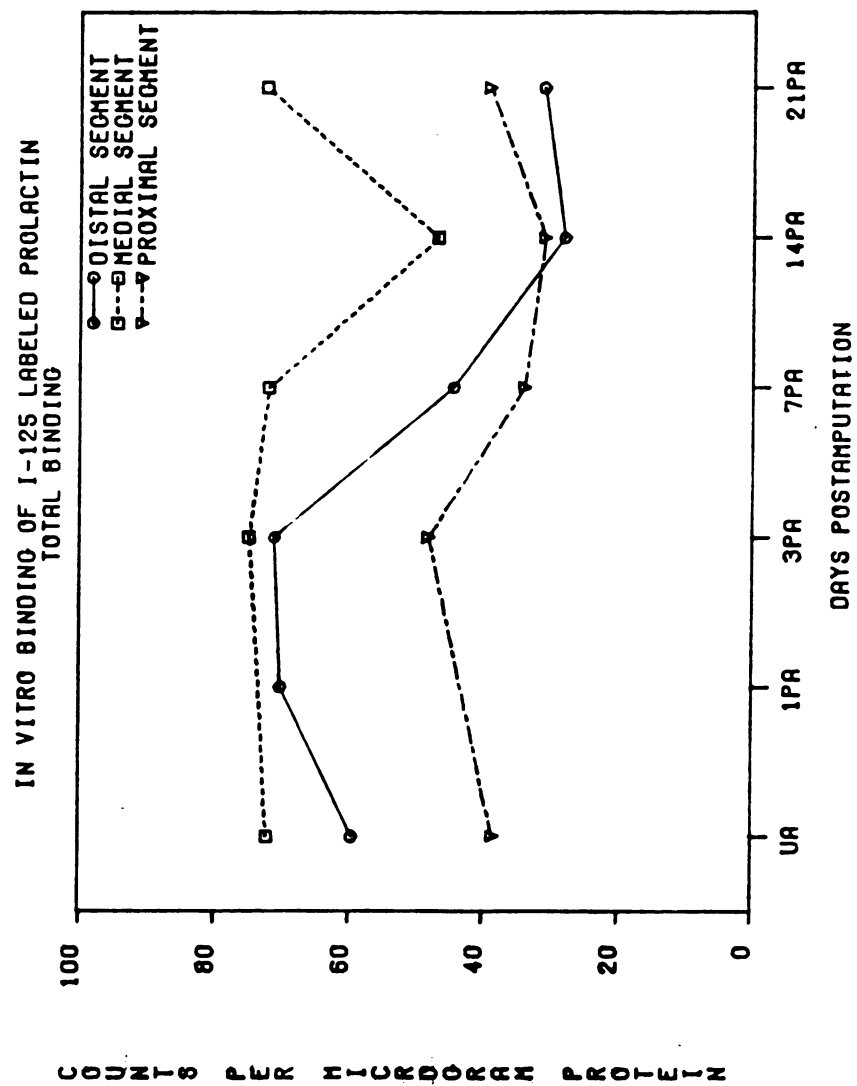


Table II

This table represents data from an experiment designed to determine the optimum labeling interval to observe the accumulation effect. Letters A - G correspond to segments illustrated in Fig. 3. Animals were injected intraperitoneally with 5 μ Ci of I^{125} labeled prolactin (specific activity 100 Ci/ g). Following the appropriate labeling interval limb tissue was cut in to the designated segments placed in an omnivial with 0.5 mls, 1.0 N NaOH, incubated at 53 degrees until solubilized and counted in a gamma counter. Protein was precipitated with TCA and quantitated by the Coomassie blue G dye binding assay. In all subsequent accumulation experiments a six hour labeling interval was used..

$$\text{relative accumulation} = \frac{\text{counts per microgram for individual segment}}{\text{total counts per microgram for all segments}}$$

TABLE II

3 Hour Labeling Interval						
Relative Accumulation						N=5
A	B	C	D	E	F	G
$\bar{x}$ 1.21	1.18	0.79	0.91	1.17	0.81	0.92
SD 0.16	0.56	0.08	0.19	0.25	0.24	0.24

6 Hour Labeling Interval						
Relative Accumulation						
A	B	C	D	E	F	G
$\bar{x}$ 1.31	1.14	0.79	0.85	1.07	0.91	1.01
SD 0.32	0.26	0.08	0.12	0.09	0.07	0.13

12 Hour Labeling Interval						
Relative Accumulation						
A	B	C	D	E	F	G
$\bar{x}$ 1.10	1.10	0.96	0.94	1.13	1.01	0.88
SD 0.19	0.16	0.16	0.08	0.09	0.15	0.16

Table III

Accumulation of I^{125} prolactin in regenerating limbs at different stages of regeneration. Experiments were performed as in Table II using a six hour labeling interval.

TABLE III

Unamputated

Relative Accumulation			N=5					
	A'	A	B	C	D	E	F	G
$\bar{x}$	1.09	0.76	1.27	0.79	0.83	1.10	0.84	1.26
SD	+0.30	+0.14	+0.49	+0.21	+0.10	+0.17	+0.19	+0.23

Unamputated

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	1.05	1.24	0.95	0.86	1.0	0.9	0.99
SD	+0.18	+0.5	+0.2	+0.13	+0.13	+0.19	+0.14

1 Hour Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	0.94	0.17	0.97	0.98	1.12	0.80	0.98
SD	+0.06	+0.32	+0.20	+0.18	+0.13	+0.12	+0.15



TABLE III (cont'd)

3 Hours Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	1.24	1.10	0.90	0.93	0.91	0.89	1.03
SD	+0.23	+0.17	+0.04	+0.11	+0.11	+0.23	+0.17

6 Hours Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	0.94	1.3	0.87	0.93	0.93	0.85	1.12
SD	+0.17	+0.18	+0.11	+0.10	+0.15	+0.11	+0.07

12 Hours Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	1.0	1.09	0.96	0.96	1.11	0.91	0.98
SD	+0.16	+0.17	+0.15	+0.12	+0.14	+0.13	+0.10

24 Hours Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	0.96	1.37	0.93	0.90	1.06	0.88	1.03
SD	+0.21	+0.65	+0.16	+0.07	+0.11	+0.10	+0.23

TABLE III (cont'd)

3 Days Postamputation

Relative Accumulation		N=4					
	A	B	C	D	E	F	G
$\bar{x}$	1.24	1.37	0.79	0.75	1.07	0.74	1.05
SD	+0.26	+0.14	+0.11	+0.16	+0.13	+0.11	+0.24

7 Days Postamputation

Relative Accumulation		N=6					
	A	B	C	D	E	F	G
$\bar{x}$	1.54	1.06	0.80	0.85	0.85	0.86	1.06
SD	+0.31	+0.07	+0.17	+0.10	+0.14	+0.04	+0.22

14 Days Postamputation

Relative Accumulation		N=11					
	A	B	C	D	E	F	G
$\bar{x}$	1.36	1.12	0.97	0.87	0.89	0.80	1.07
SD	+0.21	+0.29	+0.18	+0.12	+0.11	+0.15	+0.28

20 Days Postamputation

Relative Accumulation		N=4					
	A	B	C	D	E	F	G
$\bar{x}$	1.34	1.04	0.91	0.90	0.98	0.90	0.93
SD	+0.26	+0.07	+0.14	+0.14	+0.07	+0.07	+0.09

TABLE III (cont'd)

28 Days Postamputation

Relative Accumulation			N=2				
	A	B	C	D	E	F	G
$\bar{x}$	1.44	1.39	0.79	0.77	0.94	0.77	0.90
SD	+0.63	+0.25	+0.09	+0.01	+0.15	+0.18	+0.16

29 Days Postamputation

Relative Accumulation			N=2				
	A	B	C	D	E	F	G
$\bar{x}$	1.32	0.90	0.85	0.95	0.82	0.84	1.30
SD	+0.19	+0.13	+0.06	+0.17	+0.08	+0.09	+0.29

