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A QUANTITATIVE ANALYSIS OF HORMONE ACTION IN AMPHIBIAN METAMORPHOSIS

by

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I. General Survey of Literature

A. Amphibian Metamorphosis.

In Ranidae, the transformation of an aquatic fish-like tadpole into a land-dwelling frog occurs during a specific period of post embryonic development called "metamorphosis". This transition follows a sequential and temporal pattern and entails radical morphological and biochemical changes that are dependent upon hormonal control (see Lynn and Wachowski, 1951; Etkin, 1955, 1964 & 1968; and Weber, 1967). It is not surprising therefore that this phenomena has been of particular interest to endocrinologists, developmental biologists and biochemists alike.

Etkin (1932) divided metamorphosis in the typical anuran into the following stages: Premetamorphosis - a period of growth in which the tadpole form (oval head-body and muscular tail nearly twice the body length) is maintained without essential change. Prometamorphosis - a period characterized by differential hind leg growth with continued body growth at a reduced rate. During this period a definite sequence of relatively inconspicuous changes also occurs; resorption of the anal canal piece, formation of the skin window in the opercular skin over the gill chamber and reduction of the tubular wall of the spiracular opening. The emergence of the tadpole's forelegs through the opercular skin (this event is designated as the day of E) denotes the end of the prometamorphic period and signals the start of the final dramatic phase of metamorphosis, the climax period. During this period, the animal sheds its horny teeth and beaks and widening of the mouth follows. The complete resorption of the tadpole's tail marks the end of this period.

B. Role of Thyroid in Amphibian Metamorphosis.

Gudernatch (1912, 1914) demonstrated that amphibian metamorphosis was under endocrine control. He fed mammalian thyroid material to tadpoles and found that their rate of metamorphosis was accelerated. Allen (1916, 1917) and Hoskins and Hoskins (1917, 1918) provided direct proof for the role of the tadpole's own thyroid gland in metamorphosis; thyroidectomized tail bud embryos failed to metamorphose; instead they developed into giant tadpoles. Thyroidectomized tadpoles treated with thyroid material underwent metamorphosis.

1) Development and Function of the Thyroid During Metamorphosis

In the period following these initial investigations, thyroid development and function during metamorphosis has been extensively examined. Studies based on qualitative and quantitative characteristics of thyroid structure during metamorphosis have provided us with a basic picture of the thyroid's activity throughout this period of development.

(a) Morphological studies: -- Allen (1919) first attempted to correlate gross measurements of the thyroid with different stages of the toad's metamorphosis. Mayerowna (1922) studied the histology of the thyroid and observed changes in thyroid epithelia and colloid during metamorphosis. From histological studies by (Sklower, 1925; Etkin, 1930, 1936a; Clements, 1932; Woitkewitsch, 1937; D'Angelo and Charipper, 1939; D;Angelo, 1941; Saxén et al., 1957a & b; Fox, 1966; and Iwasawa, 1966) a morphological picture of the thyroid has emerged indicating that in early premetamorphosis, following the gradual organization of the gland, the thyroid is small and appears inactive (flat follicular epithelium and little colloid in the follicular lumen).

Later in premetamorphosis the thyroid's growth rate increases and the gland consists of colloid-filled follicles with squamus epithelium. During prometamorphosis the thyroid becomes more active, colloid is secreted rapidly, vacuolization of the colloid occurs and the follicular epithelium undergoes transformation from squamus to the columnar form. At climax, the colloid stored hormone is discharged and follicles may show partial collapse. By the end of climax the thyroid shows a low level of activity. Recently, electron microscopic studies of the changes in ultrastructure of the thyroid gland of X. laevis during metamorphosis have also demonstrated that there is a correlation between thyroid activity and metamorphic activity (Coleman et al., 1968).

Etkin (1964) pointed out that the morphological picture of the thyroid is observed to pass through a sequence of changes as gauged by height of epithelia, condition of follicles and colloid, that can be correlated with the various stages of metamorphosis of the tadpole. This picture has been interpreted as indicating the activation of the gland at the beginning of prometamorphosis and with the level of activity increasing to a maximum at climax.

(b) Radioiodine studies: -- Thyroid activity during metamorphosis has been studied quantitatively by means of radioactive isotopes of iodine (iodine -131). Experiments have been conducted on the uptake, incorporation and turnover of I^{131} by the thyroid at different stages of metamorphosis (Gorbman and Evans, 1941; Money et al., 1955; Dodd, 1955; D'Angelo, 1956; Saxén et al., 1957a & b; Kaye, 1961; Hanaoka, 1966). Other investigators (Shellabarger and Brown, 1959, and Flickinger, 1964) have attempted to establish what the synthetic pattern of this gland is, in relation to its ability to synthesize thyroid hormone and they have shown that a sequential pattern in the synthesis of organic compounds occurs in which monoiodothyronine (MIT) and diiodothyronine (DIT) are the first to appear; triiodothyronine (T_3) and tetraiodothyronine (T_4) are synthesized later. In general the use of I^{131} has demonstrated that during prometamorphosis there is an increase in the capacity of the thyroid to bind iodine and to synthesize thyroid hormone. These studies on thyroid function support qualitatively the morphological picture of activity presented. However, the quantitative refinement of these experiments does not appear to have been done to date. As an example: Saxén et al. (1957 a & b) determined protein bound iodine (PBI) in tadpoles after the removal of their thyroids and found that there was no increase in activity of the prometamorphic gland. They interpreted the activity of the thyroid at this time to be that of storage rather than secretion. However, it has already been made clear that prometamorphic changes are dependent upon increased thyroid activity. In addition, they reported only about a three fold increase in the level of PBI in the body tissues at climax. It has been suggested (Etkin, 1964) that their methods were not sensitive enough to trace the subtle change in secretory activity of the thyroid at these early stages of metamorphosis. Kaye (1961) studied I^{131} uptake by the thyroid and reported a ten fold increase in I^{131} uptake during prometamorphosis and a forty fold increase prior to climax. These studies are in closer agreement with the morphological findings already presented and with the studies of thyroxine-induced metamorphosis to be discussed in section 4. Employment of I^{131} has not clarified in a quantitative way the changes in level of effective thyroid hormone in circulation during metamorphosis; particularly, it fails to define the effective hormone level responsible for climax changes (Etkin, 1968). In addition, little evidence has been presented concerning the actual distribution of iodinated compounds in larval tissues during metamorphosis. Data of this type could be helpful in our understanding of the relationship between thyroid hormone synthesis, storage, release, and its subsequent action at the level of target tissues.

(c) Effect of goitrogens on metamorphosis: -- In recent years chemical thyroidectomy has been used effectively to inhibit metamorphosis. Antithyroid drugs and goitrogenic substances such as thiourea, thiouracil, and thiocyanate, known to interfere with thyroid function in higher vertebrates by inhibiting the formation of active thyroid hormone, have been reported to inhibit metamorphosis of amphibians (Gordon et al., 1943, 1945; Hughes and Astwood, 1944; Gasche, 1946; Iwasawa, 1956, 1957). The demonstration that interference with the thyroid's capacity to synthesize and release functional thyroid hormone inhibits metamorphic progress verifies the fact that this gland plays a decisive role in this process. The action of goitrogens on tadpoles will be discussed again in connection with other aspects of metamorphosis.

(d) Artificially induced metamorphosis: -- The availability of purified and synthetic thyroid hormones has provided another means of pursuing a quantitative approach to the role of the thyroid in metamorphosis. Etkin (1935, 1964) treated either thyroidectomized or normal tadpoles with exogenous hormone by maintaining tadpoles in thyroxine solutions of different concentrations. It was anticipated that the normal pattern of metamorphic change could be simulated by such treatment. A correlation could then be made between the level of exogenous thyroid hormone used and the pattern of metamorphosis observed as a means of estimating what the normal levels of circulating thyroid hormone might be in the tadpole at different stages of metamorphosis. Treatment of tadpoles with 1 ppb of thyroxine induced hind leg growth at a rate higher than that normally found in premetamorphic animals. Increasing the level of exogenous thyroxine to 3 ppb resulted in a rate of hind leg growth normally found in early prometamorphosis. Maximum hind leg growth was produced by treatment with 27 ppb T_4. In contrast, the induction of tail resorption in premetamorphic tadpoles at a rate comparable to that observed during normal metamorphic climax requires a solution of 243 ppb of T_4. From these results, Etkin concludes that during premetamorphosis the rate of hormone production is equivalent to less than 1 ppb of T_4. During prometamorphosis the rate of hormone production rises from the equivalent of 3 ppb T_4 to about 20 ppb T_4 until about the time of E when the rate of hormone production rises to about 200 ppb T_4. This high level is maintained until tail resorption is completed (E+5).

Etkin (1935) found that no single concentration of thyroxine produced a normal pattern of metamorphosis but a normal pattern could be simulated by regulated increases in hormone concentration during the course of the treatment. Treatment with single, high, concentrations of hormone produced animals whose pattern of development was distorted.

The results of Etkin's experiments are consistent with the picture that was presented by histological and radioiodine studies. The thyroid during early stages of prometamorphosis demonstrates low activity and this activity increases gradually until the beginning of climax when there is a dramatic increase in thyroid activity.

2) The Response of Tissues to Thyroxine

It has been suggested (Etkin, 1935 and 1964) that the sequential pattern of metamorphic events is determined by the changing levels of thyroid hormone that are synthesized and released by the thyroid during metamorphosis. Evidently, the thyroid gland controls metamorphosis by supplying thyroid hormone in amounts required by each tissue of the tadpole undergoing metamorphic change and each tissue responds to this hormone in a specific manner. If this conclusion is valid three questions must be asked. When do tissues first respond to thyroid hormone? What is the relationship between the level of thyroid hormone in circulation and the responses of tadpole tissues? How does the same stimulus, circulating thyroid hormone, induce such diverse and indeed opposite morphogenetic events, as leg growth, tail resorption and changes in the digestive system involving both growth and regression?

(a) The earliest response of tissues to thyroid hormone: -- The question of appearance of tissue sensitivity to thyroid administration was discussed by Alphonse and Baumann (1933). They noted that administration of thyroid to young tadpoles with external gills did not affect the later appearance of hind legs. Etkin (1950) and Moser (1950) demonstrated that sensitivity to thyroxine appears in tadpoles at embryonic stage #23 (Pollister and Moore stages).

(b) The differential sensitivity of tissues to thyroxine: -- From the earliest studies it became apparent that different tissues of the larval amphibian body show specific differential sensitivity to thyroid hormone. Romeis (1923) and Alphonse and Baumann (1935) drew attention to the fact that different organs of tadpoles respond differently to the same concentration of thyroid substance. Immersion experiments by Allen (1932a) and Etkin (1935) demonstrated that each tissue possesses differential sensitivity to the same concentration of thyroxine. Certain organs, hind legs, respond to low concentrations of hormone whereas other tissues, tail, required relatively high concentrations for a normal, rapid, response.

(c) Specificity of tissue response to thyroid hormone: -- Specificity of tissue response to thyroxine has been demonstrated by means of transplantation experiments carried out on anuran larvae. Fukai (1934) transplanted tails of tadpoles to different positions on the host. These transplants underwent resorption when the host's tail resorbed. Reis (1930) transplanted back skin to the tail and tail skin to the back of tadpoles. Each tissue followed its characteristic development; tail tissue underwent histolysis at the appropriate time while back skin did not. Similar experiments were conducted by Schubert (1926) who transplanted a hind limb bud to the tail of a tadpole and Schwind (1933) who transplanted optic vesicles to the tail of an embryo. In each case when the tail underwent resorption the transplanted tissue was observed to move towards the sacral region of the animal and did not undergo resorption. From such experiments it can be concluded that tissues possess intrinsic properties that determine their response to thyroxine and these properties are retained despite topographical translocations of the tissue.

3) Mode of Thyroid Hormone Action in Metamorphosis

(a) The concept of threshold and stoichiometry: -- The views regarding the mode of action of thyroid hormone in regulating the pattern of metamorphic events is at the present time controversial. Kollros (1959, 1961) has interpreted the action of thyroxine in metamorphosis to be based on a threshold concept. He suggests that the tissues involved in metamorphosis possess different thresholds and that stimulation of metamorphic events occurs to the extent that the increasing concentration of circulating thyroid hormone matches the threshold levels. He also proposes that tissues may change in their threshold sensitivity to thyroxine at different stages of metamorphosis. Kollros bases his interpretation of the mode of thyroxine's action in metamorphosis on experiments in which hypophysectomized tadpoles had been exposed to low concentrations of T_4 (0.002µg/liter) for several months without producing a metamorphic response. Raising the concentration would bring the animal to a particular point in metamorphosis at which they remained for as long as the concentration was not raised. He regards this as evidence for a true threshold below which no response occurs. In Kollros' view not only does thyroxine act as a release "mechanism" but the continued function of this "mechanism" requires the maintenance of the threshold level of thyroid hormone.

Etkin's interpretation of the role of thyroxine in metamorphosis is a quantitative concept in which thyroxine is thought to act in a fashion common to many chemical reactions, that is, in a stoichiometric manner, i.e., the higher the concentration the faster the reaction proceeds. Each tissue is characterized by a different specific total requirement of thyroid hormone to complete a metamorphic event. The rate of response of the tissue varies in proportion to the concentration available to it (Etkin, 1964; Weber, 1967). This interpretation was proposed initially by Allen (1932). In his experiments tadpoles were immersed in thyroxine solutions of different concentration. All tissues responded but the rate of response varied; some tissues did not respond as rapidly as others in weak solutions. Allen concluded that this response is not a question of "threshold" but is a question of "time of response". Etkin (1935) found that treatment of thyrodectomized tadpoles with effective concentrations of thyroxine would induce both early and late metamorphic events if given enough time to operate. The normal pattern of metamorphosis could be achieved by starting the treatment of tadpoles with low concentrations of T_4 and then gradually increasing the concentration of hormone. This process simulates the pattern of activation of the thyroid gland, during metamorphosis, already described.

Both of these theories are based upon the knowledge that the level of thyroid hormone increases during metamorphosis but the threshold concept does not suggest, as does the stoichiometric concept, that thyroxine enters directly on a molar basis into the biochemical activities of the tissues involved to produce morphological changes.

(b) Dependence of metamorphic changes on the continuous presence of thyroid hormone: -- A point that has been recognized to be of importance in understanding the mode of thyroid hormone action in metamorphosis is the relationship between tissue response and the continuous presence of the hormone. Animals treated with thyroxine become static when hormone is removed (Moser, 1950; Blaizot and

Blaizot, 1951; Kollros and Kaltenbach, 1952). Etkin (1964) studied this phenomena in Rana pipiens by two methods: (1) Temporary immersion of tadpoles into solutions of T_4. (2) Thyroidectomy of tadpoles at different stages of metamorphosis. Hind leg growth stimulated by treatment with 3 ppb T_4 ceases in 2-4 days following the change from thyroxine solution to spring water. When higher concentrations of thyroxine were used (81-243 ppb) tail resorption is initiated but when these animals are transferred to spring water tail regression ceases. However, mouth widening and leg growth persist for a few days longer. It appears that the level of thyroxine present in the animal drops gradually after removal of thyroxine so that the more sensitive tissues persist in metamorphic activity for additional periods. If animals are thyroidectomized on E - 1, or E + 0 only slight resorption of the tail occurs with some changes in the mouth parts occurring. If thyroidectomy is performed on E + 2 (when tail resorption has already begun) tail resorption advanced towards completion and widening of the mouth is completed. These experiments demonstrate that the continuous presence of thyroid hormone is required for metamorphic processes to be completed and also suggests that this hormone takes part, directly, in the chemical changes involved in the metamorphic transformation taking place in the tadpole.

4) The Direct Effect of Thyroid Hormone on Tissues

Although it has been shown that metamorphosis is dependent upon thyroid hormone, it is necessary to determine if these changes result from the action of thyroxine directly on the tissues of the tadpole, or whether they are second or third order effects.

(a) Local response to thyroid hormone: -- An approach to this problem that has successfully demonstrated this point has been the use of implanted thyroxine-cholesterol pellets. These pellets have been used to induce local tissue responses. This method of local application of hormone to specific sites in an animal was first introduced by Hartwig (1940). Slow diffusion of hormone from a pellet produces a local area in which the concentration of hormone is high and a clear distinction in metamorphic status is established between the local area and the general body tissues (Kaltenbach, 1968). Kaltenbach (1953a, b, c, 1959) produced local changes of the skin, forelimbs, hindlimbs, eyelids, nictating membranes, cornea, extrinsic muscles of the eyes, and local tail resorption. In addition to these tissues, thyroxine has been shown to have a direct effect on neural tissue; the corneal reflex center, the mesencephalic V nucleus, Mauthnerian and nonMauthnerian cells in the medulla, and the lateral motor columns in the spinal cord (Kollros, 1942, 1943; Kollros and Mc Murray, 1956; Kollros, 1957; Pesetsky and Kollros, 1956; Pesetsky, 1962, 1966). Thus by applying hormone locally in the form of pellets to various structures, the direct action of thyroid hormone on these structures has been established.

(b) _In_ _vitro_ studies: -- A different approach used to demonstrate the direct action of thyroxine in inducing morphological and biochemical changes during metamorphosis is provided by culturing isolated organs or tissues _in_ _vitro_. Isolated tails of tadpoles have been treated with thyroxine and such tails undergo shrinkage in size, resorption (Weber, 1962; Shaffer, 1963; Flickinger, 1963 and Tata, 1966). It has also been demonstrated that the addition of T_4 to organ cultures of competent larval skin (body, head, or limb) leads to increased development of sub-epidermal glands (Vanable, 1965; Vanable and Mortensen, 1966; and Mc Garry and Vanable, 1967). Other amphibian organs and tissues have been maintained in culture; Ohtsu et al. (1964) studied the conversion of visual pigments in tadpole eyes *in vitro*, Stephenson (1967) studied the effects of temperature on hearts maintained *in vitro*, Foote and Foote (1965) cultured pancreas. Experiments of this type, *in vitro*, have already demonstrated that thyroxine acts directly on tissues to induce metamorphic change and as techniques improve for culturing amphibian tissues other organs and tissues will undoubtedly be studied in this manner.

C. Role of the Pituitary in Amphibian Metamorphosis.

1) Requirement of the Pituitary for Metamorphosis

Following the demonstration by Gudernatch that amphibian metamorphosis is dependent upon thyroid control, Adler (1914) Allen (1916) and Smith (1916) demonstrated that a metamorphosis inducing substance is produced by the anterior pituitary; mammalian pituitary extracts had a positive effect in stimulating the metamorphosis of salamander and frog tadpoles.

2) Effect of the Pituitary on the Thyroid Gland

In studying the thyroid glands of hypophysectomized tadpoles it was found that these glands were poorly developed and inactive (Adler, 1914; Allen, 1916; Smith, 1916, 1920; and Hoskins and Hoskins, 1919). In control animals the glands were active. In addition, hypophysectomized animals given pituitary implants also showed activated thyroids. It was concluded that the anterior pituitary exerts its effects on metamorphosis through activation of the thyroid. Etkin (1936b, 1939) autotransplanted pituitary primordium close to the thyroid or performed the reverse operation and found that the thyroids under these conditions became activated resulting in the precocious metamorphosis of these tadpoles.

3) Histology of the Pituitary During Metamorphosis

Histological examination of the pituitary during metamorphosis have on the whole been less satisfactory than similar types of studies on the thyroid gland. Allen et al. (1929) indicated that "basophils" (thyroxine cells) are scarce initially while they increase in number during metamorphosis until climax when they are the most numerous. Similar studies by D'Angelo (1940, 1941) Saxén et al. (1957a & b) Saxén (1958) Van Orrdt (1966) and Kerr (1966) have indicated that there is a good correlation between increased activity of the thyroid and the increase in basophils during metamorphosis. These basophils also demonstrate increased cytological activity with respect to secretion. It was concluded by these authors that these cells synthesize and release thyrotropic hormone (TSH).

Histological studies of the anterior pituitary have also implicated another cell type "acidophils" with the synthesis of a growth promoting substance that is released during the early growth phase of tadpole development.

4) Pituitary-Thyroid Axis

In mammalian studies of the relationship between the thyroid and pituitary it has been established that there is a feedback or "thyrostat" mechanism by which the level of thyroxine in circulation inhibits TSH production. Depending upon the "setting" of the thyrostat a particular level of thyroxine and TSH can be maintained (Brown-Grant, 1966;

Reichlin, 1966).

Hoskins and Hoskins (1918, 1919) observed hyperplasia and hypertrophy of cells in the pars anterior of thyroidectomized tadpoles. Similar cells that appear in thyroidectomized mammals are referred to as "thyroidectomy cells". It is believed that due to the absence of thyroxine, thyrotropic cells of the pituitary are no longer under feedback inhibition, therefore, these cells appear to be highly activated. Goitrogens have been shown to inhibit metamorphosis. These drugs inhibit the formation of active thyroid hormone resulting in decreased levels of thyroxine in circulation. In turn, more TSH is released to stimulate further the thyroid; inducing hyperplasia and hypertrophy of the thyroid. Under these conditions the thyroid will eventually atrophy as the capacity of the cells of the anterior pituitary to continue to produce TSH ceases. Treatment of tadpoles with exogenous thyroxine induces metamorphic change but it also results in inhibition of the function of the tadpole's thyroid as measured by I^{131} uptake (Bowers, 1959 and Kaye, 1961). Exogenous thyroxine is believed to depress TSH secretion resulting in less activation of the animals own gland.

Although the thyroid can develop independently of the pituitary, treatment of premetamorphic tadpoles with goitrogens (Gordon et al., 1945; Blackstad, 1949; Delsol, 1952; Iwasawa, 1956) indicates that the pituitary-thyroid system is already operating by this early stage. Furthermore, the production of TSH has been shown to begin in the undifferentiated pituitary since it has been demonstrated that a locally effective thyrotropic field exists around the embryonic pituitary gland (Etkin, 1939; Kaye, 1961). It appears that the low level of thyroid hormone present in premetamorphic tadpoles results from the extreme sensitivity of the pituitary thyrotrophs to thyroxine inhibition and that this condition maintains the low level of hormone characteristic of this stage of metamorphosis. The increase in thyroid activity during prometamorphosis and climax must, therefore, be a reflection of the change in sensitivity and activity of the pituitary. It has been concluded (Etkin, 1964) that the sensitivity of thyrotrophs to thyroid hormone feedback undergoes moderate reduction during prometamorphosis and that at climax a major release of cells from negative feedback occurs. Following climax the reduction of thyroid activity implies that thyrotrophs regain sensitivity to feedback control.

D. Neural Control of Metamorphosis.

The question that presents itself next is; What is the mechanism that regulates or sets the thyrostat and causes it to change during metamorphosis?

1) Hypothalamic-Pituitary Relationships

In vertebrates, universally, the neurohumeral theory of hypothalmic control of the anterior pituitary has been generally accepted. According to this concept nuclei in the hypothalamus produce secretory products (releasing or inhibiting factors) that are transported to the median eminence by neurosecretory axons where their products are released. These products are then transported to the anterior pituitary by the hypothalamohypophysial portal system where they influence the synthesis and or release of pituitary hormones.

2) Hypothalamic Control of Metamorphosis

It has been demonstrated that the pituitary is capable of releasing enough TSH to induce metamorphic advance but in the absence of its connection to the hypothalamus it cannot produce enough TSH to complete metamorphic climax. Therefore, it has been proposed that control of the pituitary-thyroid axis involves two mechanisms; (1) direct negative feedback of thyroxine on the anterior pituitary, (2) hypothalamic control of the anterior pituitary by means of a thyrotropic releasing factor, TRF.

The dual control of anuran metamorphosis just described raises the following questions: when does the development of these two mechanisms take place? What coordinates their activity? Voitkevich (1962) and Etkin (1965/66) demonstrated that the median eminence and the portal system differentiate in parallel with other metamorphic events. However, during prometamorphosis differentiation of these areas occurs so that by climax the median eminence is well developed. The important point that has been demonstrated is that the hypothalamic mechanism for regulating the pituitary-thyroid axis is itself controlled by thyroxine. This hypothalamic system operates by positive feedback; it develops slowly but is self-accelerating (Etkin, 1963).

3) Etkin's Theory of Metamorphosis

The theory of anuran metamorphosis presented by Etkin (1965, 1966) is as follows. During larval stages, the thyroid develops independently of the pituitary. In premetamorphosis, the hypothalamic control of the pituitary-thyroid axis is minimal with the thyrostat set by negative feedback of thyroxine on the anterior pituitary. The anterior pituitary thyrotrophs are extremely sensitive to thyroxine and therefore TSH levels will be low, this in turn leads to low thyroxine concentrations. During prometamorphosis the hypothalamic elements become sensitive to thyroxine and differentiation of these parts occurs so that TRF is now transported directly to the anterior pituitary where it desensitizes these cells to thyroxine and more TSH is released. This in turn leads to more thyroxine being released which causes further differentiation of the hypothalamus and the median eminence. Thus a self-accelerating positive feedback system is established that rapidly builds up the thyroxine concentration. This initiates climax and the completion of metamorphosis. It is suggested that at the end of climax, the hypothalamic mechanism loses its sensitivity to thyroxine and that the pituitary thyrotrophs are once again subject to negative feedback by thyroxine. As a result the level of T_4 drops again to a low level.

E. Role of Prolactin in Amphibian Metamorphosis.

Recently, Etkin and Lehrer (1960) Berman et al. (1964a & b) Etkin and Gona (1967) and Bern et al. (1967) have suggested that another hormone, prolactin, may influence the metamorphic process. Etkin and Lehrer (1960) demonstrated that pituitaries implanted into hypophysectomized tadpoles produced excessive growth of these animals. The authors suggested that these grafts might be releasing prolactin and that this substance acts as a growth factor in tadpoles. It has been demonstrated by Everett (1956 and 1966) that in the rat prolactin secretion is normally under hypothalamic inhibition and that grafts released from inhibition produce excesses of this hormone. In a similar manner, a transplanted tadpole pituitary would be freed from hypothalamic inhibition and as a result release high levels of prolactin. Ball et al. (1965) Masur (1962) Nicoll and Bern (1964) Meites and Nicoll (1966) Riddle (1963) and Peyrot et al. (1966) have indicated that isolated pituitaries *in vitro* and grafts *in vivo* are capable of releasing prolactin. Berman et al. (1964b) Bern et al. (1967) Rémy and Bounhiol (1965) and Etkin and Gona (1967) injected mammalian prolactin preparations into normal and hypophysectomized tadpoles and observed that prolactin produced increases in growth. In addition, these authors have reported that administration of growth hormone also increased growth in tadpoles but it is not as effective as prolactin. Part of this growth effect is thought to be due in part to contamination of growth hormone preparations by prolactin. It has also been demonstrated by Etkin and Gona (1967) Gona (1967) and Bern et al. (1967) that prolactin can inhibit metamorphosis. Etkin et al. (1968) observed that pituitary grafts placed into the tails of tadpoles near climax interferes with their normal climax pattern so that tail resorption and other climax events are partially delayed. The area around the graft stands out conspicuously as a lobe of unresorbed tissue. The local action of pituitary grafts leading to inhibition of tail resorption could be explained by assuming that prolactin released by these grafts interferes with the action of thyroxine peripherally, at the level of target tissues. Support for this concept comes from the work of Bern et al. (1967). They injected tadpoles with prolactin and then placed these tadpoles into solutions of DL-thyroxine. At the concentration of thyroxine used tail length and depth of tail fins was reduced in tadpoles treated only with thyroxine. However, simultaneous treatment with prolactin counteracted the tail resorbing influence of thyroxine. Therefore, these authors have suggested that prolactin functions peripherally to antagonize the action of thyroxine. The experiments of Etkin and Gona (1967) and Gona (1967) have suggested that prolactin inhibits metamorphosis in yet another manner, by acting as a goitrogenic agent that interferes with thyroid function. As a result of the action of prolactin the levels of functional hormone in circulation are reduced and these tadpoles enter metamorphic stasis. The more recent work of Gona (1968) and Etkin et al. (1968) suggests that prolactin may function in both capacities. These recent demonstrations of the action of prolactin in metamorphosis suggest that the concept of metamorphosis previously presented (Etkin, 1964) will be modified to include the role of this hormone in this phenomena.

II. Introduction to the Present Study

A. Aims.

It is the aim of this investigation to study the role of two hormones (thyroxine and prolactin) in metamorphic change. Specifically, the study is designed to answer the following major questions. Is the response of target tissues to thyroxine quantitative in nature? Do target tissues of metamorphosing animals change in their sensitivity to thyroid hormone? Answers to these first two questions would be of significance in understanding the mode of action of thyroid hormone in metamorphosis. The last major question concerns the role of prolactin in metamorphosis. There is now evidence that prolactin may be a growth promoting factor in amphibians, and prolactin has been reported to inhibit metamorphic change. The question that arises is; does prolactin inhibit metamorphic change by interfering with the action of thyroxine peripherally, at the level of target tissues?

B. Method of Approach.

In formulating an approach to this problem, it was decided to pursue this study by means of *in vitro* experiments. Attempts to analyze the mechanism of hormone action *in vivo* have certain inherent difficulties. *In vivo* studies of hormone action generally rely on one of several procedures; either additional supply of exogenous hormone or removal by surgical or chemical means of glands to produce an animal lacking a particular hormone. In the intact animal withdrawal or addition of a single hormone is immediately accompanied by many changes in the whole endocrine balance of that animal and may inaugurate a sequence of secondary and tertiary physiological events so that it is often difficult to determine whether the effect observed on target tissues is due to the direct or indirect action of the hormone in question. In vitro experiments may therefore provide a superior means for studying the mode of hormone action on target structures.

The tail was chosen as an in vitro test object because numerous experiments have been performed on intact animals demonstrating that resorption is one of the most dramatic events in metamorphosis and this event appears to be a direct response to thyroxine (Kaltenbach, 1968). Tail resorption has been extensively studied from both morphological and biochemical points of view. Equally important is the fact that these tissues are capable of surviving in culture for long periods of time. Hauser and Lehman (1962) used isolated tails for a study of tail regeneration; Weber (1962) and Flickinger (1963) demonstrated that addition of T_4 to the culture medium resulted in dramatic shortening of the tail by resorption. Shaffer (1963) and Tata (1966) using T_3 demonstrated similar effects.

In recent years, biochemical analysis of tail resorption both in vivo and in vitro have been conducted. These experiments demonstrated that tail resorption is accompanied by increased levels of acid hydrolases and cathepsins (Weber, 1963; Kubler and Frieden, 1964; Eeckhaut, 1964; and Tata, 1966). Saltzman and Weber (1963) demonstrated that these enzymes were associated with macrophages of the subepidermal connective tissue. Treatment of whole animals (Weber, 1965) or isolated tails (Tata, 1966) with Actinomycin D, an inhibitor of RNA synthesis, inhibits the action of thyroxine in inducing increases of these enzymes. Furthermore, Tata (1966) has shown that cycloheximide and puromycin (inhibitors of protein synthesis) also block the action of thyroxine. These findings suggest that tail resorption is accompanied by the synthesis and turnover of RNA and proteins. Lapiere and Gross (1963) demonstrated that T_4 leads to an increased rate of collagen destruction in tail tissues as compared to the rate of collagen synthesis that continues to occur. This destruction was associated with increased levels of collagenase. Eisen and Gross (1965) have shown that breakdown of tail tissue is dependent upon the _de novo_ synthesis and release of collagenase by epithelial cells and the release of stored hyaluronidase by mesenchymal cells. These experiments illustrate that two cell types may cooperate in the process of tail resorption. All of these biochemical studies can be correlated with the morphological picture observed at this time. Usuku and Gross (1965) have shown that the basement membrane separating the epithelia from the mesenchymal cells below comes apart, with penetration of this layer by mesenchymal cells. The gelatinous core of the tail fin is also observed to undergo degeneration. At the present time, the available evidence does not indicate what the precise site of thyroid hormone action is in inducing tail resorption.

C. Regional Response of Tail Tissues to Thyroxine.

In more classical experiments conducted by Clausen (1930) it was demonstrated that different regions of the tadpole's tail undergo histolysis at different rates. Autoplastic transplants of tail tissue were made to the back of _R. pipiens_ larvae. It was found that there was an anterior to posterior gradient of response of these tissues with respect to their rate of hystölysis. Anterior tissues responded most rapidly. In observing tail resorption it becomes apparent that different regions of the tail do undergo resorption at somewhat different rates.

Most obvious is the resorption of the anal canal piece during late prometamorphosis. During climax when rapid resorption of the entire tail occurs the anterior dorsal fin appears to be resorbed first followed by a general flattening of the dorsal and ventral fins. As a result of these observations it was decided that this aspect of metamorphosis would be included in this study. That is, do different areas of the tail possess differential sensitivity to the same concentration of thyroid hormone.

D. Experimental Design.

The experimental design that was chosen as a means of answering the major questions asked at the outset of this study was as follows: Isolated pieces of tadpole tail fin would be employed to test the role of hormones in metamorphic change. The mode of thyroid hormone action in tail resorption would be studied by treating isolated tail fins, taken from animals at different stages of metamorphosis, with different concentrations of thyroxine and studying their rate of resorption. The concept that prolactin inhibits metamorphic change by interfering with the action of thyroxine peripherally would be tested by treating tail fins with prolactin or pituitary grafts and thyroxine simultaneously, and observing the rate of tail resorption under these conditions as compared with tail fins treated with thyroxine only.

III. Materials and Methods

A. General Experimental Procedures.

1) Raising and Maintenance of Animals

Tadpoles of Rana pipiens were obtained by induced ovulation (pituitary implantation) and fertilization (Rugh, 1934). Eggs were maintained at room temperature (23°-25°C) in finger bowls containing spring water. Tadpoles were maintained one or two to a finger bowl until they were to be used. In addition, some animals were placed on a cold water table (6°-10°C) as a means of slowing down their rate of development. These animals were transferred to room temperature prior to use. Animals were fed a daily diet of canned spinach with just enough spinach given so that little excess remains. This procedure reduces the chances of the water becoming foul. When tadpoles begin eating, spring water is no longer used as a medium for tadpoles, at this point "artificial" spring water is used (approximately 6 grams of coarse salt is added to a 5-pint bottle of distilled water). The tadpole's metamorphic progress was determined by observing the total length:body length:hind leg length ratio and evaluated in terms of progress toward metamorphosis according to the criteria of Etkin, (1968). Tadpoles that were at the appropriate stage of metamorphosis were placed into .05% sodium sulfadiazine solution, without any food, prior to use in experiments. In the first series of experiments animals were treated in this manner overnight. However, this period was reduced to about an hour in subsequent experiments.

2) Preparation of Tail Discs

A modification of Weber's (1962) method for culturing tail tips of Xenopus laevis was devised following several preliminary experiments in which tail tips of Rana pipiens tadpoles were cultured. In these preliminary experiments it was observed that in many instances such preparations would breakdown in culture as shown by degeneration of tissues. It was felt that this breakdown resulted in part from the inability of many of these preparations to heal over well. Specifically, the epithelium covering the cut musculature did not close over this underlying tissue. However, it was observed that both the dorsal and ventral fins would readily heal over completely. Accordingly, it was thought that culturing pieces of tail fin alone would suffice as a test object. Pieces of tissue were cut out of the dorsal fin of a tadpole with a sterile cork borer and these pieces, discs, were placed into a sterile petri dish containing culture medium, amphibian Tyrodes solution (balanced salt solution). It was observed that discs would indeed heal over rapidly and completely to form a self enclosed system that survived well in culture for long periods. Discs immersed in thyroxine solutions responded by undergoing reduction in size, resorption.