35 Days Postamputation

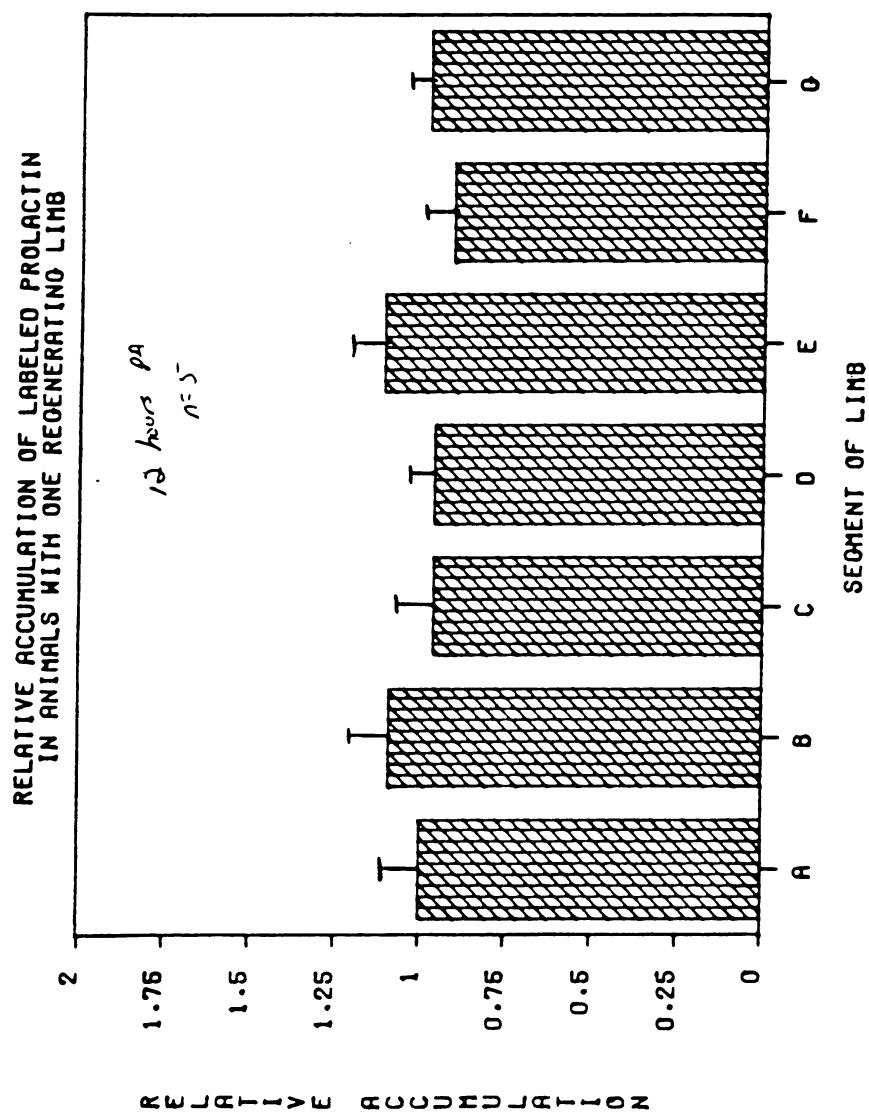
Relative Accumulation			N=3				
	A	B	C	D	E	F	G
$\bar{x}$	1.29	1.27	0.87	0.87	1.01	0.82	0.89
SD	+0.16	+0.15	+0.15	+0.15	+0.05	+0.05	+0.09

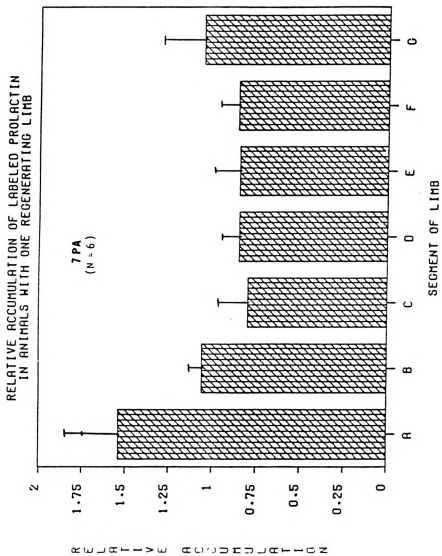
42 Days Postamputation

Relative Accumulation			N=3				
	A	B	C	D	E	F	G
$\bar{x}$	1.31	1.13	0.85	0.94	0.89	0.81	1.03
SD	+0.08	+0.15	+0.08	+0.24	+0.08	+0.08	+0.15

Figures 18 - 20

Graphic representation of selected data from Table III.







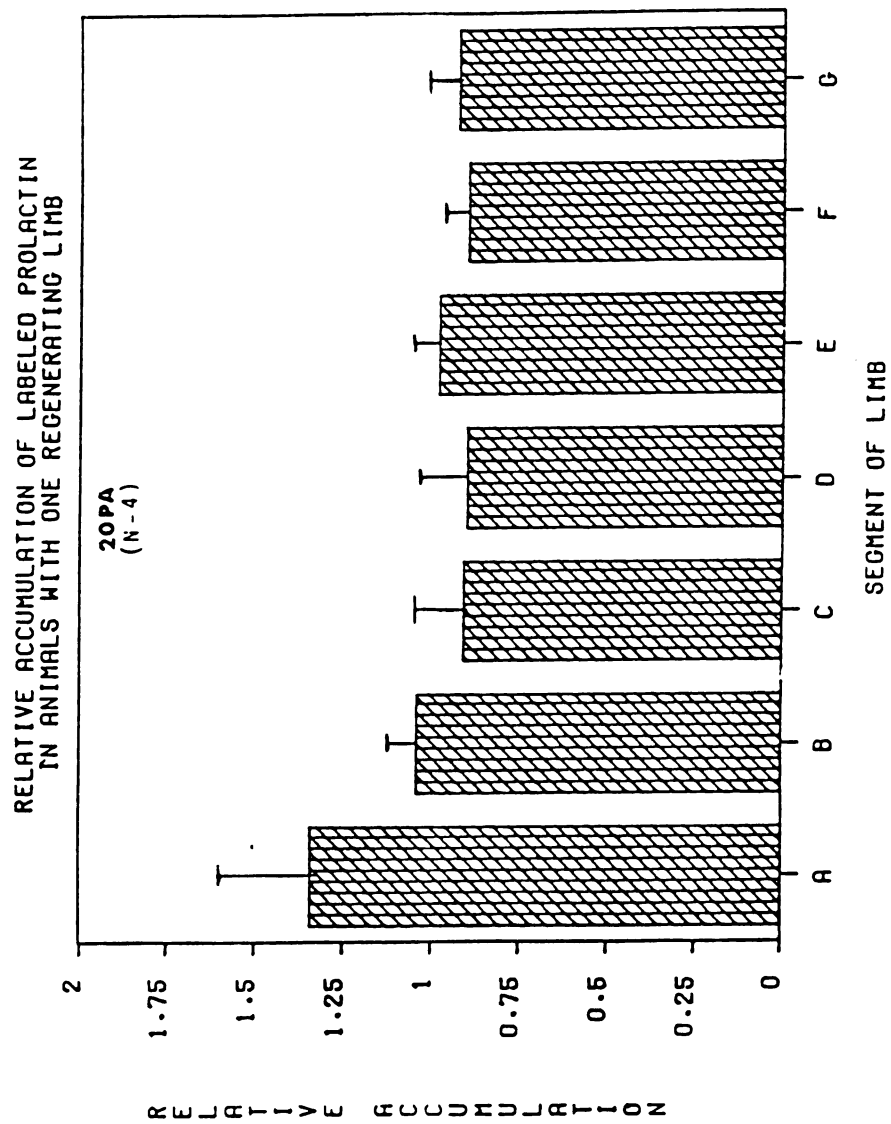


Table IV

Accumulation of I^{125} prolactin in amputated, denervated limbs. Experiments were performed as described in Table II using a six hour labeling interval. Limbs were denervated by making a small incision in the shoulder region in cutting spinal nerves IV, V, and VI.