The following procedure was then developed to culture tail fins of R. pipiens tadpoles. Tadpoles that have been pretreated with .05% sodium sulfadiazine were rinsed in spring water. A tadpole was then placed on a paper towel and its tail was cut off, with a pair of scissors, near its base. This tail is immediately transferred to a petri dish (60x15mm) containing culture medium. Care was taken to handle the tail by the cut edge of the musculature so that the fins were not damaged. Following this rinse, the tail was placed into an operating dish. This dish is a large petri dish (100x20mm) filled with control medium and has an operating platform (a foam rubber pad attached to the bottom of the dish by paraffin). The tail was placed on top of the platform and was held in place by a pair of watchmakers forceps or by a pin that was placed through the musculature and into the pad below. This procedure stabilizes the position of the tail. Then using a pair of fine scissors the fin was carefully cut off by cutting above and parallel to the tail muscle. Care was taken to exclude any muscle tissue. Rectangular pieces (approximately 4x5mm in size) were cut from the fin and generally 3 or 4 pieces were cut from the mid-portion of a fin. The term disc has been retained for convenience despite the fact that these pieces were no longer cut out with a cork borer and therefore their shape was more rectangular than discoidal. Except where indicated, only dorsal fins were used. This was done as a means of maintaining greater uniformity in the experimental population. This entire procedure was conducted under a dissecting microscope. Discs were then transferred to petri dishes by picking them up with a pair of fine curved forceps in stretcher fashion. Discs were initially cultured en masse (15 discs per dish) in large petri dishes containing approximately 35ml. of medium. Under such conditions discs undergo healing (complete enclosure by the epithelia). In many instances healing took place within hours but to ensure their complete healing discs were usually kept in these mass cultures for a two day period. A small percentage of discs did not heal over completely but in these experiments only completely healed discs (as determined by examination under a dissecting microscope) were selected for use. Finally, discs were assigned to experimental categories at random and during the experimental period were maintained singly in small petri dishes (60x15mm) containing 10ml. of the appropriate medium.

The medium used in the experiments to be described was Hanks balanced salt solution (GIBCO) diluted with sterile distilled water (two parts Hanks:one part distilled water). Preliminary studies indicated that discs may do well in other balanced salt solution such as amphibian Tyrodes. Hanks medium was used in the present experiments for convenience. Penicillin-streptomycin was added to the medium, 100 units or mcg/ml. Dishes and medium were changed every third day and all discs were maintained in an incubator at 20°C ± 2°.

All glassware used was sterile and all petri dishes were sterile, disposable, plastic dishes. Instruments were maintained sterile by dipping into 70% alcohol and flaming prior to use. The operating dish was sterilized prior to use by soaking in 70% alcohol and then rinsing in sterile distilled water. The medium in the operating dish was changed frequently during the cutting of tail discs.

3) Quantification of Tail Resorption

The following method was devised for quantifying tail disc resorption. A culture dish containing a tail disc was placed on the stage of a Bausch and Lomb Tris-simplex microprojector. The projector was set so that it projects the magnified image of the disc downwards onto a recording sheet where the outline of this disc was copied. In this manner a daily record was kept of each disc. The area of these drawings was then measured with a planimeter and the data was expressed in terms of percent shrinkage per day. Figure 1 demonstrates discs undergoing resorption as recorded by this method.

4) Statistical Treatment

The results of these experiments were evaluated according to the standard statistics employed in comparing the significance between populations.

IV. Experimental Series

A. The Response of Tail Discs to Thyroxine. (Experiments 1-5)

1. Procedure

Experiment 1 - The response of tail discs taken from tadpoles in early prometamorphosis to thyroxine.

This experiment was performed to study quantitatively the response of tail discs to thyroxine. Tail discs were cut from animals in early prometamorphosis (hind legs approximately 5mm in length) and were treated with six concentrations of L-thyroxine, 3, 9, 27, 81, 250, and 750 parts per billion (ppb) of thyroxine. A solution of 1ppb T_4 is approximately $.9 \times 10^{-9}$ m. Discs placed in Hanks salt solution (control medium) without hormone supplement served as controls. Each group consisted of 14 discs. The rate of tail resorption in the different solutions of T_4 was compared.

Experiment 2 - The response of tail discs taken from animals in late prometamorphosis to thyroxine.

In order to determine whether the responsiveness of discs to T_4 changes with advancing stages of metamorphosis, tail discs were cut from animals in late prometamorphosis (animals were chosen that were close to the day of emergence of the forelegs, E-3 to E+0). These discs were treated with the same six concentrations of thyroxine used in experiment 1. In this experiment there were 10 discs in each group.

Experiment 3 - Tail resorption during climax.

This experiment was designed to study tail resorption during climax. In *R. pipiens*, tail resorption begins after the emergence of the forelegs and the fins show rapid resorption on the second and third days after this event. Discs were cut from animals at E-1 (32 discs) E+0 (39 discs) and E+1 (18 discs). These discs were not placed en masse for healing; instead each was placed singly into a petri dish containing control medium. As will be seen in the results, the preliminary healing period of these discs had to be eliminated since the E+1 discs resorb rapidly.

Experiment 4 - The specificity of the thyroxine response.

This experiment was performed to examine whether the response to thyroxine can only be elicited by the intact thyroxine molecule. Early prometamorphic discs (7 per group) were placed into solutions of potassium iodide, DL-thyronine and DL-diiodothyronine at molal concentrations equivalent to 27 and 250 ppb T_4 (i.e. 24 and 217 x $10^{-9}\underline{m}$). Since L-thyroxine had been used in these experiments, the molality of the solutions of DL-thyronine and DL-diiodothyronine was doubled to expose discs to equimolal concentrations of the L forms of these molecules.

Discs used in experiments 1-4 were taken from tadpoles derived from the same batch of eggs.

Experiment 5 - The response of discs to thyroxine treatment for varying periods of time.

This experiment was designed to study the dependence of resorption on the continuous presence of thyroid hormone. Discs were taken from animals in early prometamorphosis and placed into solutions of 3 ppb T_4 for 1, 3, 5, and 7 days. At the end of these periods, discs were rinsed twice in control medium and then maintained in control medium for the duration of the experiment. Two control groups were employed; discs in control medium only and discs in 3 ppb T_4 continuously. The object of this experiment was to examine the pattern of resorption of discs subjected to low concentrations of thyroxine for specified periods of time and then removed from their source of hormone. In this experiment there were 14 discs in each group.

2. Results

Experiment 1 - The response of tail discs taken from tadpoles in early prometamorphosis to thyroxine.

Early prometamorphic discs placed into control medium maintained themselves well for at least 15 days in culture (figure 2). After this period these controls follow one of several courses: 1) breakdown, a rapid process (1-2 days) in which the disc appears to disintegrate and fall apart, 2) resorption, steady shrinkage of a disc in a period of 3-4 days, 3) slow shrinkage, gradual resorption of the disc over an extended period of a week or more. Control discs may maintain themselves for as long as 30 days.

Early prometamorphic discs treated with different concentrations of thyroxine demonstrate that a quantitative relationship exists between hormone concentration and the rate of tissue resorption. Discs treated with 3 ppb of T_4 begin to show resorption after about 8-9 days in culture and resorption is completed about 3-4 days later. At this concentration some variability exists as to the day when shrinkage begins. The majority of discs start this process on days 8 or 9, however, a few may begin shrinkage on day 7 and in one instance a disc only began to shrink on day 12. Discs treated with 9 ppb T_4 initiate resorption about days 5-6 and also complete this process in about 3-4 days. At 9 ppb the variability with reference to what day shrinkage begins is reduced. Discs treated with 27 ppb T_4 begin resorption on days 3-4 and this process is also completed in 3-4 days. Higher concentrations do not differ significantly from 27 ppb. Thus discs treated with 81, 250, and 750 ppb T_4 show rates of resorption similar to that observed for 27 ppb T_4. At these concentrations (27-750) there is little variability and resorption is initiated and completed uniformly by almost all discs. Only rarely do experimental discs undergo breakdown or slow shrinkage instead of resorption in 3-4 days.

Experiment 2 - The response of tail discs taken from animals in late prometamorphosis to thyroxine.

Control discs from animals in late prometamorphosis show resorption from day one in culture; this is in contrast to the control discs taken from animals in early prometamorphosis (experiment 1) that do not show resorption for at least 15 days. The resorption of these discs from late prometamorphic donors tends to level off after several days and the discs persist at this reduced size (figure 3).

Addition of exogenous thyroid hormone to discs of this experiment also demonstrates that a quantitative relationship exists between hormone concentration and the rate of resorption of tail discs. Treatment with 3 ppb T_4 results in complete resorption by day 9. This rate of resorption is similar to the rate obtained by giving 9 ppb to discs taken from animals in early prometamorphosis (see figure 2). In 9 ppb T_4 discs show a resorption curve similar in form to that observed with 27 ppb T_4 in early prometamorphosis; resorption is completed in 6-7 days. This dosage (9 ppb) appears to be the lowest concentration giving the maximum response at this stage of metamorphosis. The curves for 27, 81, 250, and 750 ppb T_4 all show rates of resorption similar to that of 9 ppb T_4.

Experiment 3 - Tail resorption during climax.

If the level of hormone rises around the day of E it is expected that tail discs cut on specified days near this time might show different rates of resorption, in control medium, based on their higher endogenous level of hormone content. Discs that were cut from animals at E+1 show a dramatic increase in the rate of resorption as compared to discs from animals at E-1 and E+0 that behaved as did the controls (that were essentially the same, i.e., E-3 to E+0) in experiment 2 (figure 4). The rate of resorption of discs at E+1 is about the same as that observed for the fins of intact animals during climax.

Experiment 4 - The specificity of the thyroxine response.

No acceleration of response over control discs was obtained with the concentrations of KI, or DL-diiodothyronine used and only high levels of DL-DIT gave any response. This response was extremely weak, that is, less than the response elicited by treatment with 3 ppb of T_4 (Table 1).

Experiment 5 - The response of discs to thyroxine treatment for varying periods of time.

In this experiment, early prometamorphic discs maintained in 3 ppb T_4 continuously show a curve for resorption similar to that obtained in experiment 1 (figure 5). Discs placed in 3 ppb T_4 for just one day do not differ from the controls maintained in Hanks continuously. Discs placed into 3 ppb T_4 for 3 days show initiation of resorption on day 5 but this rate is low and soon disc size levels off and the discs maintain themselves at their reduced size. Discs placed into 3 ppb T_4 for 5 days give a similar response but the degree of resorption is slightly greater. Discs placed into 3 ppb T_4 for seven days show further resorption than the previous groups, these discs give an initial rate of resorption that parallels that of discs maintained in thyroxine continuously. However, this group then shows a sudden leveling off in disc size whereas discs maintained in thyroxine continuously are completing their resorption.

3. Conclusions

The Response of Tail Discs to Thyroxine - Experiments 1-5.

One of the major intents of these experiments was to study, quantitatively, the response of tail tissue, taken from animals at different stages of metamorphosis, to thyroxine and to investigate whether a quantitative relationship exists between thyroid hormone and its mode of action. The demonstration of such a relationship would be of significance in distinguishing between the two concepts that have been proposed for the mode of thyroid hormone action in metamorphosis (threshold or stoichiometric).

The results of experiment 1 and 2 demonstrates that a quantitative relationship does exist between the level of thyroid hormone and the rate of resorption of tail discs. The range of this relationship is related to the stage of metamorphosis being tested. In early prometamorphosis this range is observed over 3-27 ppb of T_4 while with discs taken from animals in late prometamorphosis the range is reduced so that 9 ppb T_4 appears to be the lowest dosage tested that gives the maximum response. The results of experiment 5 provides further evidence that such a quantitative relationship exists, for when early prometamorphic discs are placed into 3 ppb T_4 for varying lengths of time (1, 3, 5, and 7 days) and then removed from this medium and placed into control medium these discs initiate but fail to complete resorption.

A quantitative relationship is observed between the amount of time the tissue had been in contact with hormone and the degree of resorption achieved.

In addition, the results of experiment 5 indicate that the action of this hormone is dependent upon the continuous presence of the hormone. It can be concluded therefore that the hormone does not merely act as a triggering mechanism.

In these experiments, it is important to note that changing the concentration of thyroxine does not affect the length of the period in which resorption occurs (generally 3-4 days). Changing the concentration of thyroxine changes the length of the period that precedes resorption (latent period). The quantitative nature of these results indicates that we may be dealing with a stoichiometric phenomenon involving molecular interactions of hormone and some other molecule. Perhaps, thyroxine T interacts with a component P that is present in a cell, to form a relatively stable product PT. This product, PT, would then be responsible for inducing the events that lead to tail resorption. Furthermore, tail resorption would only occur when a specific amount of PT has accumulated. In addition, PT must be continuously present for resorption to proceed to completion. The latent period of hormone action (period during which PT is formed) would be dependent upon the amount of thyroxine and component P present and also on the stability of the product PT. Treatment with low concentrations of thyroxine would require a longer latent period than treatment with higher concentrations of T_4. Also, if very low concentrations of T_4 were employed sufficient levels of product PT might not be able to accumulate because of the half-life of product PT. In the case of tail resorption, the period of resorption (3-4 days) would be identical in all cases since this event only occurs when the required level of PT is present. According to such a scheme it can be seen that the primary action of thyroxine involves a stoichiometric relationship between T and P while the secondary action of this hormone is dependent upon the action of PT and this event demonstrates a threshold-like effect.

Additional support for the concept that thyroxine exerts its effects on metamorphosis by taking part in the molecular changes of these tissues can be provided by the studies on the effects of low temperature on metamorphosis. Frieden et al. (1965) studied the effect of low temperatures (5°C) on thyroxine (T_3) induced tail resorption. No T_3 effect was observed after up to 100 days incubation at 5°C. In more recent work, Frieden (1968) and Ashley et al. (1968) studied the effects of temperature on tail decrease and shifts in ammonia to urea excretion by the liver. Both of these responses are induced by thyroxine and both can be arrested by shifting thyroxine treated animals from 25° to 5°C. These studies indicate that the action of thyroxine involves the formation of an intermediary product that is responsible for inducing metamorphic change. It also appears that it is the formation of this intermediary product that is dependent upon temperature. The arrest of metamorphosis by low temperatures should prove to be a valuable clue to understanding the basic molecular changes accompanying metamorphosis. The demonstration of such temperature effects may be used to support Etkin's quantitative concept for the action of thyroxine in metamorphosis.

The next major question asked at the outset of this study was; do target tissues change in their sensitivity to thyroxine during metamorphosis? The results of experiment 1 and 2 and those of previous workers indicates that tail tissues do change in their(sensitivity) response to thyroid hormone. If discs are taken from tadpoles in early prometamorphosis and are treated with the same concentrations of T_4 as discs taken from tadpoles in late prometamorphosis the latter group exhibit a more rapid response. It can be seen that the sensitivity of tissues to thyroxine increases during the prometamorphic period.

The important question that arises is; Why does the same tissue change in its sensitivity to thyroid hormone during metamorphosis? The answer to this question, may be related to one's interpretation of what the mode of thyroid hormone action is in metamorphosis. In Kollros' interpretation, tissues are more sensitive to T_4 during metamorphosis because the threshold level is lowered. However, this interpretation does not offer any explanation of how or why the threshold level is changed and how thyroxine acts in determining the threshold level? Etkin's quantitative interpretation of the role of thyroxine in metamorphosis involves thyroid hormone directly in determining the rate at which metamorphosis occurs by proposing that thyroxine takes part in a stoichiometric fashion in the activities of these tissues.

Perhaps an answer to the question of why tissues change in their sensitivity to thyroid hormone during metamorphosis can be found in a further examination of the results of these experiments. In experiment 1, early prometamorphic discs maintained in control medium do not demonstrate resorption of any kind until day 15 in culture. It appears that these tissues were not previously exposed to a concentration of thyroid hormone that was equivalent to or greater than 3 ppb of T_4;

because the results of experiments 1 and 5 indicate that if these discs had been exposed to 3 ppb T_4 for only 3 days some resorption would have occurred prior to day 15. The response of these control discs is therefore in accord with the generally accepted concept that the level of circulating thyroid hormone is extremely low during early prometamorphosis (see introduction). This level is less than the equivalent of 3 ppb T_4 in the external medium.

Late prometamorphic discs, experiment 2, placed into control medium demonstrate partial resorption. This is interpreted to mean that these tissues had been exposed to or possessed a sufficient level of hormone at a time of isolation to program them for some resorption. The leveling off observed suggests that removal of these tissues from a further source of hormone (cutting off the tail and maintaining them in control medium) was responsible for the cessation of further resorption. The resorption demonstrated by these control discs would be in agreement with the previously discussed concept that the level of thyroid hormone in circulation increases during the prometamorphic period.

The results of experiment 3 demonstrate that discs taken from animals at E+1 undergo rapid and complete resorption in control medium while discs taken from animals at E-1 and E+0 do not. This indicates that at about the time of emergence of the forelegs (beginning of climax) there is a rise in the level of thyroid hormone in circulation so that by E+1 these tissues have been exposed to or possess sufficient hormone to program them for complete resorption. These results are also in agreement with the general concept of thyroid activity during metamorphosis.

In accord with a quantitative interpretation for the mode of thyroid hormone action in metamorphosis it can be seen that discs taken from animals in early prometamorphosis do not show resorption <u>in vitro</u> because they have not been exposed to a sufficient level of thyroid hormone to induce resorption. The resorption of discs taken from animals at late prometamorphosis and during climax is interpreted to mean that these tissues had been exposed to sufficient thyroid hormone during the prometamorphic and climax periods to program them, at the time of isolation, for resorption.

It is suggested that the observed increase in sensitivity of tail discs to thyroxine at later stages of metamorphosis results from the fact that such tissues possessed or had been previously exposed to more thyroid hormone than identical tissues isolated at earlier stages of metamorphosis. Therefore, treating tail discs from tadpoles in late prometamorphosis with thyroxine is actually equivalent to adding these amounts of hormone to the previously existing level so that resorption would result from the sum of these two factors

The results of experiment 5 provide evidence to support this interpretation. Early prometamorphic discs placed into T_4 for varying periods of time and then maintained in control medium produced a series of curves showing increased degrees of resorption dependent upon the exposure of these discs to T_4. The curve for discs placed into T_4 for 5 days simulates the curve produced by the controls in experiment 2 (late prometamorphic discs in control medium) in which resorption occurred initially but then levelled off.

An interesting observation is that tail discs <u>in vitro</u> appear to be more sensitive to thyroxine than the same tissues <u>in vivo</u>. Tail discs give a pronounced response to 3, 9, 27, and 81 ppb T_4 whereas intact tadpoles generally require concentrations of hormone, greater than 200 ppb T_4. In addition, responses of tail tissues, in vivo, to even 81 ppb T_4 are very slow and do not reach maximal levels until higher concentrations of hormone are used (Etkin 1935, 1964). This increased sensitivity of tail discs to T_4 will be analyzed in greater detail later in the discussion.

Finally, the results of experiment 4 demonstrates that the effect of T_4 on tail resorption is due to the specific action of thyroid hormone. Neither KI nor DL-thyronine gave any effect. The slight effect of DL-DIT is in conformity with the observed action of this molecule on amphibian metamorphosis (Kaltenbach, 1968). These results strengthen the presumption that the action of T_4 on tail discs in vitro is comparable to its action in vivo and is a specific property of thyroid hormones (T_4 and T_3). Flickinger (1963) reported similar results in experiments in which whole tail tips were tested with thyroxine, DIT, MIT, and Iodide.

In summary, these experiments demonstrate that: (1) A quantitative relationship exists between thyroxine and its mode of action. (2) This action is dependent upon the continuous presence of this hormone. (3) Tail tissue changes in its sensitivity to thyroxine at progressive stages of metamorphosis. It is suggested that this change in sensitivity of tail tissues to T_4 results from the previous exposure of these tissues to a low but rising level of thyroid hormone during prometamorphosis and climax.

B. The Regional Response of Tail Fins to Thyroxine.

1. Procedure

Experiment 6 - The regional response of tail fins taken from animals at early prometamorphosis.

This experiment was performed in order to study the regional sensitivity of tail tissues to thyroxine and to find out whether different geographical areas of the tail respond identically to the same concentration of T_4? In this experiment, early prometamorphic tadpoles were used. Initially, 5 regions of the tail were tested; the anterior dorsal fin, the mid-dorsal fin, tail tips, the mid-ventral fin and the anterior ventral fin. In subsequent runs of this experiment a sixth region was added, the portion of the anterior ventral fin that includes the anal canal piece. After healing, these discs were placed into either 9 ppb T_4 or control medium. This experiment consisted of three runs and there were 10 discs in each group per run (total of 340 discs). In the first run only the first 5 areas mentioned were tested but in runs two and three all six areas were examined. All of these animals were from the same batch of eggs.

2. Results

The results of the three runs of experiment 6 were similar and therefore these data have been grouped together. The controls all maintained themselves equally well and they are represented by a single line (figure 6). However, regional differences in sensitivity to thyroxine was observed for two areas, the anterior dorsal fin and the anal canal discs. These two regions exhibited a more rapid response to thyroxine than did the other regions tested. The anal canal discs are the most sensitive to T_4, by day 5 (figure 6) these discs have undergone 58% shrinkage, anterior dorsal fin discs have undergone 47% shrinkage and the other regions tested vary from 24-28% shrinkage. All of these differences between the anal canal discs and the less sensitive regions are statistically significant at the $<.001$ level, while the differences between the anterior dorsal fin and these regions varies from $<.05$ to $<.01$.

3. Conclusions

The results of this experiment are in general agreement with studies performed in the past (Clausen, 1930). It was shown that the anterior portion of the fin is more sensitive to T_4 than the more posterior regions. It would be of interest to pursue these experiments further to see if this same pattern of sensitivity to thyroid hormone persists later on in metamorphosis. One area of particular interest would be the anal canal region. This region normally resorbs prior to the emergence of the forelegs while the other regions of the fin resorb after this event. Therefore, one might expect that differences in sensitivity to T_4 between these areas might be greater if animals in later stages of prometamorphosis were tested. In addition, it might be worthwhile to repeat such experiments using different concentrations of T_4. It might be that at other concentrations regional differences in sensitivity might be more obvious. However, the main intent of this experiment was to test whether dorsal and ventral fins could be used as equivalent test objects so that it would be possible to have each donor animal supply more discs for use in experiments. Based upon the results of these experiments it was determined that discs taken from either fin could be used as equivalent test objects provided that the more anterior regions of the fins were not included.

Finally, it becomes evident that the tail, like the entire animal represents a mosaic with respect to its response to thyroid hormone. However, in the tail we are not faced with explaining how thyroid hormone induces a different type of metamorphic response but rather, what is the mechanism that is responsible for inducing the same response, resorption, at different rates in the same type of tissue.

C. The Activity of Discs After a Long Period In Vitro.

1. Procedure

Experiment 7 - The response of discs to thyroxine after long periods in vitro.

This experiment was performed to study the response of discs that had been in culture for long periods of time to subsequent treatment with thyroxine. In the previous experiment (experiment 6) T_4 treated discs completed resorption by 10 days *in vitro*. At this time control discs appeared to be in good condition. The question was raised, how would discs that had been in culture for long periods of time respond to treatment with thyroid hormone? To answer this question, the control discs (cut from tadpoles in early prometamorphosis) from the first run of experiment 6 were divided into two groups (approximately 25 discs per group). One group was placed into 9 ppb T_4 and the other kept in control medium.

2. Results

Discs maintained *in vitro* for long periods are still capable of responding to thyroxine by undergoing resorption (figure 7). These discs responded to thyroxine in a predictable fashion based on the concentration of T_4 used. The rate of resorption of these discs was similar to that of the original experimental discs (experiment 6) right after healing. In experiments 6 and 7 thyroxine induced resorption of tail discs was completed after approximately 8 days.

3. Conclusion

It has been assumed based upon the general appearance, maintenance of size and some histological examination of tail discs that discs survive in control medium for long periods of time. At the present time nothing is known about the biochemical status of discs during this period. The results of this experiment indicate that discs maintained in culture for 11 days are still capable of responding to the addition of T_4 to their medium by undergoing resorption. This resorption follows the same pattern observed for discs treated with the same concentration of hormone right after healing. Therefore, it may be suggested that discs maintained *in vitro*, under these conditions, are still capable of performing the biochemical processes involved in resorption. It would be of interest to know more about the metabolic status of these tissues *in vitro*. Another point of interest is to consider how these discs are capable of maintaining themselves *in vitro*, in view of the simplicity of the culture medium. It may be that since most of the disc is composed of loose connective tissue this material possesses a pool of nutrients sufficient to maintain the relatively few cells that are present in a disc. Also, the thinness of discs could allow for adequate gas exchange.

D. Thyroxine Interactions With Pituitary Grafts (Experiments 8 - 10).

1. Procedure

Experiment 8 - Inhibition of T_4 induced tail resorption by whole pituitaries.

Tadpoles in early prometamorphosis were decapitated and their pituitary glands were rapidly dissected out, in control medium, and were implanted into discs in the following manner: After decapitation the tadpole's tail was cut off and treated in the usual manner. The tail was then placed onto an operating platform where a sharpened needle was pushed into the fin parallel to the long axis of the tail. In this fashion, a tunnel was produced in the central gelatinous core. One point of fine watchmakers forceps was then inserted into the tunnel to widen the entrance. In making the tunnel care was taken not to penetrate the sides thereby creating a hole through which the implant could escape. Following the completion of this process, the pituitary or other tissues tested were inserted into the tunnel and pushed along to its final position in the fin. The discs containing implants were then cut out and treated in the usual manner.

Spinal cord, liver, pars intermedia and cerebrum were tested for use as control tissue. For reasons to be discussed later the cerebrum was chosen. In addition, sham-operated discs were used as controls (discs had a needle inserted into them but no tissue was implanted).

In this experiment the whole pituitary was implanted into one fin (dorsal or ventral) and control tissue was implanted into the fin on the opposite side of the tail. The use of dorsal and ventral fins for controls and experimentals was alternated from animal to animal. After healing, discs were chosen at random and placed into either control medium or medium containing thyroxine. Two concentrations of thyroxine were tested, 3 ppb and 6 ppb. There were ten discs in each group.

Experiment 9 - Inhibition of thyroxine induced tail resorption by the pars anterior.

In this experiment pituitary glands were split into two parts, pars anterior and the posterior lobe (this piece consisted mainly of pars intermedia and will be referred to as pars intermedia) and these pieces were implanted into separate discs. This experiment was performed to evaluate the contribution of each lobe of the pituitary to the observed effect (inhibition of T_4 induced tail resorption). In this experiment there were 8 pars anterior discs treated with T_4 and 7 similar controls. Cerebrum-implanted and sham-operated controls were included.

Experiment 10 - Inhibition of spontaneous resorption of climax discs by pituitary grafts.

This experiment was performed to determine whether an implanted pituitary can inhibit the spontaneous resorption of discs cut from animals at E+1 of climax. The pituitaries and tails used in this experiment came from the same E+1 animals. The results of experiment 3 demonstrated that discs cut from the tails of tadpoles at this stage of metamorphosis will undergo rapid resorption without exogenous thyroid hormone. The procedure used in this experiment was necessarily somewhat different from that used in the previous two experiments (8 and 9). At this stage of metamorphosis tail resorption has begun and as a result the ventral fin is not wide enough to be used as a site for implants. A set of three discs, pituitary, cerebrum, and sham-operated, were prepared from each dorsal fin. The sequence of assignment of discs was changed from animal to animal. Each disc was then maintained, singly, from the start in control medium and measurements were taken from day one in culture. As previously discussed (experiment 3) discs taken from animals at this stage of metamorphosis show rapid spontaneous resorption from day one *in vitro*. Eighteen animals were used in this experiment.

2. Results.

The results of several preliminary experiments had indicated that discs containing pituitary grafts failed to undergo resorption when such discs were maintained in low concentrations of thyroxine. However, if similar discs were maintained in higher concentrations of T_4, inhibition was not observed. Pituitary implanted discs maintained in control medium did not show any increased capacity to maintain themselves *in vitro* as compared to control discs. In these experiments spinal cord and liver were used as control tissues. It was found that even in the absence of T_4, discs containing these tissues would show a rapid rate of shrinkage or breakdown in culture. It appears that these tissues had a toxic effect on discs. Thyroxine treated and control spinal cord discs would shrink between days 4-7 in culture. T_4 treated discs were more uniform in their response but the rate of resorption of both groups was more rapid than that of the sham-operated controls maintained in the same concentration of thyroxine. Liver implanted discs gave similar results although not all liver implants maintained in control medium underwent rapid resorption. Cerebrum implanted discs did not exhibit rapid resorption in control medium. Cerebrum implanted discs maintained in T_4 underwent resorption at the same rate as sham-operated discs. This tissue was then used in subsequent experiments as the control implant tissue.

Experiment 8 - Inhibition of T_4 induced tail resorption by whole pituitaries.

The results of this experiment indicate that pituitary, cerebrum and sham-operated discs maintained in control medium all survived equally well for at least 17 days in culture (figure 8). Sham-operated and cerebrum implanted discs placed into 3 ppb T_4 completed resorption by day 12 in culture. Pituitary discs in 3 ppb T_4 maintained their size showing virtually no resorption during this period. Sham-operated and cerebrum discs placed in 6 ppb T_4 completed resorption by day 9 in culture. Pituitary discs in 6 ppb T_4 demonstrate a three day delay in the initiation of and completion of resorption so that these discs were resorbed by day 12. It should be pointed out that this is the day when sham-operated and cerebrum discs placed into 3 ppb T_4 completed resorption. It would appear that the implanted pituitary had the capacity to block for the action of approximately 3 ppb T_4.

Experiment 9 - Inhibition of thyroxine induced tail resorption by the pars anterior.

Discs containing implanted pars anterior and maintained in 6 ppb T_4 demonstrated a four day delay in completion of resorption (figure 9). Implanted pars intermedia (not shown in figure 9) do give a one day delay in resorption but it is thought that this might be due to the incomplete removal of all cells of the pars anterior or to some mixing of cells from the pars anterior with the pars intermedia. This experiment demonstrates that the pars anterior alone is capable of inhibiting thyroxine induced tail resorption.

Experiment 10 - Inhibition of spontaneous resorption of climax discs by pituitary grafts.

The results of this experiment show that an implanted pituitary can also inhibit the spontaneous resorption of tail discs cut from animals at E+1 (figure 10). All discs undergo some initial resorption, part of this occurs regularly and is associated with the process of healing but only unimplanted and cerebrum implanted discs completed resorption. Discs containing pituitary implants show initial resorption but this (resorption) levels off. At day 6 in culture, pituitary implanted discs have only undergone 41% reduction while the other two groups have undergone 80% reduction in size.

3. Conclusions

Thyroxine Interactions With Pituitary Grafts - Experiments 8 - 10

These experiments were conducted to test the concept that a pituitary graft releases a substance, (possibly prolactin) that acts peripherally to inhibit the action of thyroxine. Such a concept was suggested by the previous demonstration of Etkin et al. (1968). They demonstrated that pituitary glands implanted into the tails of tadpoles near climax resulted in retardation of metamorphosis. Bern et al. (1967) demonstrated that injections of mammalian prolactin could inhibit metamorphic changes. It was anticipated that if the pituitary graft acted in this fashion *in vivo*, thyroxine induced resorption of tail discs *in vitro* might be retarded.

The results of these experiments show that a pituitary implant inhibits thyroxine induced tail resorption *in vitro*. In addition, it was shown that the capacity of such a graft to inhibit resorption is dependent upon the concentration of T_4 present in the medium. The inhibitory effect of the implant appears to be quantitatively equivalent to reducing the level of thyroxine in the medium by about 3 ppb of T_4.

These findings suggest that an antagonism exists between the action of a pituitary factor, released by the graft, and thyroid hormone on tail tissues. As discussed in the introduction, there is much evidence to support the concept that a pituitary factor promotes growth and maintenance of tail fins while T_4 induces resorption of the same structure. The status of these tissues at any stage of metamorphosis may be dependent upon the relative concentrations of the two hormones in the animals circulation.

Other lines of evidence also suggest that amphibian metamorphosis may be dependent upon the reciprocal action of these two hormones. If tadpoles are thyroidectomized they are known to attain gigantic sizes without showing signs of metamorphic progress (Allen, 1917, and Hoskins and Hoskins, 1919). On the other hand, treatment of tadpoles with exogenous thyroxine inhibits growth and stimulates metamorphosis (Steinmetz, 1952, 1954). This treatment results in the development of a small frog. Steinmetz (1954) treated tadpoles with thiourea (a goitrogen) and found that these tadpoles achieved a larger size than controls and in addition their metamorphosis was inhibited. These experiments demonstrate that modifications of the thyroid half of the reciprocal relationship between a pituitary growth factor and thyroid hormone results in emphasis of the appropriate side of this scale.

Histological studies by D'Angelo (1940 & 1941), Van Oordt (1966), and Kerr (1966) can be interpreted to support the concept that the pituitary of premetamorphic tadpoles is actively secreting a growth factor. Their studies demonstrated that acidophils, generally believed to be responsible for the secretion of growth hormones, are present in significant numbers during premetamorphosis and at the same time the number of basophils (TSH producing cells) is low. As has already been described, the morphological picture of the thyroid during this period appears to be the reciprocal of that for tadpole growth.

The concept of pituitary-thyroid antagonism is not unique to anurans; for studies of the "second metamorphosis" (land to water) of Triturus viridescens, a urodele, have demonstrated that the pituitary hormone, prolactin, is the "water drive" factor (Grant and Grant, 1958). Primary metamorphosis of the newt are also believed to be under the control of a prolactin-thyroid axis.

The concept of a reciprocal relationship between the pituitary and thyroid in controlling amphibian metamorphosis is similar to the principle that has been presented many times before in developmental biology that growth and differentiation tend to be mutually exclusive.

Finally, the results of these experiments demonstrated that pituitary implants were also capable of inhibiting the spontaneous resorption of discs cut from animals at E+1. It has been proposed (experiment 3) that such discs were already programmed for rapid resorption because of previous exposure to thyroid hormone and the level of thyroid present in these tissues at this stage of metamorphosis. The question; How does the pituitary growth factor accomplish this inhibition? will be explored later.

As an interesting sidelight to these experiments it should be pointed out that discs containing pituitary implants (whole lobes or separated lobes) demonstrate expansion of the pigment cells present in these tissues. This expansion is first evident around the graft and then spreads from the implant towards the periphery of the disc. Pigment cells usually maintain this expanded state throughout the experiment. Such an effect indicates that the substances (presumably MSH possibly ACTH) released by these implants are capable of rapid diffusion through the tissues. Pituitary containing discs have been cut in half so that only one half contains the implanted pituitary. Within a period of about a half an hour the pigment cells in the half disc without the pituitary implant show contraction while pigment cells in the pituitary containing half maintain their expanded state (figure 11). These results indicate that the life of such a hormone in discs is not more than a half hour or so. Histological examination of discs containing pituitary implants for two weeks indicates that these pituitaries are maintained in good condition as compared to pituitaries freshly dissected from a tadpole. The effect of a pituitary graft on the pigment cells present in discs provides a visible indication of the functional integrity of the graft. Similar effects on pigment cells may be seen when a pituitary is simply placed into the culture dish with a freshly cut disc, but the pattern of pigment cell expansion now starts from the periphery and spreads towards the center of the disc. However, when such a disc heals the pigment cells contract indicating that the hormones released by the pituitary in the medium are not capable of penetrating the intact epithelium of the tail fin. This observation was of importance in planning the subsequent experiments of this study.