TABLE IV

Control7 Days Postamputation

Relative Accumulation			N=5				
	A	B	C	D	E	F	G
$\bar{x}$	1.16	1.00	0.85	1.02	1.07	0.84	1.05
SD	+0.09	+0.14	+0.02	+0.17	+0.12	+0.10	+0.02

Denervated Animals7 Days Postamputation

Relative Accumulation			N=5				
	A	B	C	D	E	F	G
$\bar{x}$	1.24	1.01	0.92	0.95	0.92	0.82	1.17
SD	+0.05	+0.09	+0.12	+0.16	+0.10	+0.14	+0.14

Sham Denervated7 Days Postamputation

Relative Accumulation			N=4				
	A	B	C	D	E	F	G
$\bar{x}$	1.26	1.85	0.88	0.67	0.97	0.64	0.74
SD	+0.28	+0.75	+0.70	+0.12	+0.32	+0.04	+0.07

Denervated Animals7 Days Postamputation

Relative Accumulation			N=9				
	A	B	C	D	E	F	G
$\bar{x}$	1.64	1.84	0.64	0.74	0.89	0.53	0.72
SD	+0.50	+0.71	+0.12	++0.27	+0.20	+0.14	+0.22

TABLE IV (cont'd)

Control10 Days Postamputation

Relative Accumulation			N=5				
	A	B	C	D	E	F	G
$\bar{x}$	1.36	1.23	0.96	0.96	0.85	0.70	0.98
SD	+0.13	+0.39	+0.21	+0.21	+0.10	+0.15	+0.22

Denervated Animals10 Days Postamputation

Relative Accumulation			N=6				
	A	B	C	D	E	F	G
$\bar{x}$	1.15	0.88	0.94	0.99	0.76	1.06	1.32
SD	+0.13	+0.13	+0.11	+0.15	+0.11	+0.32	+0.21

Table V

Accumulation of ^{125}I prolactin in amputated limbs from hypophysectomized animals. Experiments were performed as described in Table II using a six hour labeling interval. Animals were hypophysectomized three days prior to amputation by reflecting the sella turcica and removing the pituitary with a pair of watchmakers forceps.

TABLE V

Sham Hypophysectomized7 Days Postamputation

Relative Accumulation		N=4					
	A	B	C	D	E	F	G
$\bar{x}$	1.43	1.83	0.78	0.68	0.84	0.61	0.83
SD	<u>+0.45</u>	<u>+0.81</u>	<u>+0.10</u>	<u>+0.08</u>	<u>+0.15</u>	<u>+0.09</u>	<u>+0.20</u>

Hypophysectomized Animals7 Days Postamputation

Relative Accumulation		N=10					
	A	B	C	D	E	F	G
$\bar{x}$	1.48	1.36	0.82	0.80	0.92	0.70	0.93
SD	<u>+0.30</u>	<u>+0.23</u>	<u>+0.08</u>	<u>+0.14</u>	<u>+0.16</u>	<u>+0.17</u>	<u>+0.12</u>

Table VI

Accumulation of β -lactoglobulin in regenerating limbs. Experiments were performed essentially as described in Table II with the exception that I^{125} labeled β -lactoglobulin was substituted for labeled prolactin.

TABLE VI

 β -lactoglobulin3 Days Postamputation

Relative Accumulation		N=4					
	A	B	C	D	E	F	G
$\bar{x}$	1.31	1.23	0.64	0.90	1.10	0.88	0.96
SD	<u>+0.30</u>	<u>+0.36</u>	<u>+0.17</u>	<u>+0.29</u>	<u>+0.10</u>	<u>+0.14</u>	<u>+0.26</u>

 β -lactoglobulin7 Days Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	1.20	1.35	0.96	0.74	0.92	0.92	0.91
SD	<u>+0.37</u>	<u>+0.87</u>	<u>+0.41</u>	<u>+0.16</u>	<u>+0.38</u>	<u>+0.22</u>	<u>+0.38</u>

 β -lactoglobulin14 Days Postamputation

Relative Accumulation		N=5					
	A	B	C	D	E	F	G
$\bar{x}$	1.41	1.46	0.86	0.76	1.04	0.68	0.77
SD	<u>+0.34</u>	<u>+0.50</u>	<u>+0.16</u>	<u>+0.25</u>	<u>+0.28</u>	<u>+0.23</u>	<u>+0.18</u>

Table VII

Accumulation of I^{125} in regenerating limbs. This experiment was performed essentially as described in Table II with the exception that $0.5 \mu\text{Ci } I^{125}$ was substituted for $5.0 \mu\text{Ci } I^{125}$ prolactin.

TABLE VII

I-125 Alone - 0.5 Ci7 Days Postamputation

Relative Accumulation							N=6
	A	B	C	D	E	F	G
$\bar{x}$	1.05	0.89	0.98	0.99	0.95	0.85	1.28
SD	<u>+0.32</u>	<u>+0.25</u>	<u>+0.23</u>	<u>+0.22</u>	<u>+0.29</u>	<u>+0.28</u>	<u>+0.38</u>

Plate I

Figure 21 Brightfield light micrograph showing localization of silver grains in outside of wound epithelium from animal 21 days postamputation. 438 x.

Figure 22 Brightfield light micrograph showing localization of silver grains in underlying cells of wound epithelium from animal 7 days postamputation. 438 x.

Figure 23 Brightfield light micrograph showing localization of silver grains both on outer and inner layers of wound epithelium. 438 x.

Figure 24 Brightfield light micrograph of distal end of muscle bundle from animal three days postamputation. Note concentration of grains at end of center muscle bundle. 438 x.

Figure 25 Brightfield light micrograph showing distribution of grains in underlying tissue. 438 x.

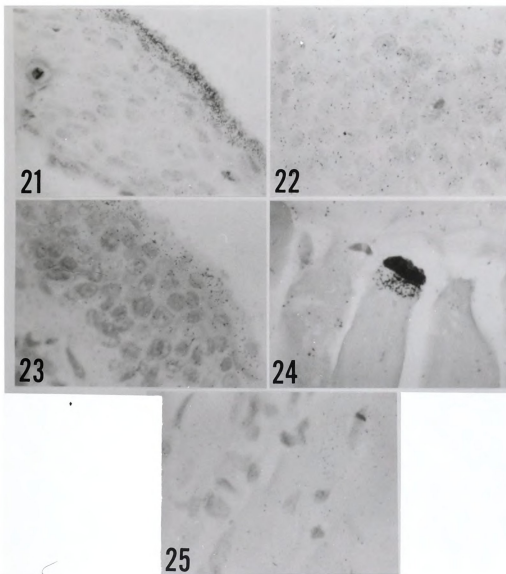




Plate II

Figure 26 Phase contrast micrograph showing localization of grains in wound epithelium. Tissue from animal seven days postamputation. Sections in all three of following micrographs from same limb. Incubated in 80,000 counts prolactin. 239 x.

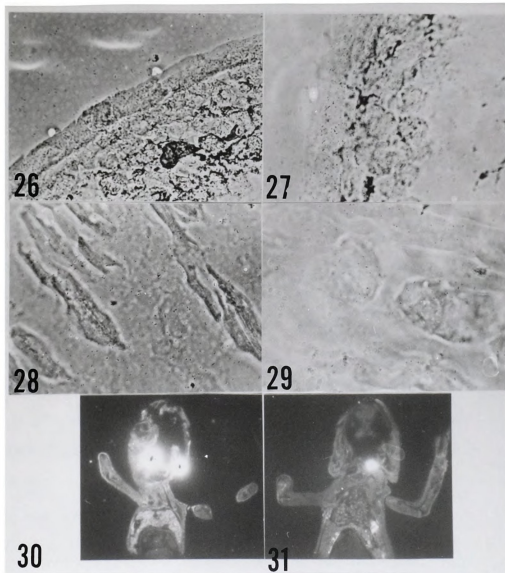
Figure 27 Phase contrast micrograph showing localization of grains in wound epithelium. Incubated in 80,000 counts plus prolactin excess. 478 x.

Figure 28 Phase contrast micrograph showing localization of grain in tissue beneath wound epithelium. Incubated in 80,000 counts prolactin. 478 x.

Figure 29 Phase contrast micrograph showing localization of grains in tissue beneath wound epithelium. Incubation 80,000 counts I^{125} prolactin plus prolactin excess. 478 x.

Figure 30 Whole body autoradiogram from a newt 14 days postamputation injected with I^{125} prolactin. 3.2 x.

Figure 31 Whole body autoradiogram from a newt 14 days postamputation injected with I^{125} alone. 3.2 x.