E. Effect of Mammalian Hormones (Prolactin and Growth Hormone) on Thyroxine Induced Tail Resorption - (Experiments 11 and 12).

1. Procedure

Experiment 11 - Inhibition of T_4 induced tail resorption by pituitary hormone preparations.

The tails from tadpoles in early prometamorphosis were cut off, placed in an operating dish and injected with hormone according to the following procedure. A Hamilton syringe with a 30 gauge needle was inserted into either the dorsal or ventral fin of the tail. As the needle was withdrawn from the fin, hormone or control medium was injected, to fill the space left by the withdrawal of the needle. Each disc was injected with .1mg of hormone in a volume of .01 ml. Control medium, containing NaOH in amounts equivalent to that used in dissolving the hormones tested was injected into control discs. Dorsal fins were injected with one hormone preparation and ventral fins with the other. Control discs were cut from both fins. As in previous experiments the distribution of hormones was alternated from dorsal to ventral fin in succeeding animals. In addition to injection, hormone was also added to the medium in which discs were healing to allow for additional penetration of hormone. Previous experiments with pituitary glands *in vitro* indicated that hormones, affecting pigmentation, readily penetrate discs until healing occurs but after this time large molecules (such as intermedin) do not pass into the disc. The concentration of hormone in the medium was equivalent to that injected into the discs. After two days, discs were selected and placed singly into petri dishes containing control medium or solutions of 3 ppb T_4. There were 14 discs in each group.

Experiment 12 - Inhibition of spontaneous tail resorption by pituitary hormone preparations.

This experiment was designed to test the effect of three anterior pituitary hormone preparations in inhibiting the spontaneous tail resorption demonstrated by discs cut from tadpoles on E+1. In this experiment discs were not injected with hormone, instead 3 discs were cut from the dorsal fin of tadpoles and each disc was placed, singly, into a solution containing either ovine, prolactin, growth hormone, FSH or control medium. The sequence of assignment of discs was changed from animal to animal. This experiment was conducted in two groups of nine animals in each group. In group one growth hormone was tested and in group two FSH was tested. FSH was used in group two as a means of testing the specificity of the responses observed with group one.

The hormones used were obtained as a gift from the Endocrine Section of NIH. Ovine prolactin (NIH-p.s. -8) Ovine GH (NIH -GH-s8) and Ovine FSH (NIH-FSH-1).

2. Results

Experiment 11 - Inhibition of T_4 induced tail resorption by pituitary hormone preparations.

Early prometamorphic discs injected with prolactin, GH or control medium maintained themselves well in culture in control medium for at least 13 days. Similar discs placed into 3 ppb T_4 show different rates or resorption. Initially there was an adverse effect of prolactin and GH on discs maintained in thyroxine and these discs underwent some shrinkage during the first three to four days of the experiment but then recovered (did not shrink further). This initial shrinkage was not observed for discs treated with thyroxine only. By day seven, all three thyroxine treated groups had undergone about 30% resorpbion, but by day eight thyroxine treated discs had undergone more resorption than discs treated with thyroxine and prolactin or GH. This difference in response continued so that by day ten, out of the original fourteen discs in each group, only five thyroxine treated discs were left and these discs were 67% resorbed. Seven discs were left in the thyroxine-

GH treated group and these discs had undergone only 37% shrinkage. The thyroxine-prolactin treated group had five discs left but these discs had undergone an average increase in size of 22%. This increase in size is about the same as that of the three control groups in this experiment whose average increase in size at day ten was 14%. These results provide some evidence that mammalian prolactin inhibits thyroxine induced tail resorption. Growth hormone also possessed this capacity but to a lesser extent. However, the data were too few to permit a meaningful statistical analysis so they do not permit definite interpretation.

Experiment 12 - Inhibition of spontaneous tail resorption by pituitary hormone preparations.

Treatment of discs cut from E+1 animals with prolactin and GH demonstrates that both of these hormones reduce the degree of spontaneous resorption normally exhibited by such discs (figure 12). However, the effect of prolactin is more uniform than that of GH for the variance in resorption exhibited by discs treated with GH is greater than that for discs treated with prolactin. With prolactin the difference between experimental and control discs gives a p value of $<.01$ while the GH effect has a p value of $<.05$). Discs treated with FSH by contrast underwent resorption at the same rate as discs treated with control medium.

It should be mentioned that treatment of discs with hormones also induces expansion in the pigment cells present in the discs. This effect may be due to the contamination of these hormones by MSH or ACTH. This expansion often persists overnight and in some cases, varying degrees of expansion may continue for as long as 24-36 hours.

This effect does not continue for days as in the case of implanted pituitary glands. Also, the hormones tested do not all produce the same degree of expansion of the pigment cells; GH gives the greatest effect followed by prolactin and FSH.

3. Conclusions

Effect of Mammalian Hormones (Prolactin and Growth Hormone) on Thyroxine Induced Tail Resorption - Experiments 11 and 12.

The results of these experiments have not on the whole been helpful in clarifying the question as to the identity of the pituitary factor; specifically, whether it is similar to mammalian prolactin or GH. In this study both hormones were capable of inhibiting thyroxine induced tail resorption. The fact that FSH does not exert this action demonstrates that the inhibition is specific and not simply the result of adding protein hormones to these tissues.

The experiments of Berman et al. (1964b) and Bern et al. (1967) and Etkin and Gona (1967) indicated that prolactin was more effective in promoting general body growth in tadpoles than GH. Therefore these authors believed that the tadpole's growth factor is probably prolactin. In addition, prolactin but not other pituitary hormones tested have been shown to inhibit metamorphosis although the precise mode of action of mammalian prolactin in accomplishing this is still not clear. Prolactin has been thought to act as a goitrogen (Gona, 1967) and also to operate peripherally (Bern et al. 1967, Etkin et al. 1968). Perhaps it works at both sites as suggested by the more recent radioiodine work of Gona (1968) and some of the findings of Etkin et al. (1968).

It is possible that both prolactin and GH are capable of exerting an antagonistic effect to thyroid hormone *in vitro* as the present experiments indicate. At the present time it is recognized that these two mammalian hormones are similar in chemical structure and function and it is believed that these hormones have arisen from a common parent molecule. However, this does not mean that both of these molecules possess equivalent functional capacities with respect to tadpole tissues. It is also possible that the growth factor of tadpoles is, in a similar manner derived from the common precursor molecule of mammalian GH and prolactin and therefore possesses certain functions in common with these mammalian hormones. It may be that the differences reported in the activity of these hormones on tadpole tissues results from contamination of these hormones by other hormones (GH has been reported to show considerable TSH activity and also contamination by prolactin). However, it is also possible that contamination of these hormones by a common substance could also account for the similar actions of these two hormones *in vitro*. Altogether, it is difficult to arrive at any meaningful physiological interpretation of the results with mammalian hormone preparations.

Finally, the results of this study demonstrate that pituitary implants were more effective in inhibiting tail resorption than are mammalian hormones. However, such results are difficult to interpret. The hormones tested were mammalian hormones and as such may not possess activity completely equivalent to that of the tadpoles own growth factor. A more serious problem may be that of hormone administration and maintenance of hormone levels in these tissues. It is evident that hormones did reach these tissues, as evidenced by pigment cell response. However, discs were only exposed to a limited amount of hormone and the duration of this effect is not likely to have been long, if the effect observed on the pigment cells present in these tissues is any indication of the half life of these hormones in vitro. It should be pointed out that experiments in which pituitary glands were implanted into discs do not appear to be limited by this problem of hormone administration and maintenance of hormone levels in the tissue. As judged by the effect on pigment cells, these pituitaries release hormone continuously, for the duration of the experiment. It is of course not possible to quantify the release of hormone by such implants. In addition, at the present time nothing is known about the physiological levels of such hormones in the tadpole. Therefore, it may be that the methods of hormone administration employed to date have not supplied the appropriate amount of hormone to inhibit tail resorption more dramatically. It was with this idea in mind that discs cut from animals at E+1 were used. It was anticipated that any variance from the rapid spontaneous resorption normally shown by these discs could be attributed to the effects of the hormone administered. Indeed, use of these discs did provide more significant results than treatment of early prometamorphic discs that required two weeks in vitro before any significant differences between experimentals and controls could be seen.

In summary, it has been demonstrated that the pars anterior of the tadpole's pituitary releases a substance that interferes with the action of thyroxine on tail discs in vitro. This substance may be a prolactin-like or GH-like molecule since both of these mammalian hormones exert actions similar to that of the tadpole's own pituitary. In addition, it has been demonstrated that a quantitative, reciprocal relationship exists between the pituitary factor and thyroid hormone with respect to their effects on tail resorption. It is proposed that the interaction of these hormones is important in controlling amphibian metamorphosis.

V. General Discussion

A. Hypothalamic Control of Metamorphosis.

In the introduction, a general theory, proposed by Etkin (1963, and 1965/66), was presented to explain the endocrine control of amphibian metamorphosis. This theory was formulated with the understanding that metamorphosis was dependent upon the changing levels of thyroid hormone as determined by pituitary and hypothalamic control. With the demonstrations of the possible role of a pituitary factor in metamorphosis this new component should be fitted into the overall picture.

According to the neurosecretory concept of hypothalamic control of the pituitary, it was postulated that pituitary thyrotropin release is dependent upon the influence of thyrotropin releasing factor (TRF) produced by the hypothalamus (Guillemin et al., 1965; Reichlin, 1966; Schally et al., 1966). Etkin (1963, 1964) proposed the concept that neurosecretory control over TSH production by the tadpole's pituitary begins to raise the TSH level at the end of prometamorphosis and this level reached its maximum during metamorphic climax. If this is the case, then the concept developed for TRF should also apply to the prolactin inhibiting factor (PIF). In anurans, the release of a prolactin-like hormone is generally believed to be under hypothalamic inhibition. The level of PIF reaching the pituitary during premetamorphosis should be minimal, thus allowing for active production of prolactin and the stimulation of growth of the tadpole during this period. During prometamorphosis the level of PIF would increase resulting in the decrease of prolactin and a decreased growth rate of the tadpole. Finally, at climax the prolactin levels would be minimal, due to the maximum levels of PIF. In summary, as hypothalamic control of the pituitary develops the levels of both TRF and PIF would increase; in turn the levels of prolactin would decrease and the levels of TSH and consequently thyroxine would increase, resulting in decreased growth but increased metamorphic transformation of the tadpole.

This concept does not imply that a 1:1 relationship exists between the effects of the pituitary factor and thyroxine but rather that relative changes occur in the concentration of these hormones. It may be that changes in the level of one hormone might be greater than that of the other. As an example, it may be that the levels of pituitary factor (prolactin) are reduced more than the increases in thyroid hormone. If the pituitary factor inhibits thyroid action, reducing the level of this factor without change in the level of thyroid hormone in circulation could result in greater activity of thyroid hormone present in circulation. By the same token the opposite relationship could apply, that is, increasing the level of thyroid hormone while maintaining a constant level of growth factor. Increasing the level of thyroid hormone, as has been demonstrated, can swamp out the peripheral effects of the pituitary factor. However, based upon the current understandings of hypothalamic control of pituitary function the more reasonable interpretation would be to postulate that a reciprocal effect occurs in which the levels of both hormones, pituitary factor and thyroid hormone, are changed.

It was mentioned above (but was not discussed at the time) that early prometamorphic tail discs maintained in culture are more sensitive to T_4 than these tissues <u>in vivo</u>. Several explanations are possible. It may be that intact animals require higher levels of thyroid hormone because of the capacity of these animals to bind, metabolize or excrete this substance. This capacity would probably be greatly reduced in tail discs *in vitro*. However, another possibility exists based on the role of growth factor in metamorphosis. In the intact animal this factor is probably released by the pituitary continuously. It may be that in isolating pieces of tail from the tadpole the levels of growth factor in the tissues is now greatly reduced and as a result, an agent that normally interferes with the action of T_4 peripherally is no longer present. Therefore these tissues become more sensitive to thyroxine. These two explanations are not offered as alternatives, for the increased sensitivity of tail discs to T_4 *in vitro* could result from a combination of both of these factors.

A similar interpretation could be advanced to account for the difference in sensitivity of tail fins to T_4 at different stages of metamorphosis. Discs taken from animals in early prometamorphosis might be less sensitive to treatment with thyroxine than similar pieces taken from animals in late prometamorphosis because tissues from animals in early prometamorphosis were exposed to or possessed a higher level of prolactin. Therefore, higher levels of T_4 would be required to induce resorption of early prometamorphic discs as compared to discs taken from animals at late prometamorphosis. Finally, it appears reasonable to expect that the differences in sensitivity of tail discs to thyroxine at these two stages of metamorphosis result from the reciprocal changes that are believed to be occurring in the levels of both of these hormones, that is, the decreasing concentration of prolactin and the increasing levels of T_4.

B. Hormones in Development - Primary Mechanisms.

In studying the processes of growth and differentiation it has been demonstrated that hormones play a vital role. Hormones influence development by directing the realization of alternative paths of development; activating processes of differentiation at specific times in the developmental history of an organism and thus controlling developmental events in different parts of the body; finally, hormones can control growth and maintenance of the organism (Frye, 1967).

To date, many theories have been proposed to explain the action of hormones. A theory that has become popular in recent years (based on considerable evidence) has been that hormones exert their effects on cells by interacting with the cell's genetic material and the systems associated with the transcription and translation of this material. These theories are based on the present concepts of gene action (DNA-RNA-proteins) and gene regulation (induction and repression). Hormones have also been shown to exert their influence on cells by affecting the permeability of various cellular membranes. In this way substrates and cofactors could become more, or less, available. Other theories propose that hormones exert their action on cells by affecting preexisting proteins, i.e., changing their activity (allosteric changes) or by acting as co-enzymes or co-substrates thus increasing the enzymatic activity of a reaction. Finally, hormones may act by displacing a cofactor that is acting as a suppressor of an enzyme (Villee, 1967).

Regardless of the cellular processes involved, it becomes clear that hormone action is dependent upon two events; first, a cell must possess a receptor site with which the hormone interacts causing a change in the biological function of the receptor and this can be considered as the primary action of the hormone. Amplification of primary function occurs and this is followed by the secondary and tertiary actions of the hormone that affect other metabolic events in the cell. It is generally believed that up to now the primary actions of hormones have not been demonstrated but rather secondary actions have been described (Gorski et al., 1965).

In studying the mechanism of hormone action it has been observed that a single hormone may possess multiple actions, depending upon the target tissue tested. The important question that arises is, does this hormone possess the same primary action in each case and are the secondary and tertiary changes produced dependent upon the different intrinsic properties of the specific target cells? Alternatively, the same hormone could possess different primary actions in each cell type that induce specific changes in those cells. Finally, it has been demonstrated that in many instances the action of hormones is dependent upon the concentration of hormone tested (Tata, 1966).

C. Hormonal Control of Metamorphosis.

1) Thyroid Hormone in Metamorphosis

The present study has been particularly concerned with the role of thyroid hormone in metamorphosis. One method of studying hormone induced changes during metamorphosis that may be expected to provide significant insight into the mode of thyroid hormone action in these processes is the biochemical approach. In recent years, two events in amphibian metamorphosis have received considerable attention, changes in liver metabolism and tail resorption.

During metamorphosis there is a change in the manner by which nitrogenous waste products are eliminated. There is a transition from ammonotelism to ureotelism. Munro (1939, 1953) demonstrated that ammonia excretion exceeds that of urea during premetamorphosis and prometamorphosis while the situation is reversed during climax and adult stages. This change can be induced by administration of thyroid hormones. Paik and Cohen (1960) demonstrated that thyroxine specifically induces the synthesis of carbamyl phosphate synthetase, a key enzyme in the urea cycle. Brown and Cohen (1959) also found that the activity of this enzyme may be correlated with the degree to which the adult stage is morphologically further developed than the larval stage. Cohen (1966) has shown that several enzymes involved in urea biosynthesis increase during metamorphosis and other enzymes decrease. In view of the present concept of the role of DNA and RNA in the control of differentiation and development, several laboratories have been investigating the effect of thyroid treatment on these molecules. As an example, Kim and Cohen (1966, 1968) state that thyroxine treatment increases the rate of synthesis of various types of RNA in tadpole liver. Tata (1965, 1966b) has confirmed that there is an early increase in nuclear RNA synthesis following thyroxine treatment. These changes in RNA precede changes in protein synthesis thus accounting in part for the lag period generally observed for the action of thyroid hormone. Kim and Cohen (1966, 1968) have also reported that chromatin prepared from the nuclei from tadpoles treated with thyroxine was more efficient in promoting the synthesis of DNA dependent RNA by RNA polymerase than chromatin from untreated tadpoles. These studies have indicated that thyroxine exerts its action on metamorphic change by affecting RNA synthesis (specific m-RNA and also general RNA synthesis). This affect on RNA synthesis would be expected to be followed by the synthesis of proteins. In addition, Kim and Cohen (1968) indicate that thyroxine also exerts an affect on protein synthesis by operating at the level of RNA translation. Specifically, thyroxine stimulates the step in protein synthesis involving the transfer of amino acids from aminoacyl s-RNA to ribosomes.

As previously discussed, tail resorption is one of the most dramatic events that occurs during metamorphosis and as such has been investigated by many workers. Recent experiments have demonstrated that protein synthesis is a prelude to tail regression (Weber, 1965, 1967; and Tata, 1966a). Thyroid hormone increases the rate of protein synthesis and DNA dependent RNA synthesis in tail tissues.

It has been demonstrated that both tail resorption and changes in liver metabolism are preceded by changes in RNA and protein synthesis. These results have enabled some investigators to propose the following model for the action of thyroid hormone in inducing the multiple changes that occur to the tadpole during metamorphosis. It is proposed that thyroxine exerts its action on metamorphosing tissues by affecting the systems associated with the transcription and translation of the cell's genetic material. However, it must be stated that although these experiments indicate that thyroxine does indeed stimulate these processes it has not been shown that this is the primary site of this hormone's action (Tata, 1966b). This model is significant because all of the diverse changes of metamorphosis can now be explained by a single common mechanism. That is, metamorphic change appears to be dependent upon protein synthesis that is in turn preceded by DNA dependent RNA synthesis.

The results of this experiment could be interpreted in terms of the model just described. Thyroxine could affect the specificity or rate of transcription of DNA so that either a particular region of DNA is transcribed or the efficiency of DNA directed RNA synthesis in a particular region of the cells DNA is affected. This could be accomplished by having T_4 combine with a repressor molecule (Jacob-Monod) that is now activated and is capable of affecting the synthesis of RNA. There could be a quantitative relationship between the number and stability of such molecules and the synthesis of the RNAs necessary for metamorphic change to occur. Such a concept would be in keeping with the model just proposed and with the results of the present experiment. This scheme is basically the same as that proposed earlier for the interaction of thyroxine with component P to form active product PT.

During metamorphosis there is a gradual loss of body water and at the same time there is an increase on both organic and inorganic substances. Fletcher and Myant (1959) stressed the importance of the regulation of body water and electrolytes in metamorphosis. Etkin (1930) and Urbani (1962) demonstrated that during climax there is about a 60% loss of body substances: this loss represents intestinal evacuation and atrophy of tissues. This desiccation is postulated to the result from the action of thyroxine in inducing the disappearance of acid muccopolysaccharides from larval connective tissue. It is tempting to speculate that the loss of water, induced by thyroxine, might be the primary site of thyroid hormone action. This in turn could influence the pattern of nitrogen excretion and also the regression of tail tissues.

The results of the present study could support such a mechanism.

That is, thyroxine affects molecules that are involved in the regulation of water and ion transport through cellular membranes. Based upon the model already proposed (action of PT) thyroxine could act by interacting with such molecules to induce a change in their structure and or function that would affect the permeability of that membrane. As previously indicated, this interaction would be relatively stable. A quantitative relationship would then exist between the number of such molecules and their effects on metamorphosis. That is, a certain number of molecules would have to be altered by interacting with thyroxine before a significant change in water or electrolyte balance would occur. Following these changes, other metabolic systems could be affected (RNA synthesis, protein synthesis, carbohydrate metabolism, etc.). The specific changes induced would be dependent upon the intrinsic responses of the particular tissues in question.

2) Prolactin in Metamorphosis

In homiotherms, prolactin has been demonstrated to act as a growth factor and recent experiments have now implied that this hormone may function in a similar capacity in amphibia. It is, however, important to note that some investigators (Nicoll and Bern, 1964) have shown that prolactin produced by different classes of vertebrates does not possess equivalent properties. That is, the vertebrate prolactins tested to date all appear to be able to stimulate the "water drive" reaction in T. viridescens, however, not all prolactins (i.e., fish) are capable of stimulating the crop sac response in pigeons. This is of importance in experiments where purified mammalian hormones are tested on non-mammalian test objects and with inferences then being made as to the action of the non-mammalian hormones on these same tissues.

At the present time, little work has been done in analyzing the biochemical effects of prolactin on amphibian metamorphosis. Etkin and Gona (1967) and Gona (1967) have presented evidence that mammalian prolactin inhibits metamorphosis by acting as a goitrogenic agent. This mechanism would act to control (lower) the level of T_4 in circulation and thus regulate the rate of metamorphosis. Other workers, Bern et al. (1967) and Etkin et al. (1968) have presented evidence which indicates that prolactin may exert its action peripherally, at the level of target tissues to regulate metamorphosis. In connection with this point, Paik and Cohen (1961) indicated that certain antithyroid substances (thiouracil) assumed to interfere with the biosynthesis of T_4 in the thyroid also seems to interfere with the affect of T_4 peripherally. In the liver, this substance interferes with the synthesis of liver enzymes that are normally stimulated by T_4. In the tail, the activity of hydrolytic enzymes is low during the growth phase of metamorphosis (prolactin period) and this activity increases during prometamorphosis and climax (thyroxine period). However, no work has been done as yet to study the effect of prolactin on these enzymes. Does prolactin suppress the synthesis of such enzymes? and if so at what level in the cell's synthetic apparatus does this inhibition occur? Another question that arises is; do prolactin and thyroxine affect metamorphosis by operating at the same sites or different sites in the cell? For example, Lapiere and Gross (1963) demonstrated that thyroxine leads to an increased rate of collagen breakdown as compared to the rate of collagen synthesis that continues to occur. Prolactin might act by suppressing the increased rate of collagen destruction induced by T_4. It is also possible that prolactin does not interfere, directly, with the action of thyroid hormone but may operate at other sites. In the example just cited, prolactin might influence tail resorption by inducing an increase in the rate of collagen synthesis and as a result cancel out the effect of thyroxine induced collagen breakdown.

Other mechanisms of prolactin action can be proposed. Prolactin may stimulate the rate of breakdown or excretion of T_4 by the tadpole. Prolactin might act by binding thyroxine in circulation or by affecting the capacity of cells to bind T_4. This would be of importance in view of the demonstration that tail resorption is dependent upon the continuous presence of thyroid hormone. Lowering the level of effective hormone present by such mechanisms could result in the failure of these tissues to complete resorption. Schwartz et al. (1968) have shown that rabbit red blood cells change in their capacity to bind T_4 at different stages of their maturation. Hasen et al. (1968) have shown that in rats the liver and kidney can bind thyroxine that is then maintained in these cells and this T_4 can be released again to the plasma. They point out that degradation and net metabolic effects of a hormone might be related to the intracellular content of exchangeable T_4 and therefore tissue binding and plasma binding must be considered in any analysis of the factors governing the distribution, kinetics, and turnover of a hormone. Finally, it has been shown that ions that block thyroid iodide transport (perchlorate and thiocyanate) reduce the level of plasma bound thyroid hormone. This may be dependent upon two processes: (1) augmented uptake of T_4 by muscle cells, or (2) these ions may compete with T_4 for sites on serum albumin and thus account for more free T_4 and lowered PBI.

Finally, prolactin has been shown to enhance the survival of salt water fish in hypotonic solution. This is accomplished by preventing the fall of Na levels. This action is unique to prolactin and exhibits a dose response relationship (Ball and Ensor, 1967 and Pickford and Philips, 1959). These studies have not indicated what the primary action of this hormone is. Presumably it could be the cell membrane or systems associated with cell membranes that are involved in controlling transport. It has already been mentioned that thyroxine stimulates the loss of water during amphibian metamorphosis. In view of the action of prolactin in fish (osmoregulation) it might be speculated that prolactin could inhibit the loss of water by tadpole tissues. Perhaps, the primary relationship between prolactin and thyroid hormone in metamorphosis is concerned with the process of water and electrolyte balance. At the present time, these proposals only represent speculation and as such must await future investigation.

VI. Summary and Conclusions

1. An *in vitro* system, consisting of tadpole tail fins maintained in Hanks balanced salt solution, has been employed to study the effects of hormones (prolactin and thyroxine) on metamorphic change.

2. The results of these experiments demonstrate that a quantitative relationship exists between thyroxine and its action in inducing tail resorption. The response of tail fins to thyroxine varies, over a given range, directly with the concentration of thyroxine tested. The action of thyroid hormone in this process is dependent upon the continuous presence of this hormone.

3. Tail discs change in their sensitivity to thyroxine at progressive stages of metamorphosis; sensitivity to hormone increases during the prometamorphic period. The results support the concept that the increased sensitivity of tail discs to thyroxine is due to the previous *in vivo* exposure of these tissues to a low but rising level of thyroid hormone during prometamorphosis and a sudden increase in hormone one to two days before the emergence of the forelimbs. In addition, changes in sensitivity of tail discs to thyroxine might reflect changes in the level of a pituitary factor (prolactin) present in these tissues at the time of isolation.

4. Pars anterior of the tadpole's pituitary implanted into a tail disc release a substance that interferes with the action of thyroxine *in vitro*. A quantitative, reciprocal relationship exists between the pituitary factor and thyroid hormone with respect to their effects on tail resorption.

5. Simultaneous treatment of tail discs with prolactin-or growth hormone and thyroxine also results in inhibition of thyroxine induced tail resorption. These results make it difficult to determine what the substance is that the tadpole's pituitary is releasing. Is it a prolactin-like or GH-like molecule? The results of the present investigation and of other works would favor the concept that the pituitary factor is a prolactin-like hormone.

6. Finally, it is proposed that the interaction of these two hormones is important in controlling amphibian metamorphosis.

VII. Appendix

A. Technical Advantages of Tail Discs Over Tail Tip Preparations

1) Uniformity of Response.

In these experiments unlike those of other previous studies only completely healed discs were selected for use. Unhealed tail tips demonstrated extreme variability in their response to thyroxine treatment. Therefore, the quantitative interpretation of the results of these studies was very unsatisfactory. In some initial experiments of the present work discs were used that were not completely healed and variability was observed in their response to a given concentration of thyroxine. It was observed that unhealed discs responded more rapidly to thyroxine treatment than healed discs. For this reason only healed discs were tested. It is suggested that the variability in response demonstrated in previous experiments on isolated tails by certain investigators results from their use of tails that varied in their degree of healing and that the results of these experiments reflects the differential penetration of hormone into these tail tissues. Completely healed tail discs are free of difficulties of this kind and give a reliable, uniform response.

2) Simplicity of System

Tail discs are composed of a simple integument enclosing a loose connective tissue and they are completely devoid of the striated muscle that constitutes the bulk of tail tip preparations. Therefore, tail discs represent a simpler test object than tail tips; that is, in terms of the tissues that it is composed of.

3) Quantification of Results

Previous investigators, (Weber, 1962; Shaffer, 1963; Tata, 1966;

Lindsey et al., 1967) determined tail resorption by measuring the tail's length at periodic intervals and the data were then expressed as percentage of initial length. These measurements are generally difficult to make since tail tips may assume twisted or curved shapes. In addition, during tail resorption it is observed that tail fins which make up a large area of the tail are the tissues that are most sensitive to treatment with thyroxine and as such respond first to such treatment. The previous methods of quantifying tail resorption did not include this factor and therefore the previous studies only represent a rough approximation of the changes in size that these tissues undergo during resorption. It is believed, that the method used in this study (microprojection and measurement of areas) for quantifying tail resorption is superior to that previously used.

4) Dosage

In the present study, six concentrations of thyroxine (T_4), ranging from approximately $3x10^{-9}\underline{m}$ to $7.5x10^{-7}\underline{m}$, were tested. The medium employed was Hank's balanced salt solution. The lowest concentration used by previous workers in the order of $1x10^{-6}M$ to $2x10^{-7}M$ and generally solutions of higher concentration were used. In addition, several workers have used T_3 instead of T_4 (Shaffer, 1963 and Tata, 1966). This analog, T_3, of thyroxine is generally more active in inducing metamorphic changes than T_4 so that in these experiments higher concentrations of an even more active compound were used. The use of such high concentrations can be explained in part; the culture media used in these experiments contained plasma, a substance that is capable of binding thyroid hormone. Plasma could then act to dilute the level of effective, free thyroxine present so the real level of hormone acting on the tail tissues was actually lower than the concentration reported. However, plasma has not been used in the media of all previous workers. Weber (1962) employed T_4 in Holfreters solution and obtained tail resorption with concentrations of $1x10^{-6}M$ and $2x10^{-7}M$. This is approximately 65-300 times higher than the lowest concentrations used in this study. In a recent paper, Lindsey et al. (1967) using Niu Twitty's solution, claimed that resorption of tail tips in vitro required at least $1.3x10^{-7}M$ T_4 or $1.5x10^{-8}M$ T_3. However, when they tested lower concentrations of hormone they only maintained their cultures for 9 days. The results of the present experiments demonstrate that if discs are cut from animals at stages comparable to those used in the experiments of Lindsey et al. and if low concentrations of T_4 are used (concentrations that this group reported could not induce tail resorption), resorption was observed to begin at about day 9 in culture and was completed about day 12. Thus it appears that these workers did not continue their experiments long enough to produce a thyroxine response. Presumably they could not maintain their controls adequately for periods longer than 9 days. This could be due to the fact that they used whole tail tips. Finally, it is evident that the present study has produced the most sensitive demonstration of thyroxine induced tail resorption, in vitro, reported to date.

B. Immersion as a Method of Thyroxine Administration

In the experiments of this study, the immersion technique was chosen as the means of administering thyroxine since it is the simplest method, involves minimal manipulation of discs and since Kollros (1963) demonstrated that " The immersion technique can be asserted to provide a continuous and relatively unvarying hormone source."

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Fig. 1. Early prometamorphic discs treated with different concentrations of thyroxine as recorded by projection.

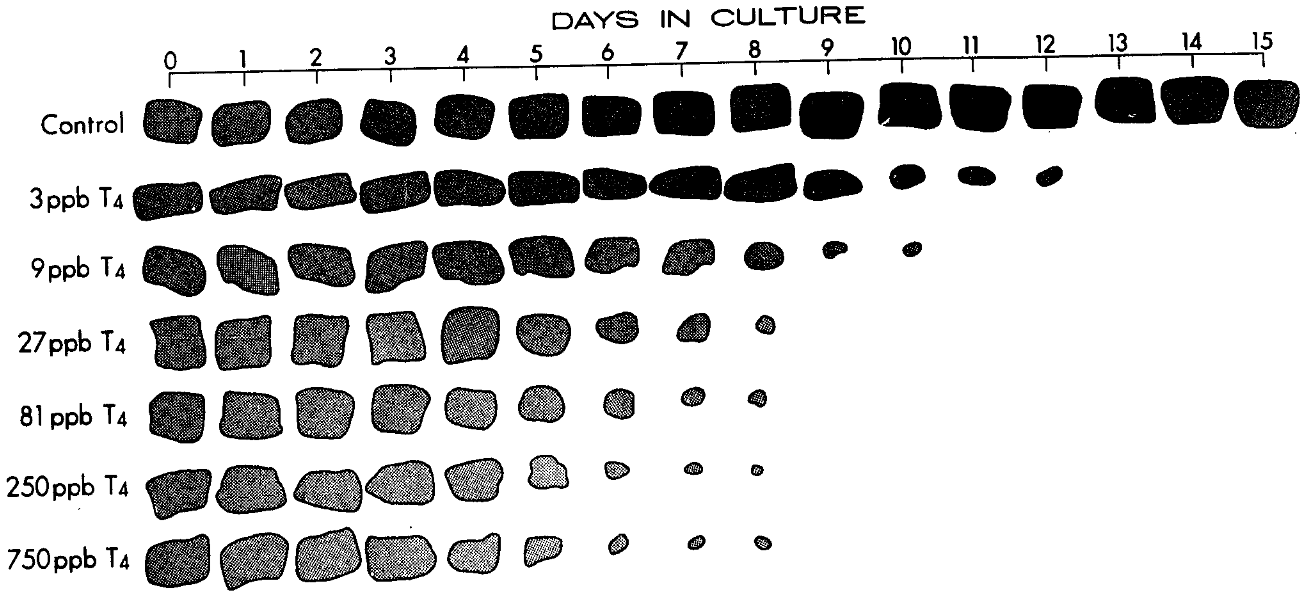
DAYS IN CULTURE
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Control
3ppb T4
9ppb T4
27ppb T4
81 ppb T4
250ppb T4
750ppb T4

Fig. 2. Experiment 1, early prometamorphic discs treated with thyroxine. The standard error of the mean is indicated by the vertical lines and the numbers above these lines indicates the number of discs still present out of the original 14 discs in that group.