Discussion

In the past, quantitative estimates of amphibian pituitary hormones have suffered from a number of shortcomings. Heterologous radioimmunoassays (RIAs) using antisera to mammalian hormones are often uncertain in accuracy due to variability in cross-reaction and sensitivity (McKeown, 1973; Farmer and Papkoff, 1979; Nicoll, 1978). Homologous RIAs have been used relatively infrequently in non-mammalian species due to difficulties associated with isolating sufficient quantities of hormone suitable for antibody production. Recently, homologous RIAs have been developed for bullfrog prolactin and growth hormone (Clemons and Nicoll, 1977; Clemons, 1976) but the cross-reaction in this assay for hormones from other amphibian species is unknown.

The most frequently employed method for estimation of non-mammalian pituitary hormones is ND-PAGE followed by densitometry (Nicoll, et al., 1969; Nicoll and Nichols, 1971; Nicoll and Licht, 1971). It has been reported, however, that this method is also not entirely reliable (Farmer and Papkoff, 1979). One source of error is that pituitary proteins may be incompletely solubilized prior to separation (Samli, et al., 1972). In addition, bands corresponding to the major pituitary hormones show wide variation in R_f among vertebrate species (Nicoll and Nichols, 1971; Nicoll and Licht, 1971). Finally, while a protein with prolactin-like activity is generally well-defined in ND-PAGE, other pituitary proteins are not well separated (see Figure 5).

Bioassays are also not entirely reliable when estimating pituitary hormones due to the uncertainty of a measurable effect even in the presence of hormone. For example, the pigeon crop sac bioassay is a reliable indicator of the presence of prolactins originating from some species but not others. Another bioassay effective for some prolactins but not others is the plasma sodium retention assay in certain teleosts (Farmer and Papkoff, 1979). It is interesting that regardless of the species of origin, vertebrate prolactins exhibit a striking degree of sequence homology indicating that this is a very evolutionarily conservative molecule. It is equally interesting that with respect to the relationship between binding, bioassay activity and phylogeny a unidirectional specificity is evident. In general, hormones from "higher" vertebrates bind and are active in "lower" vertebrates while the reverse is not true. It has been suggested that this may be due to the development of more sophisticated regulatory processes in advanced vertebrates (Bentley, 1976).

In a direct comparison of the reliability and sensitivity of bioassay, RIA and ND-PAGE methods for quantitation of rat prolactin, Asawaroengchai and Nicoll (1977) found that bioassay and RIA were more sensitive than ND-PAGE. In general, however, there was reasonably good correlation between methods in terms of assessing relative amounts of hormone.

In the present study PAGE was used to estimate levels of newt pituitary proteins in the pituitary. This method is not suitable for examining circulating levels of hormones. In ND-PAGE (Figure 5) the electrophoretic pattern from newt pituitaries shows clear similarities to the pattern from most other vertebrates (Nicoll and Nichols, 1971).

One band, the fastest migrating major protein, is most clearly seen both by visual examination and after gel scanning. In other vertebrates protein isolated from this band is active both in bioassays and in radioimmunoassays for prolactin (Asaworoengchai and Nicoll, 1977; Nicoll and Nichols, 1971). Not surprisingly, then, it seems that newts have a pituitary protein with clear similarities to prolactin from other vertebrates. The identity of the other proteins on ND-PAGE could not be determined with any degree of certainty. Bioassays of slower migrating proteins from other urodeles have indicated both GH and TSH activity (Nicoll and Nichols, 1971; Schultheiss, 1980) and it is likely that this activity could be found among slower migrating pituitary proteins from the newt. Bioassays, however, were not performed here.

For quantitative estimate of newt pituitary proteins rather than ND-PAGE, however, SDS-PAGE was employed. SDS-PAGE was judged superior to other available methods on the basis of several criteria: 1) superior solubilization of tissue (Zanini, et al., 1974); 2) superior separation of peaks compared to ND-PAGE (Zanini, et al., 1974 and preliminary studies in this lab); 3) Uncertainty of cross-reaction between newt pituitary hormones and available antisera to mammalian hormones (for RIA) (McKeown, 1973; Hayashida, et al., 1973); 4) the ability to simultaneously examine levels for several pituitary proteins from the same set of animals.

The general pattern on 10% SDS-PAGE gels of newt pituitary proteins was very similar to that observed for rat pituitary proteins. Two bands are particularly prominent on the low molecular weight end of the gel, identified in the rat as growth hormone and prolactin (Zanini, et al., 1974). In the rat, as in other mammals, growth hormone is present in

greater quantity than prolactin (Nicoll, 1978) and this is evident in SDS-PAGE gels. In the newt this pattern is reversed, the band tentatively identified as newt prolactin being present in greater quantity than any other detectable protein. This result is consistent with other studies of amphibian pituitary hormones which indicate that, in general, amphibian pituitaries contain higher levels of prolactin than growth hormone or other pituitary hormones (Nicoll and Licht, 1971; Ensor, 1978).

In 10% SDS gels the apparent molecular weight of the bands corresponding to rat prolactin and growth hormone were 22-23,000 and 19-20,000 daltons for prolactin and growth hormone respectively. In the present study band II has been labeled "newt" prolactin on the basis of three criteria: 1) this protein is present in greater quantity than any other pituitary hormone, consistent with the ND-PAGE gels in which "newt prolactin" is the most prominent band; 2) although the calculated molecular weight is less than that observed for mammalian prolactin this value is in reasonably good agreement with that calculated for other amphibian prolactins (Ensor, 1978); 3) cultured newt pituitaries show enhanced production of protein in this band, an indication of release from hypothalamic inhibitory control (Ball, 1981; Hall and Chadwick, 1979); the general pattern of bands compared to the rat is consistent with the identification of this band as newt prolactin. The identity of bands I and III is less certain but on the basis of molecular weight and general quantities of hormone present these bands can be tentatively identified as growth hormone and thyroid stimulating hormone, respectively.

Upon examination of the three most prominent bands from animals with regenerating limbs, it appears that there are no significant differences between levels of these hormones either at different stages of regeneration or between intact animals and those bilaterally amputated. Initial studies indicated increased levels of Protein III in male animals during the course of regeneration. In subsequent studies, however, this result was not reproducible. It should be noted, however, that in the mouse at least one case has been noted in which there is a concomitant change in release and synthesis of prolactin. In cultured pituitaries from pregnant mice, prolactin release and synthesis are high on days 5-7 of pregnancy, low on days 8-16 and intermediate at 18-19 days of pregnancy. Prolactin storage in the pituitary during this period did not change appreciably during this period (Nixon and Talamantes, 1980). If an analagous situation exists in vivo for the newt the apparent lack of change in levels of pituitary hormones may actually be the result of this type of synthesis/release coupling. One other study which has bearing on the issue of levels of pituitary hormones during the course of regeneration is that of Barber and Scadding (1978). This study failed to note any hypertrophy of cells of the adenohypophysis during the course of amphibian limb regeneration.

Binding of I-125 Prolactin to Newt Limb Tissue

Typically, hormone binding experiments are performed as one indication of a direct relationship between hormone and target tissue. Numerous past experiments have indicated a biological role for prolactin in limb regeneration in the newt (Connelly, et al., 1969; Tassava, 1969; Schuable and Nentwig, 1974; Bromley and Thornton, 1974; Hessler and

Landesman, 1981) but none has indicated a direct association between hormone and regenerating tissue. In addition to a biological effect in limb regeneration prolactin has also been shown to affect structures in the intact limb. These effects include an effect on osmoregulatory activity of the skin (Lodi, et al., 1978) skin texture (Dent, et al., 1973a) and regulation of mitotic rhythm and cellular proliferation in the epidermis (Hoffman and Dent, 1977a, and b).

Previous prolactin binding studies on amphibian tissues have indicated that specific binding sites for ovine prolactin were evident in a number of amphibian tissues including kidney liver, bladder and tail fin (White, 1981; White, et al., 1981; White and Nicoll, 1979; Carr, et al., 1981; Carr and Jaffe, 1981). The present study, however, is the first to indicate specific prolactin binding in unamputated or regenerating amphibian limb tissue. This binding is proportional to the amount of tissue present and that displacement of I^{125} prolactin is proportional to the amount of unlabeled hormone added. This displacement effect is specific for prolactin and not seen when excess of other hormones are added (Figure 14).