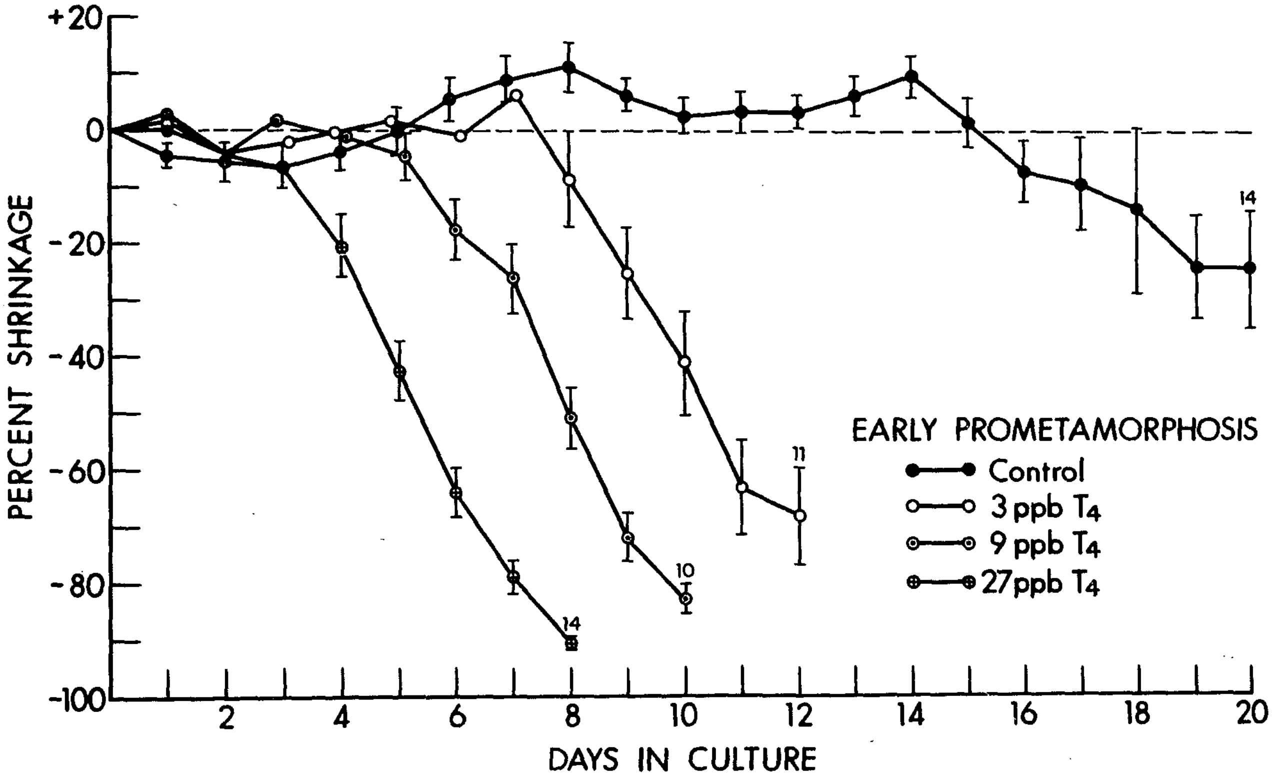
+20
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
2
4
6
8
10
12
14
16
18
20
DAYS IN CULTURE
EARLY PROMETAMORPHOSIS
Control
3 ppb T4
9 ppb T4
27ppb T4
14
10
11
14

Fig. 3. Experiment 2, discs taken from animals at late prometamorphosis and treated with thyroxine.

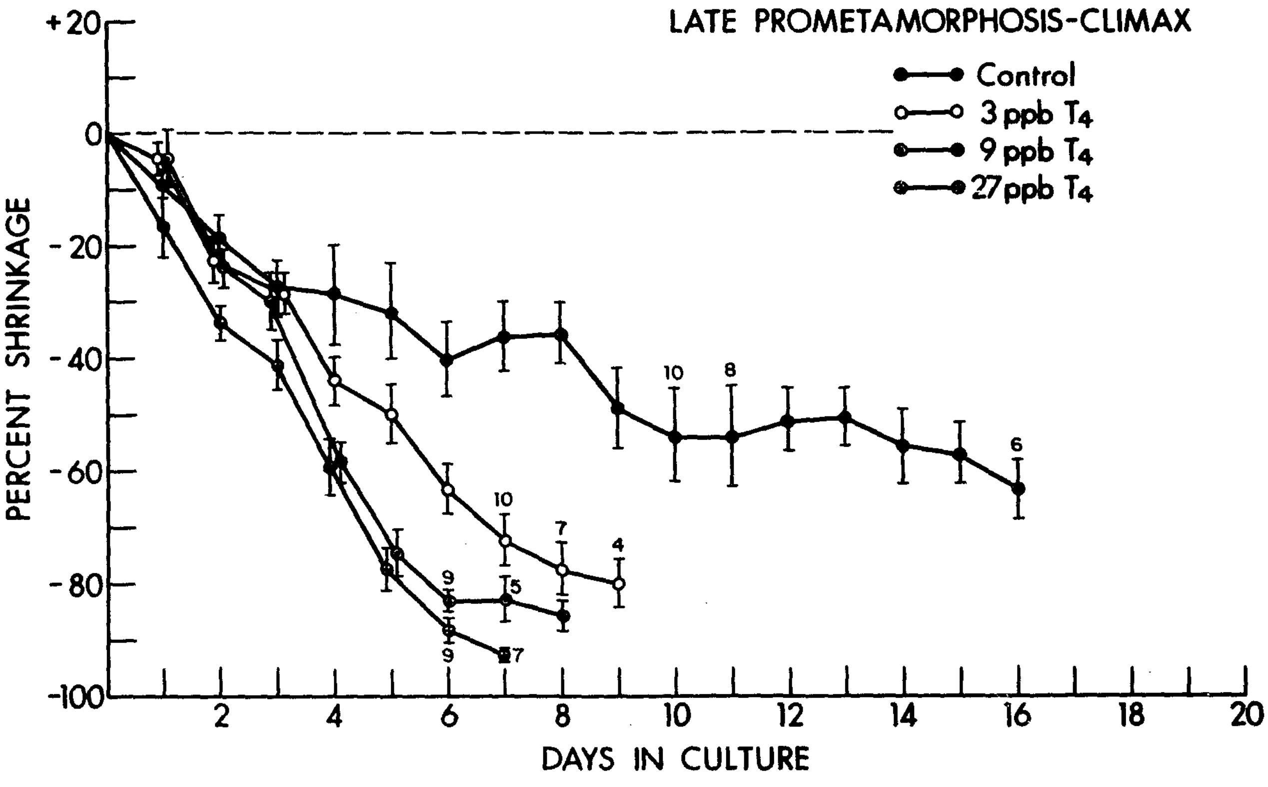
LATE PROMETAMORPHOSIS-CLIMAX
Control
3 ppb T4
9 ppb T4
27 ppb T4
PERCENT SHRINKAGE
+20
0
-20
-40
-60
-80
-100
DAYS IN CULTURE
2
4
6
8
10
12
14
16
18
20
10
8
6
10
7
4
9
5
9
7

Fig. 4. Experiment 3, early prometamorphic discs and discs cut from animals at E-1, E+0 and E+1; maintained in control medium.

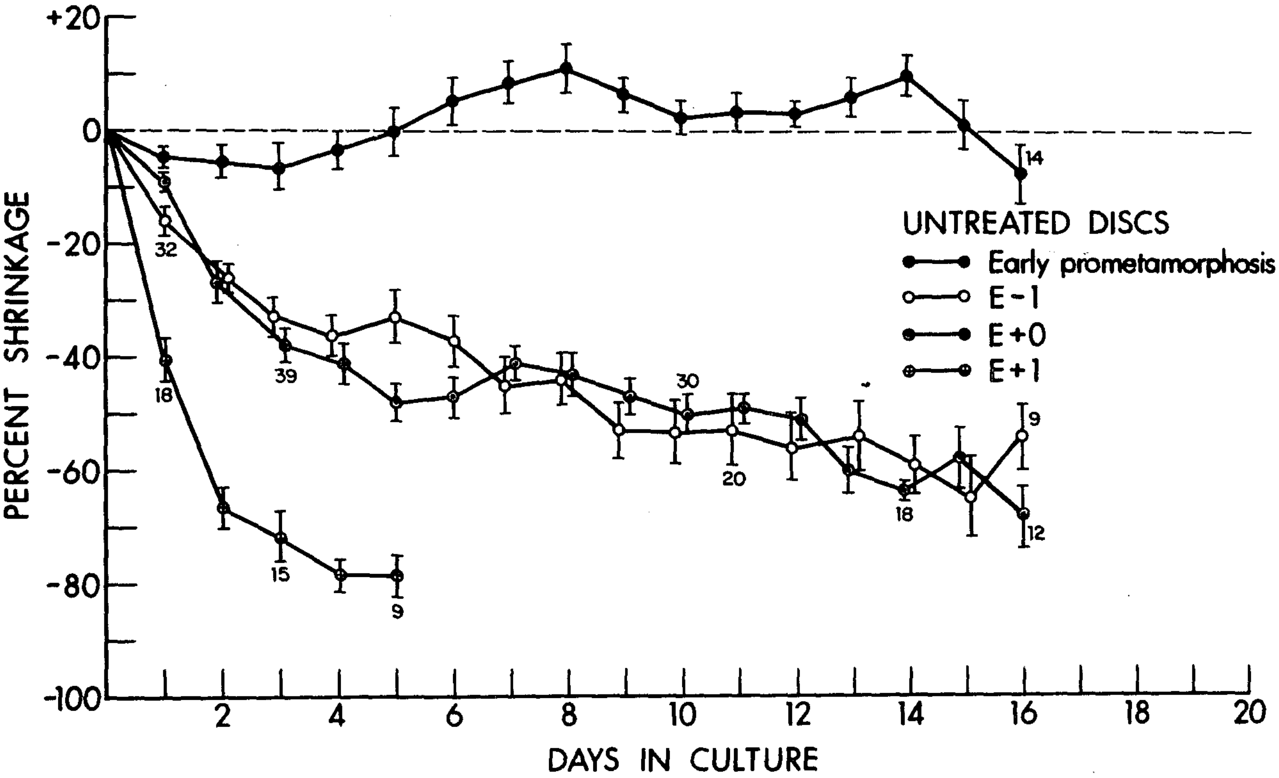
+20
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
2
4
6
8
10
12
14
16
18
20
DAYS IN CULTURE
UNTREATED DISCS
Early prometamorphosis
E-1
E+0
E+1
14
32
18
39
15
9
30
20
18
9
12

Fig. 5. Experiment 5, early prometamorphic discs placed into 3 ppb of T_4 for different periods of time and then maintained in control medium.

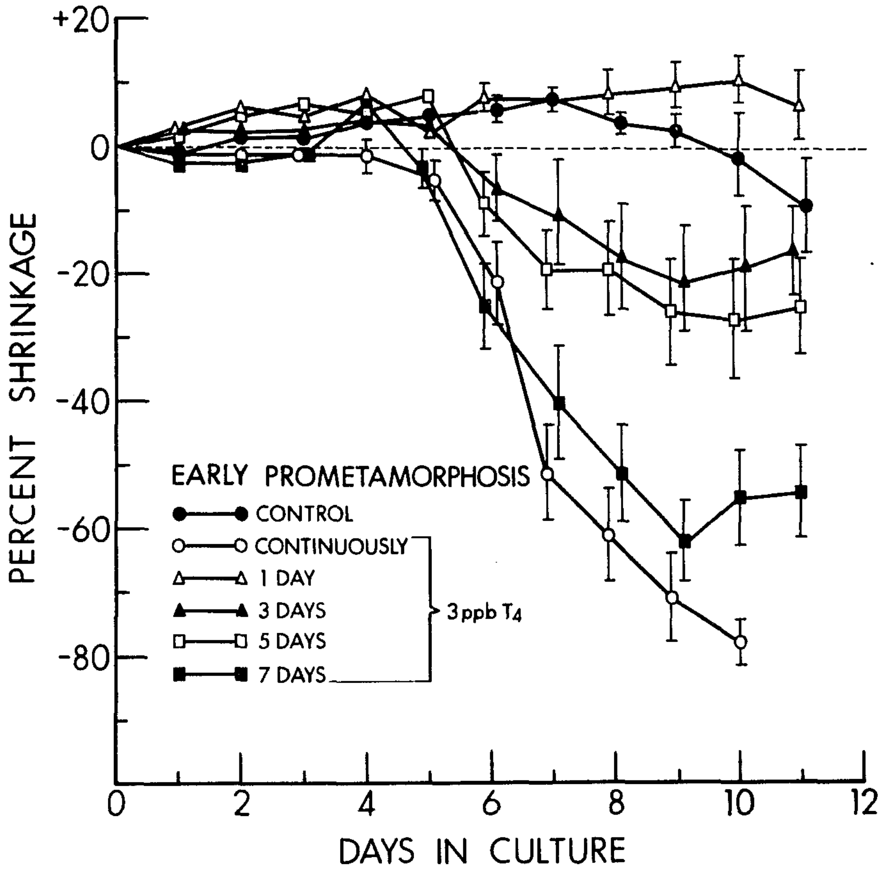
+20
0
-20
-40
-60
-80
PERCENT SHRINKAGE
0
2
4
6
8
10
12
DAYS IN CULTURE
EARLY PROMETAMORPHOSIS
● CONTROL
○ CONTINUOUSLY
△ 1 DAY
▲ 3 DAYS
□ 5 DAYS
■ 7 DAYS
3 ppb T4

Fig. 6. Experiment 6, response of six regions of the tadpole's tail to thyroxine treatment. Tissues were taken from tadpoles in early prometamorphosis.

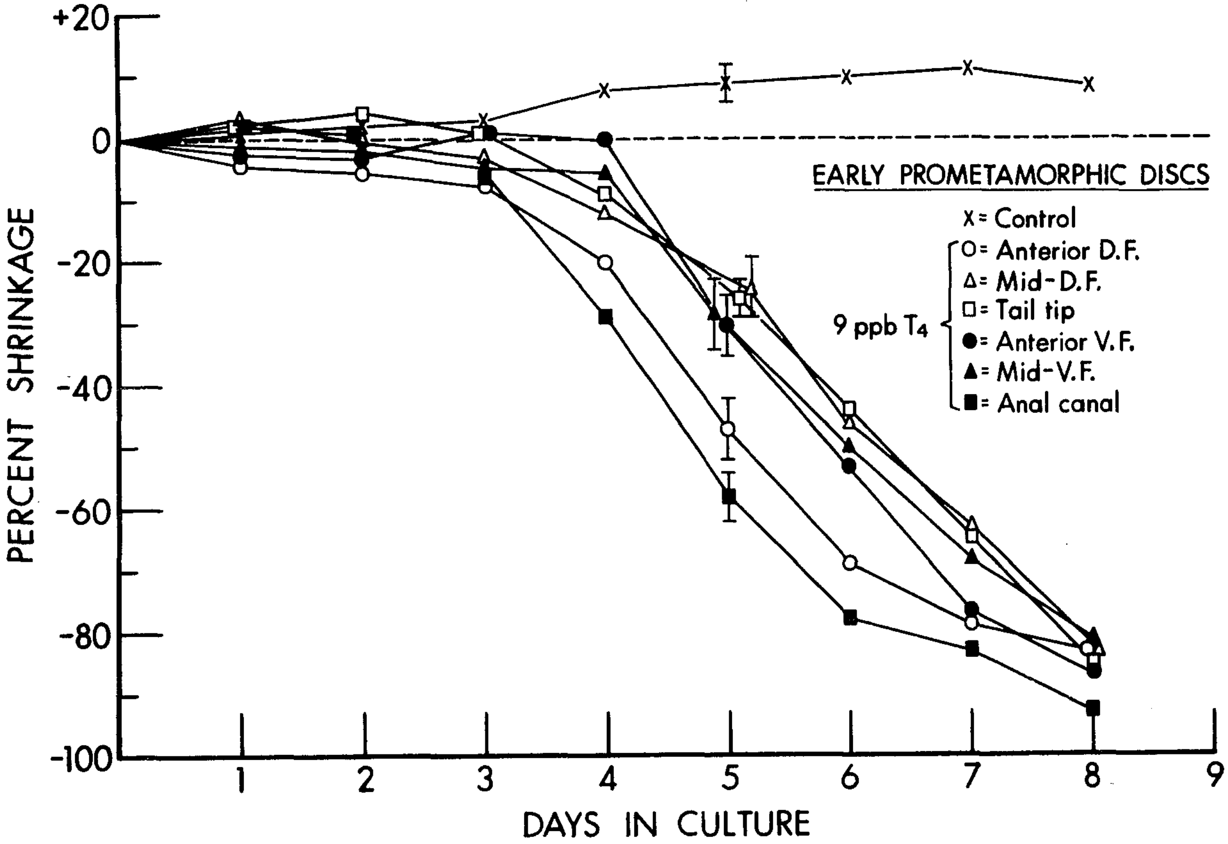
+20
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
1
2
3
4
5
6
7
8
9
DAYS IN CULTURE
EARLY PROMETAMORPHIC DISCS
X = Control
9 ppb T4
○ = Anterior D.F.
△ = Mid-D.F.
□ = Tail tip
● = Anterior V.F.
▲ = Mid-V.F.
■ = Anal canal

Fig. 7. Experiment 7, early prometamorphic discs maintained in control medium for 10 days and then treated with T_4.

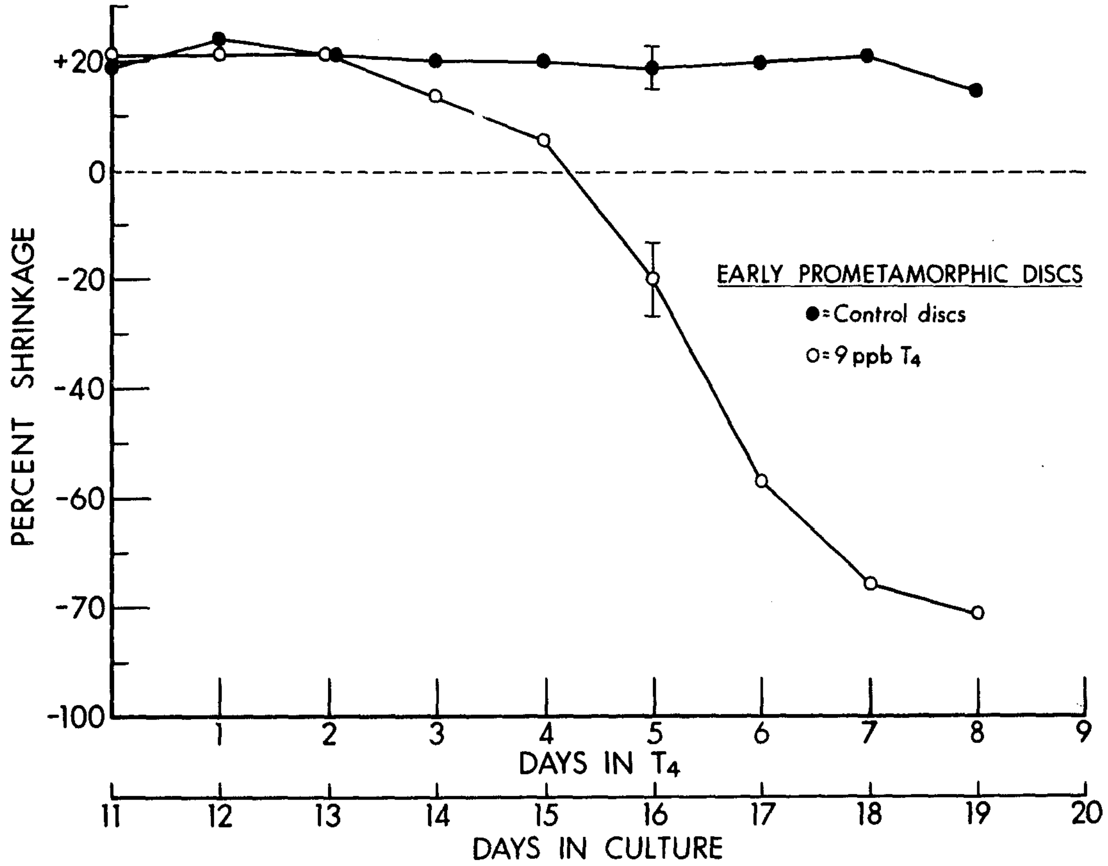
+20
0
-20
-40
-60
-70
-100
PERCENT SHRINKAGE
1
2
3
4
5
6
7
8
9
DAYS IN T₄
11
12
13
14
15
16
17
18
19
20
DAYS IN CULTURE
EARLY PROMETAMORPHIC DISCS
● = Control discs
○ = 9 ppb T₄

Fig. 8. Experiment 8, discs taken from animals in early prometamorphosis. Discs contain pituitary or cerebrum implants, the last group are sham-operated control discs. Discs are maintained in one of two concentrations of thyroxine (3 ppb or 6 ppb) or in control medium.

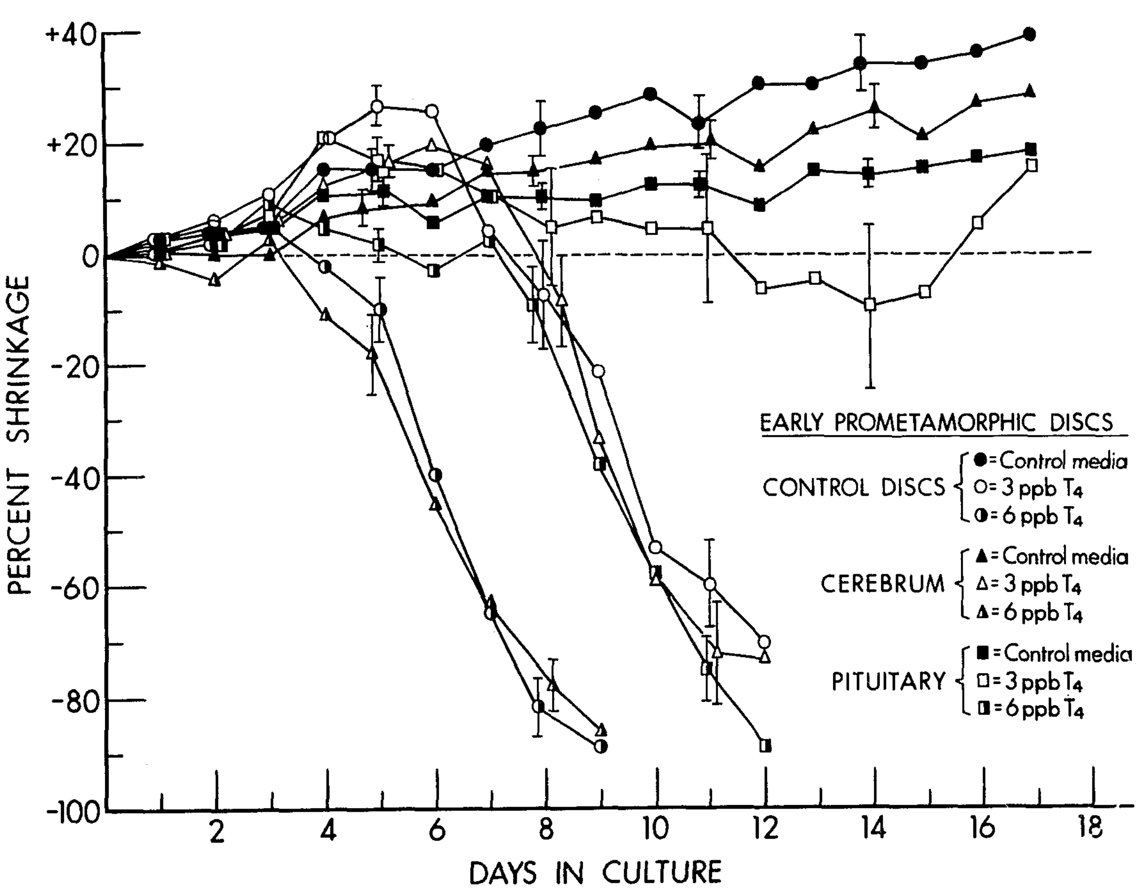
+40
+20
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
2
4
6
8
10
12
14
16
18
DAYS IN CULTURE
EARLY PROMETAMORPHIC DISCS
CONTROL DISCS
● = Control media
○ = 3 ppb T4
◑ = 6 ppb T4
CEREBRUM
▲ = Control media
△ = 3 ppb T4
△ = 6 ppb T4
PITUITARY
■ = Control media
□ = 3 ppb T4
◨ = 6 ppb T4

Fig. 9. Experiment 9, early prometamorphic discs containing pars anterior or cerebrum implants and sham-operated control discs maintained in 6 ppb T_4 or in control medium.

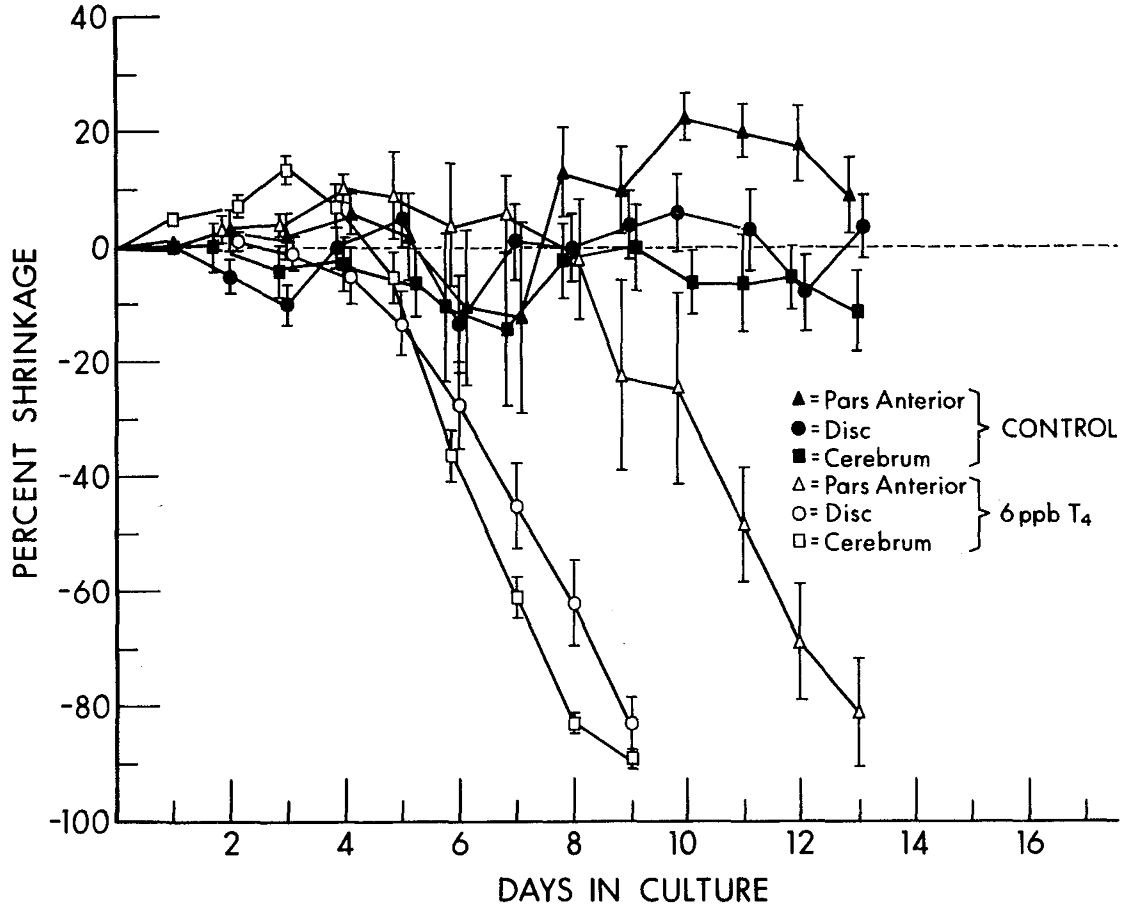
40
20
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
2
4
6
8
10
12
14
16
DAYS IN CULTURE
▲ = Pars Anterior
● = Disc
■ = Cerebrum
CONTROL
△ = Pars Anterior
○ = Disc
□ = Cerebrum
6 ppb T4

Fig. 10. Experiment 10, discs taken from animals at E+1. Each tail supplied 3 discs; a pituitary implant, a cerebrum implant and a sham-operated disc. Discs were maintained in control medium.

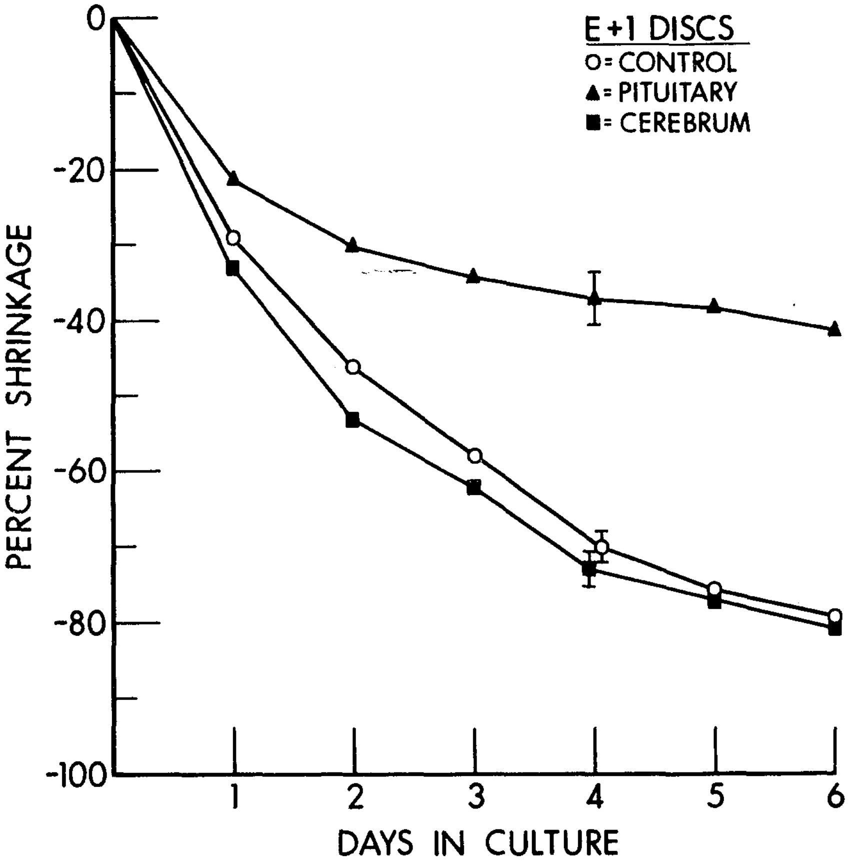
E+1 DISCS
○= CONTROL
▲= PITUITARY
■= CEREBRUM
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
1
2
3
4
5
6
DAYS IN CULTURE

Fig. 11. Tail discs from <u>Rana</u> <u>catesbeiana</u> tadpoles.

A. Tail disc without a pituitary gland implant; pigment cells contracted.

B. Tail disc with a pituitary gland implant; pigment cells expanded.

C. Same disc (B) cut in half, only one half (left side) contains the graft and the pigment cells in this half remain expanded while the pigment cells in the graftless half disc contract **(right side).**

A.

B.

C.

Fig. 12. Experiment 12, discs taken from animals at E+1 maintained in solutions containing; prolactin, GH, FSH or control medium.

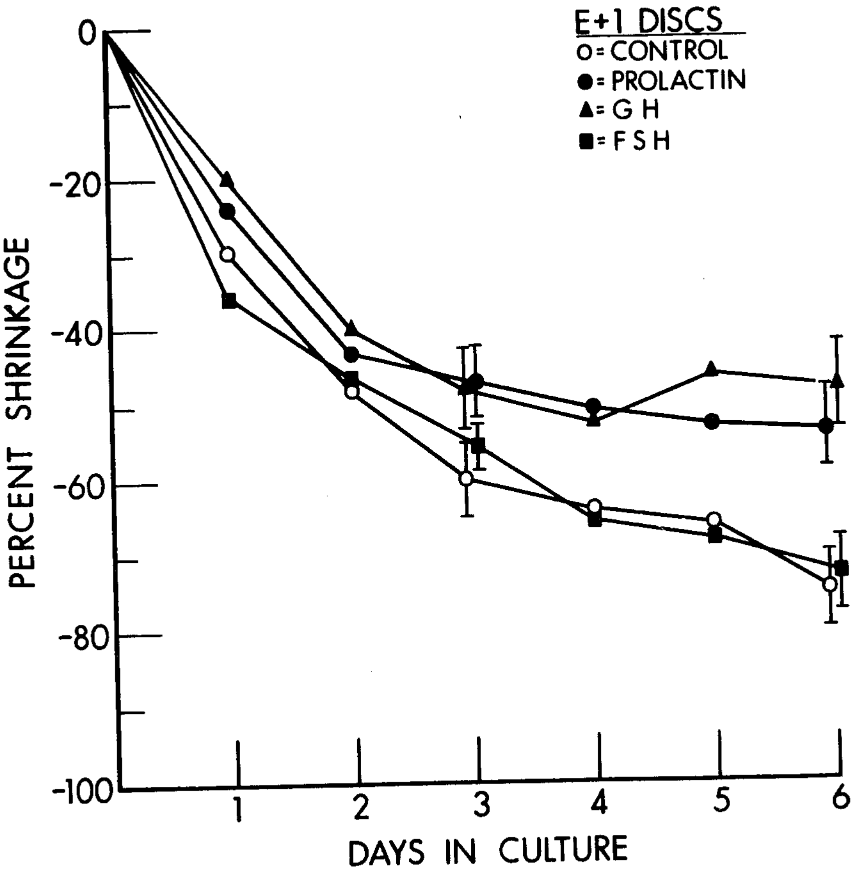
E+1 DISCS
○ = CONTROL
● = PROLACTIN
▲ = G H
■ = F S H
0
-20
-40
-60
-80
-100
PERCENT SHRINKAGE
1
2
3
4
5
6
DAYS IN CULTURE

Table 1 - Experiment 4

Specificity of the thyroxine response

Mean % shrinkage of early prometamorphic tail discs

days in culture	control medium	L-thyroxine 24x10^{-9}m*	KI 24x10^{-9}m	KI 217x10^{-9}m	DL-thyronine 47x10^{-9}m+	DL-thyronine 435x10^{-9}m+	DL-DIT+ 47x10^{-9}m	DL-DIT+ 435x10^{-9}m
1	+6.2± 1.7	+1.3± 2.4	+1.3± 1.8	+3.2± 1.5	-0.7± 2.0	+4.5± 2.8	+5.0± 2.1	+9.6± 1.8
5	+6.5± 2.2	-21.9± 12.6	-0.9± 3.0	+4.8± 1.7	+0.3± 2.8	+8.6± 3.5	+7.0± 4.6	+11.4± 4.2
9	+5.5± 3.7	-90.8± 2.0	+8.5± 2.5	+7.6± 5.6	+5.9± 2.5	+8.4± 3.5	+8.6± 4.1	+13.4± 5.6
16	+9.8± 2.0		+9.5± 8.8	+9.2± 7.6	-4.7± 10.6	-2.0± 8.3	-5.4± 3.0	-71.0± 3.8

* 27 ppb T_4

+ the molality of these racemic solutions, DL-thyronine and DL-DIT, was doubled to treat discs with equimolal concentrations of the L form of these molecules.

Autobiographical Statement

I was born on November 12, 1939 in Antwerp, Belgium. My parents left Belgium in 1940 and subsequently came to live in New York City in 1941. I attended P.S. 131 and Jamaica High School.

I graduated from the City College of New York, with a B.S. Degree in June, 1961. My major field was Biology. I participated in athletics as an undergraduate and earned three varsity letters in lacrosse; I also participated in freshman soccer and lacrosse.

I entered the graduate program in Biology at New York University in September, 1961. In October, 1964, I was awarded a Masters Degree. The title of my Thesis was, "The role of the pituitary in pigment pattern development."

I entered the graduate program in Biology at the City University in September, 1964. For the last two and one half years I have been supported by a predoctoral stipend from a National Institute of Health training grant awarded to Dr. M. Hamburgh.

I have had experience teaching at the City University as a lecturer (part time). I have instructed first level biology, comparative anatomy, and embryology laboratories.

I have been awarded a Public Health Service Post-Doctoral Fellowship from the National Institute of Child Health and Human Development to study in the laboratory of Dr. E. Boell at Yale University, New Haven, Connecticut.

Publications

Derby, A. 1967. An *in vitro* quantitative analysis of the response of tadpole tissue to thyroxine. Amer. Zool., 7: 719 (abstract).

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____________________ 1968. Prolactin-like antithyroid action of pituitary grafts in tadpoles. 5th International Symp. Comp. Endocrinol., (to be published in a Supplement of Gen. Comp. Endocrinol.).