With respect to in vitro binding of I^{125} prolactin to regenerating limb tissues several points are noteworthy. First, specific binding sites for any growth factor or hormone had not been demonstrated in regenerating limb tissue prior to this study. The results here clearly indicate that specific prolactin receptors are present in intact limbs and throughout the course of regeneration. In the distal, regenerating segment, the only evidence of increase in receptors is at three days postamputation while the other stages examined (0, 1, 7, 14, and 21 days postamputation) appear to have essentially the same levels of receptors.

Surprisingly, in intact animals receptor levels are higher in the medial segment than in either distal or proximal segments. The number of receptors in the medial segment gradually decreases, however, so that by 14 - 21 days there is essentially no difference between proximal medial and distal segments. In the proximal segment the numbers of receptors are higher than at later stages and this may indicate one of two things. Either the entire limb is influenced by the amputation trauma and hence the prolactin receptors or receptor levels in animals three days postamputation had artificially high levels of prolactin receptors due to something other than the amputation stimulus. This second possibility seems unlikely however, because all groups of animals were kept in identical bowls and treated identically in every manner with the exception of time of amputation.

That an increase in levels of prolactin receptors would be found as early as three days postamputation is consistent with previous studies on the relationship between pituitary hormones and the regenerating limb (Schotte and Hall, 1952). This work indicated that the regenerating limb is most dependent on pituitary influence during the early stages of regeneration and becomes increasingly independent of this influence in later stages. It was in this context that previous in vitro studies of hormonal influence on limb regeneration may have underestimated pituitary hormone influence owing to the "independent" stage at which they are generally explanted (Schotte and Hall, 1952).

Non-specific binding of prolactin to limb tissue is presumably not influenced by receptors. Changes were detected in the non specific binding, however, during the course of regeneration. This change is most apparent as a decrease in non-specific binding from 7 -

21 days postamputation. At this time it is not possible to determine how or if this change in non-specific binding influences the course of regeneration.

When I^{125} prolactin is injected in vivo this molecule is found to preferentially accumulate in regenerating limb segments from 3-42 days postamputation. This experiment was repeated on a number of occasions using newts from different sources and in nearly every case this effect was unmistakable. The fact that no accumulation is evident in an analagous segment of non-regenerating limb nor up to twenty-four hours suggests that accumulation is a regeneration related event and not due to experimental artifact. The mechanism for accumulation of I^{125} prolactin, however, is uncertain. On the basis of the in vitro receptor binding experiments it might be expected that if prolactin accumulation is a receptor mediated event a peak would be expected at three days postamputation with little other difference in accumulation between other stages. This is not the case. In fact, all stages examined following three days postamputation show a distinct accumulation effect. Experiments with I^{125} β -lactoglobulin were designed to determine if the accumulation effect is specific for prolactin or can be attributed to non-specific protein accumulation in the regenerating segment. Beta -lactoglobulin is a protein similar in size to prolactin with no known biological effects on regenerating limbs. The fact that this protein accumulates in similar fashion to prolactin may indicate that this effect is not mediated by specific receptors.

A mechanism of distal accumulation of hormones which seems broadly similar to the one reported here was discovered by Bromley (1977). In both cases the distal, regenerating segment shows greater accumulation

of label than other, non-regenerating segments. The accumulation effect per se, however, indicates only that these molecules have a preferential affinity for regenerating vs. other limb segments without any clue as to the nature of this interaction. Important differences do appear to exist between the two systems. Most salient is the fact that Bromley's mechanism deals with adrenocorticosteroids while a wide variety of other steroids and non-steroidal molecules do not show distal accumulation in regenerating limbs. Another important difference is that Bromley's mechanism is nerve dependent; the present one is not. Whether these two classes of results signify changes in receptor levels or some other general mechanism remains to be determined. It may, in fact, be possible that for certain molecules both specific and nonspecific components of the accumulation process are expressed. The present study indicates that in vitro, variations in both specific and nonspecific prolactin binding to regenerating segments are possible during the course of regeneration.

Tissue Localization of I-125 Prolactin

In tissue from animals injected in vivo the most striking localization of silver grains is over the outer layer of the wound epithelium. This effect can be seen both in autoradiograms prepared from histological sections for light microscopy (Figures 21 and 23) as well as whole body autoradiograms (Figure 30). This type of localization was not detected in animals injected with an equivalent amount of I¹²⁵ labeled alone (Figure 31). Based on the in vitro receptor assays discussed above it seems unlikely that increased occurrence of silver grains in that area can be attributed to changes in

local hormone receptor levels. Furlong, et al. (see appendix) have reported morphological differences between the outer layer of stump epidermis and wound epithelium and LaSalle (1980) has reported differences in permeability between these two areas. This difference in grain distribution, then, may be a reflection of morphological changes in this tissue then which in turn affect accumulation of various substances in that area. Although, as described above, it seems unlikely that the localization of prolactin in the wound epithelium is due to changes in prolactin receptors it is possible to conceive a mechanism whereby latent prolactin receptors would be exposed by the amputation stimulus. From morphological data it is clear that surfaces of cells from the wound epithelium are much more exposed than adjacent stump epidermis and these cells would presumably also be much less constrained in their interactions with hormones and other growth factors. In a similar vein it may also be that prolactin receptors are exposed by the homogenization process and that as a result the number of receptors available to a regenerating limb in vivo is greatly overestimated by the in vitro prolactin receptor assay.

Another series of experiments which have bearing on this issue comes from in vitro incubation of tissue sections in I^{125} prolactin followed by autoradiography. In these experiments grains are more heavily localized in wound epithelium and stump epidermis than subjacent tissues but the outer layer of wound epithelium generally has fewer grains associated with it than that of the underlying wound epithelium. That grain distribution would be heavily localized in wound epithelium and stump epidermis may be expected on the basis of studies by other workers. In hypophysectomized newts skin texture and cutaneous

secretion is affected (Dent, et al., 1973; Tassava, 1969a) and recently it has been suggested that pituitary hormones serve to maintain the integrity of relationship between wound epithelium and underlying tissue. In this laboratory scanning electron microscopy has shown that the continuity between stump epidermis and wound epithelium is interrupted in hypophysectomized animals and also that the outer layer of wound epithelium differs somewhat in appearance from that of animals with intact pituitary.

It seems clear then that one of the major influences of pituitary hormones in limb regeneration is in maintaining the integrity of the integument. Prolactin binding sites are found both in the wound epithelium and in the stump epidermis. When injected in vivo prolactin accumulates in the wound epithelium but the physiological significance of this effect remains unknown. Finally, this study points to the suggestion that morphological changes in regenerating limb tissue may be of consequence in their response to hormones and other factors.

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APPENDIX A



TITLE PAGE

Title:

Morphological Changes in the Wound Epithelium from the Regenerating
Limb of the Newt, Notophthalmus viridescens

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Figures

Three plates, total of 18 figures

Number of text pages

14

Abbreviated title

Fine structure of Wound Epithelium

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ABSTRACT

Fine structure of the wound epithelium was examined from a total of 42 animals from three to 21 days postamputation. Both scanning and transmission electron microscopy revealed stage dependent characteristics of wound epithelial cells. It was found that in all stages examined both the outer, exposed surface as well as the internal structure of the wound epithelium differed considerably in appearance from the stump epidermis. Distribution and type of outer cell layer surface structures were found to be dependent both on position on the limb and stage of regeneration. The inner cell layer from later stages (18 - 21 days postamputation) was notable for abundant collagen deposits which appeared concomittantly with loss of cell surface structures. In early stages (3 - 15 days postamputation) collagen was not observed and cell surface structures were abundant. Evidence is also presented for secretory activity by the wound epithelium. These observations may provide a basis for further examination of the nature of the interaction between wound epithelium and underlying tissue.



There are many examples of developmental systems in which epithelial/mesenchymal interactions play a central role. These interactions typically occur in embryonic tissues such as the developing limb, kidney, or pancreas (Deuchar, 1975; Wessels, 1977; Lash and Burger, 1977). The regenerating limb, however, represents a variation of the epithelial/mesenchymal interaction in that fully differentiated adult tissues revert to a condition resembling that of the embryonic limb after amputation. Initially, following amputation, epidermal cells migrate from the cut edge of the skin to cover the cut, distal end of the limb to form a structure known as the wound epithelium (Repesh and Oberpriller, 1978).

The contribution of the wound epithelium is not yet entirely clear, although it is known that an intact wound epithelium is required for regeneration (Singer and Saltpeter, 1961). Particularly useful explanations for the importance of the wound epithelium include direct influence on the aggregation of blastemal cells (Thornton, 1965), and influence on the traverse of blastemal cells through the cell cycle (Tassava and Mescher, 1965). Histological examination has shown that the cells of the wound epithelium differ considerably from the epidermal cells from which they originate. These differences are manifested in alterations in the characteristic staining of nucleus and cytoplasm as well as increased amounts of intercellular space in wound epithelium as compared to stump epidermis (Singer and Saltpeter, 1961.)



Although the importance of the wound epithelium to regeneration is well-known, no previous study has attempted to examine progressive, fine structural changes in this structure during the course of regeneration. We report here distinctive features of the wound epithelium on the outer, exposed surface as well as on the surface of underlying cells. These morphological changes may be a reflection of other more subtle changes in these epithelial cells as they change from stump epidermis to wound epithelium and back again to stump epidermis.

METHODS AND MATERIALS

Newts, Notophthalmus viridescens, were obtained from Connecticut Valley Biological Supply. They were kept at ambient room temperature in tap water conditioned with sodium thiosulfate and fed beef or pork liver two to three times per week. Amputations were done bilaterally, midway between elbow and wrist, at three day intervals for eighteen days. Twenty-one days after the first amputations, right limbs from all animals were removed and fixed overnight in cold Karnovsky's fixative (Karnovsky, 1965).

For scanning electron microscopy, limbs were transferred to 0.1M cacodylate buffer, washed and split bilaterally along the longitudinal axis with a razor blade. Samples were subsequently treated with 1% OsO₄ in 0.1M cacodylate for two hours, dehydrated with ethanol, critical point dried and attached to aluminum stubs. Left limbs, denervated by cutting spinal nerves III, IV and V were treated similarly but are discussed in a separate

report (Heidemann, et al., 1982). All specimens were sputter-coated with approximately 30 nm of gold and viewed with an ISI Super III scanning electron microscope at an accelerating voltage of 15 KV. Limbs from a total of forty-two animals were prepared with a minimum of three from each stage examined.

Samples for transmission electron microscopy were prepared and fixed as above. Following osmication, tissues were embedded in Epon/Araldite/Spurr's (1:1:3), cut and examined on a Philips 201 TEM. For light microscopy, specimens were fixed in Karnovsky's and embedded in Methylene Blue/Azure A/Basic Fuchsin (Humphrey and Pittman, 1979) and photographed.

RESULTS

External Surface of the Wound Epithelium

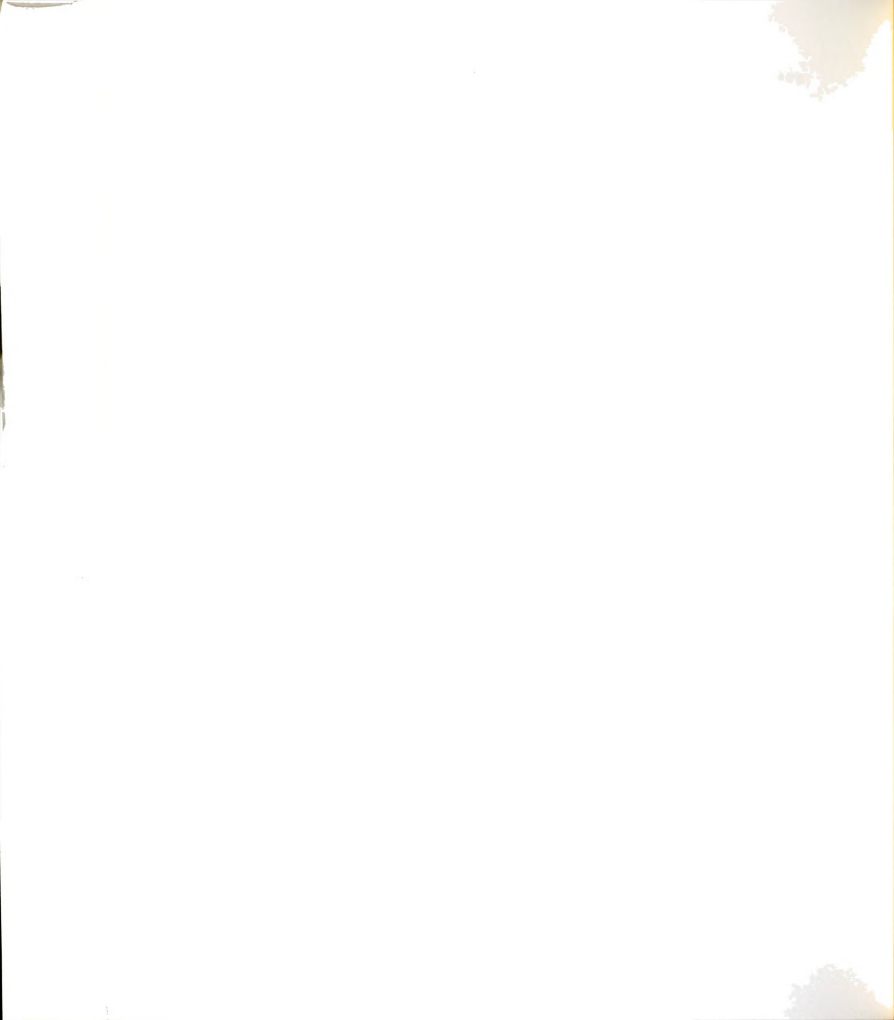
The outer surface of the wound epithelium is considerably different at all stages examined from the stump epidermis from which it originates. Papillae are commonly found in an irregular pattern on the stump epidermis (fig. 1). Higher magnification shows the surface of stump epidermal cells to be covered by a network of interconnecting microridges. In some cases, the stump epidermis appears highly convoluted which presumably can be attributed to an approaching molt. Papillae are now observed on the wound epithelium through twenty-one days postamputation although progressive changes are seen in the external aspect of the wound epithelium both in

general appearance and also with respect to microarchitecture of the cell surface.

At three days postamputation, prominent furrows are detected apically (fig. 2) that are absent more proximally. Higher magnification reveals that cell surface characteristics also vary with position on the stump. At the apex of the limb, numerous microvilli are observed (Fig. 3A) while more proximally the surface has a honeycomb appearance (Fig. 3B). From six to fifteen days postamputation the outer layer is composed of cells which are flattened and hexagonal (fig. 4). In general, higher magnification of these cells show surface structures ranging from microvilli to microridges (fig. 5) while more proximal cells are covered exclusively by microridges. By 18 - 21 days postamputation, cells continue to be hexagonal and flattened but are larger in surface area than cells from earlier stages (fig. 6). In addition, protrusions corresponding to cell nuclei are prominent and the surface of the cells are covered exclusively by dense microridges (fig. 6 inset). Examination of one limb at 34 days postamputation indicates that the outer surface changes little during the 21 - 34 day postamputation interval. It does appear, however, that cell nuclei become even more prominent

Internal Structure

Stump epidermis is typically four cells thick consisting of an outer layer of cornified squamous cells overlying two to four layers of cuboidal cells. Light microscopy shows that the cells of the stump epidermis are regularly arranged and tightly packed (fig. 7), an observation emphasized by scanning electron microscopy (fig. 8). By contrast, the cut surface of the wound epithelium at three days postamputation is a structure in which intercellular spaces are clearly evident (fig. 9). Individual cells are



flattened and covered by a latticework extracellular coating. A fibrous, fibrin-like matrix can be seen underlying the epithelium of this stage.

The epithelium from six to twenty-one day regenerates consists of two distinct cell layers: 1) an outer flattened layer; 2) an inner layer consisting of up to fifteen layers of cells more rounded than those of three day regenerates (fig. 11). Transmission electron microscopy indicates that the outer layer of cells has an extremely dense cytoplasm with the prominent external structures indicated by SEM also evident (fig. 15).

With respect to structure of underlying cells of the wound epithelium this study generally confirms and expands on the results of earlier scanning (Oberpriller and Oberpriller, 1978; Repesh and Oberpriller, 1980; Jasch, 1980; Geraudie and Singer, 1981) and transmission (Singer and Saltpeter, 1961; Norman and Schmidt, 1967) electron microscopy studies of the wound epithelium. The number of cells in the wound epithelium following amputation initially increases to form a thickened structure over the cut end of the limb which begins to thin by 18 - 21 days postamputation. The inner layer consists primarily of cells loosely arranged within the wound epithelium. Cell surfaces are nearly entirely covered by a random distribution of microvilli and blebs (fig. 13). Generally, blebbing was somewhat more prevalent in six to nine day regenerates than in later stages.

Transmission electron microscopy shows that intracellular vesicles of varying dimensions are common in cells from the inner layer of wound epithelium. The secretory nature of these vesicles (fig. 16) appears to originate from mucous granules and may be partially responsible for the blebbing seen on cell surfaces. Extensive collagen buildup in association



with cells of the wound epithelium (fig. 18) was not observed in any stage prior to eighteen days postamputation. The prominent rough endoplasmic reticulum in the cells of this stage would be consistent with the notion of the wound epithelium as a site of collagen synthesis in later stage regenerates. Earlier stages are noteworthy for their large intercellular spaces and lack of extracellular matrix.

DISCUSSION

There is no evidence of morphological difference externally between apical epithelial cells versus those elsewhere in regenerating limbs of Ambystoma mexicanum (Tank, et al., 1977). That does not appear to be the case in N. viridescens, used in this study. Distinct differences are noted between stump epidermis and wound epithelium at all stages examined and between apical and more proximal cells from three to eighteen days postamputation. These differences are generally expressed as variations in the pattern of cell surface structures. Other, progressive changes in the wound epithelium, however, are also observed during the stages examined in this study. These changes were manifested in a stretching and flattening of the outer layer of cells with a tendency for nuclei to protrude from the surface of the cell. This was particularly evident at twenty-one days postamputation. It is interesting to note there that while Ambystoma mexicanum is a neotenuous amphibian the limbs examined in this study are from adult newts (N. viridescens). Changes in the structure of the skin



during metamorphosis, then, could conceivably account for the differing results between these two species.

This study offers the first clear indication of the extend of secretory activity in the wound epithelium. Collagen production is not apparent up to fifteen days postamputation but was found in considerable amounts in subsequent stages. What significance, if any, this has relative to the relationship between wound epithelium and underlying tissue cannot be determined. It is conceivable, however, that production of collagen may signal a shift in the nature of the epithelial/mesenchymal relationship during later stages.

As noted in figure 16 secretory activity was also noted in association with intracellular vesicular structures, presumably mucous granules. This type of activity generally occurred infrequently but is undoubtedly responsible for some of the blebbing seen on epithelial cell surfaces. Judging from the preponderance of blebs in some stages it is likely that there are other causes for these structures as well. It is well-known that transformed cells in culture exhibit structures similar to those noted herd (Kessel and Shih, 1974), and may indicate release from some type of inhibitory activity. also, Sugrue and Hay, 1982 have noted that chick corneal epithelial cells exhibit blebs in the absence of extracellular matrix. As shown in figure 18, cells in close approximation with collagen were notable for their absence of surface structures. There may be similarities then between those two systems. Further examination of this structure may yield clues as to the nature of its importance in successful limb regeneration.

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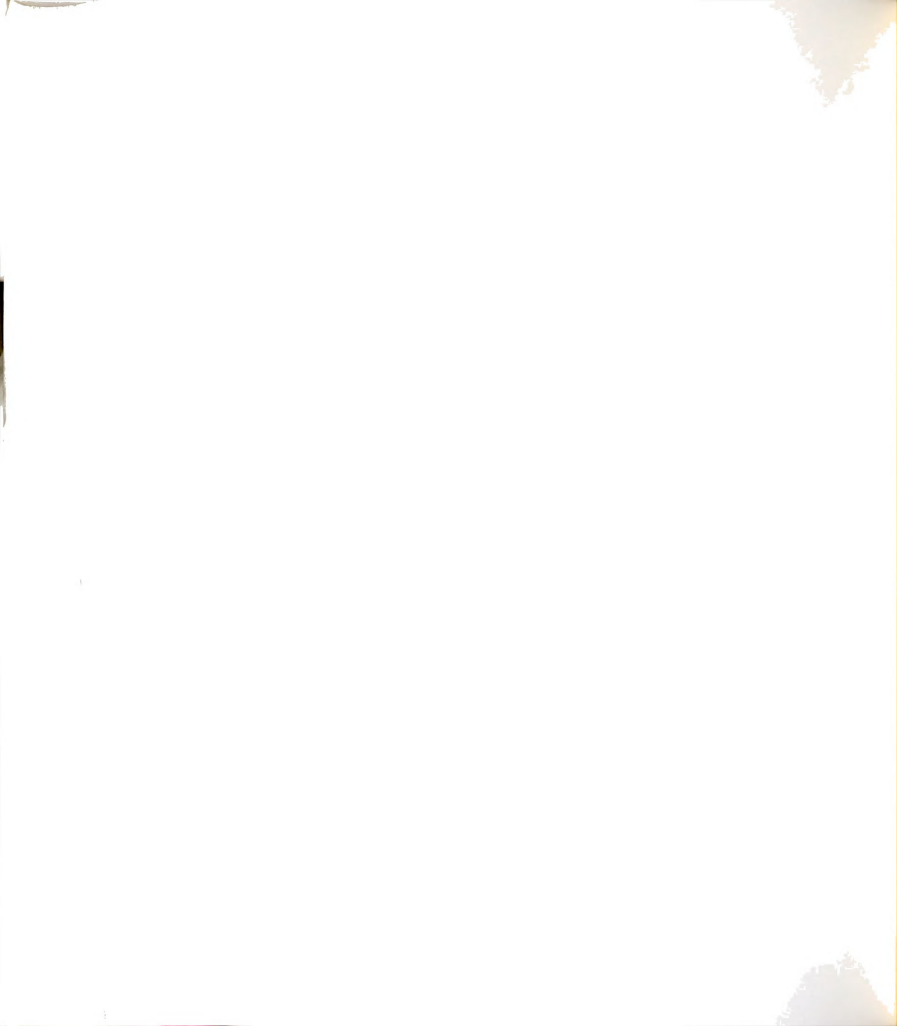
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FIGURE LEGENDS

Plate I

Fig. 1

Scanning electron micrograph (SEM) of outside stump epidermis. Specialized structures such as the papillae shown here (center) are common in stump epidermis but absent in wound epithelium. Borders of individual cells are evident (b). Bar = 11.6 μ .

Fig. 2.

SEM of outside apical wound epithelium three days postamputation. Note furrows between cells (arrows). Bar = 20.2 μ .

Fig. 3

Higher magnification (SEM) of cell surfaces shown in fig. 2 representing the different types of cell surface structures seen at this stage. Cell surface structures shown in 3a tended to be found in more proximal portions of the wound epithelium; those shown in 3b were found apically. Fig. 3a: bar = 0.3 μ ; fig. 3b: bar = 0.4 μ .

Fig. 4

SEM of apical outside wound epithelium nine days postamputation. Note fairly regular arrangement of cells and clear cell borders compared to three days postamputation (fig. 2). Bar = 5.3 μ .

Fig. 5

Higher magnification (SEM) of cell surfaces shown in fig. 4. Note microridges (mr), microvilli (mv) and intermediate structures (i). Bar = 1.2μ .

Fig. 6

SEM of outside apical wound epithelium twenty-one days postamputation. Cells are regular and hexagonal with protruding nuclei (n). Inset shows the surface of these cells at higher magnification. Bar = 6.7μ ; inset bar = 0.3μ .



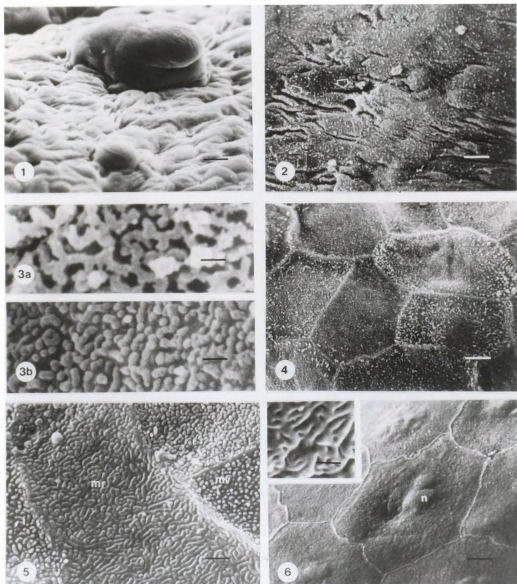


Plate II

Fig. 7

Light micrograph of cross-section through stump epidermis showing outer squamous layer with underlying cuboidal epidermal cells. Also shown in dermis (d) with skin gland (sg). Bar = $25\ \mu$.

Fig. 8

SEM of cross-section through stump epidermis. The outside of epidermis at the upper right aspect of figure. The cut epidermis typically appears very dense compared to the wound epithelium. Bar = $9.6\ \mu$.

Fig. 9

SEM of cut, inner surface of wound epithelium three days postamputation. Outside of wound epithelium extends toward upper left. Inner layer of cells are somewhat flattened with longitudinal axis arranged perpendicular to cut surface of limb. Bar = $6.7\ \mu$.

Fig. 10

SEM of overview of regenerate six days postamputation showing wound epithelium (we) covering the distal end of the limb. The cut end of the bone (b) can be seen beneath the wound epithelium. Bar = $50\ \mu$.

Fig. 11

SEM higher magnification of inner layer of wound epidermal cells shown in fig. 10. Note loose arrangement of cells with prominent intercellular spaces. Bar = $2.2\ \mu$.

Fig. 12

SEM of surface of cell from inner layer of wound epithelium six days postamputation. Note variety of cell surface structure ranging from microvilli (mv) to blebs (b). The nucleus (n) is in the lower right-hand corner. Bar = $1\ \mu$.



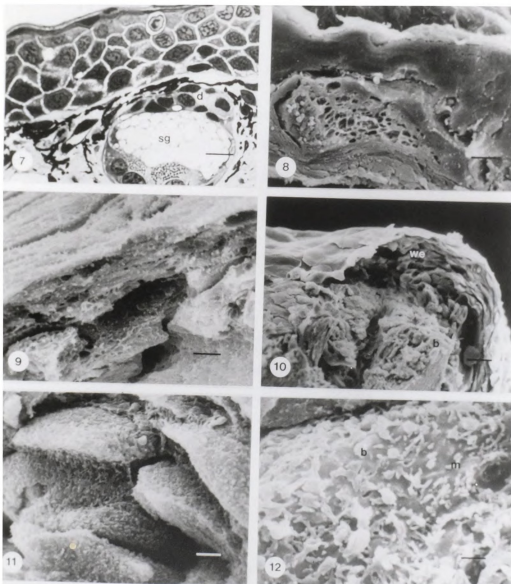




Plate III

Fig. 13

SEM of cell surface from inner layer of wound epithelium nine days postamputation. Surface structures are similar to those seen in previous figure (fig. 12) only, but are more abundant. Bar = 0.95μ .

Fig. 14

SEM of cut wound epithelium at 21 days postamputation showing outer flattened surface (top) with underlying cells. Inner layer of cells appear more tightly packed than early stages (compare with figs. 9, 10, and 11). Cell surface projections are also abundant. Bar = 13.7μ .

Fig. 15

Transmission electron micrograph showing structure of cell from outer layer of wound epithelium eighteen days postamputation. Cytoplasm is extremely dense and cross-section of structures forming microridges (mr) are evident. Numbers of secretory granules occupy the lower aspect of the figure. Bar = 0.7μ .

Fig. 16

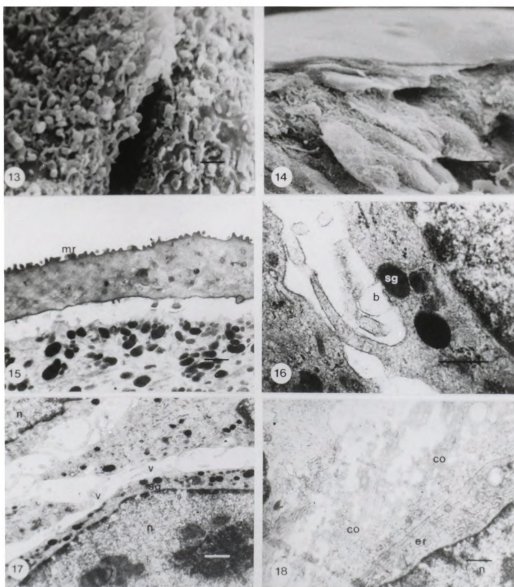
TEM of cell from inner layer of wound epithelium fifteen days postamputation. The nucleus (n) lies to the right of the figure. Secretory granule (sg) is shown releasing contents; an interconnecting bleb (b) is also evident. Bar = 0.4μ .

Fig. 17

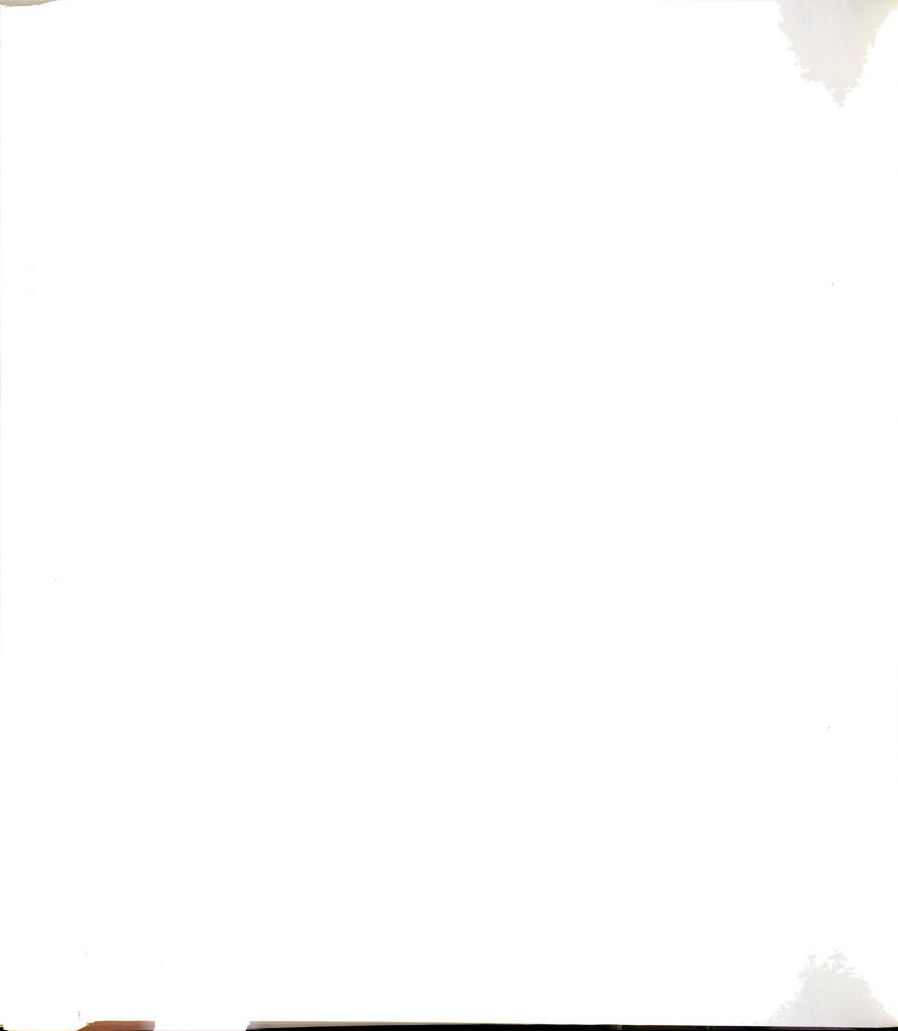
TEM of cells from inner layer of wound epithelium fifteen days postamputation shown abundant intracellular secretory granules (sg) and villous projections (v) on the cell surface. Two nuclei (n) are visible. Bar = 0.8μ .

Fig. 18

TEM of cell from inner layer of wound epithelium eighteen days postamputation showing abundant extracellular collagenous fibers (co). Cell is notable for lack of surface structures and prominent endoplasmic reticulum (er). Bar = 0.8μ .







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