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The Effects Of Thyroxine On Deoxyribonucleic Acid Content In The Brain Of Rana Clamitans Tadpoles

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GARNER

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THE EFFECTS OF THYROXINE ON DEOXYRIBONUCLEIC ACID CONTENT IN THE BRAIN OF *RANA CLAMITANS* TADPOLES

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L.A.G.

DEDICATION

This thesis is dedicated to my wife and son, Betty and Keith Garner, for their understanding, patience, and encouragement over the past months.

L.A.G.

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INTRODUCTION

The metamorphic changes transforming the tissues of the tadpole into those of the mature frog are known to be under the control of thyroid hormone, that is, they fail to occur, if the hormone is absent. However, it is becoming increasingly clear that control does not mean the determination of the specific character of the ensuing changes, but refers merely to the reactivated and further sustenance of different chains of morphogenetic events temporarily arrested in the larval stage, then continuing in each tissue reacting according to its own characteristics (Weiss and Rossetti, 1951).

The importance of the thyroid gland as a controlling factor in metamorphosis is to be credited to Gudernatsch (1912) who fed horse thyroid gland to amphibian larvae and found that they showed an increase in metamorphosis. A great amount of work has been done along the line of experimental hyperthyroidism in the amphibian. The work of Allen (1916) and Hoskins (1917) on the removal of the thyroid gland in amphibian larvae clearly indicates that this gland is normally indispensible to metamorphosis in these forms.

In more recent years investigators have been concerned with direct action of thyroid hormone on various reacting systems (Kaltenbach, 1953a). Thyroid hormone has been shown to influence a variety of systems in the amphibian, including the nervous system. The nervous system responds to thyroid hormone by an increase in cell number (Kollros and Race, 1960; Race, 1961). Thyroxine also influences cell size (Weiss and Rossetti, 1951; Pesetsky and Kollros, 1956; Reynolds, 1963). Weiss and Rossetti (1951) reported a marked spurt in brain growth in Rana pipiens during metamorphosis, which is accompanied by intensified activity during normal metamorphic climax. A similar increase in mitotic activity has been reported by Martin (1962) in studies of normal development of the cerebellum in Rana pipiens, in young tadpoles immersed in high concentration of thyroxine, and also by Reynolds (1966) in studies of the lumbo-sacral spinal cord of Rana pipiens larvae.

Various hormones, depending on their nature and degree of activity in the organism, exert a certain effect on the metabolism of mucleic acids. Mandel (1951) reported that after the injection of thyroxine (1 mg. per

day) into rats, DNA content increased from 22 to 32% in the kidney. Similar increases in spleen ranged from 35 to 60% (Mandel, 1952). A single injection of thyroxine into male mice has the effect of raising the proportion of DNA and proteins relative to DNA in the liver after 48 hours (Baxi, 1951).

Whatever is the moment when the synthesis of DNA occurs, cell division is associated with the doubling of the DNA content, so that the DNA of the nuclei in actively growing tissues must be higher than resting tissues (Thomson, 1953). The results of Moore (1952) on embryos of Rana pipiens indicate a wide range of DNA value in the liver, pronephros kidney, and fore brain. His mitotic counts showed that there is no visible correlation between DNA content of nuclei and mitotic activity.

It has been found that the DNA content of two daughter cells is the same amount of DNA as the mother cell (Tata, 1966). If this is the case, cell division must be preceded by DNA synthesis. The first observation of this came from Caspersson's ultraviolet work (1939) on the spermatogenesis of the grasshopper. He suggested that synthesis of DNA occurs during the first phases of the

division and reaches its maximum at metaphase. Swift (1950) reported an increase in DNA during the interphase stage in the nuclei of developing liver and in pronephros and erythrocyte nuclei of recently hatched Amblystoma larva. Seshachan (1949) found a similar process in the division of the ciliate micronucleus. Alfert (1950) observed that the DNA content of the nuclei is always doubled prior to the onset of nuclear division in the early development of mouse embryo. The conception of the synthesis of DNA in late interphase seems to be accepted by most authors at the present time.

The biological role of the nucleic acids is one of the most important scientific problems demanding solution today. Not enough is known about these substances to effectively utilize them for practical purposes, but the vast quantity of scientific material accumulated for more than eighty years since the discovery of nucleic acids leaves no doubt as to their fundamental biological importance. Nucleic acids are complex compounds, widespread in nature and present in various amounts in all cells. Because of their ability to combine with other compounds, particularly proteins, nucleic acids are constantly extracted from tissues together with these

substances. Such protein-nucleic acid complex is called a nucleo-protein, since it is obtained in considerable amounts from material rich in nucleo-proteins (Bradshaw, 1966).

Since the literature reveals that a marked increase in mitotic activity occurs during normal metamorphic climax in the nervous system, and following the administration of exogenous thyroxine, it seemed feasible to investigate further the role of thyroxine in stimulating nucleic acid synthesis in the brain of the Rana clamitans larvae.

METHOD AND MATERIALS

1. Immersion Method of Absorption of Thyroxine

The animals used in this investigation were Rana calmitans larvae, which were collected from ponds in the vicinity of Prairie View A. and M. College. One hundred and fifty tadpoles were selected for this study. The animals were staged according to the method of Taylor and Kollros (1946). Because of prior work done on mitotic activity, using stage IV tadpoles, stage IV was chosen as the experimental stage. They were divided into six groups of twenty-five each. Five groups were used as experimental animals, and one group served as controls. All animals were fed canned spinach. The control larvae were placed in 500 milliliters of tap water. All experimental animals were immersed in 500 milliliters of water containing 200 ug/l of dl-thyroxine. The animals were immersed in thyroxine solutions for a period of one, two, three, four, and five days, respectively. The solutions and food were changed daily.

At the end of the experimental periods, both control and experimental tadpoles were removed from their

containers and frozen in DNA buffer solution, after which the brains of the animals from each group were removed and weighed on an analytical balance in 10 milliliters of cold DNA buffer solution. After computing the weight of the brains for each group of animals the extraction technique for removing DNA was followed. The technique used was that of Bradshaw (1966).

2. Extraction of Deoxyribonucleic Acid

A Waring blender was obtained and placed in the refrigerator overnight to become chilled. After chilling the blender, 50 milliliters of DNA buffer solution was poured into the blender, and the brains from each group, previously frozen in DNA buffer solution, were broken into small cubes and dropped into the blender for a period of one minute in order to homogenize the brain tissue. Homogenizing the tissue in the frozen state serves to break the cell membranes, thereby releasing the cellular contents as a free dispersion. The homogenate was centrifuged at a speed of 5000 rpm's for fifteen minutes to sediment unbroken cells, debris, and deoxyribonucleic proteins. All soluble materials remained in the

supernatant, which contained most of the RNA as well. The sediment was rehomogenized and centrifuged at a speed of 5000 rpm's for fifteen minutes to obtain as much DNA as possible. Sodium citrate was added to the buffer to inhibit the activity of intracellular DNA hydrolyzing enzymes released from disrupted lysosomes. DNAase requires MG ions for its activity and sodium citrate has a powerful affinity for metallic ions if this type. The citrate binds Mg++ and prevents the DNAase from destroying the DNA during the extraction procedure.

The next step takes advantage of the fact that deoxyribonucleoproteins are soluble in a strong sale solution, which is true of DNA also, since most of the proteins are precipitated by such solutions dispersing the DNA sedimentin 2.6 M NaCl (about 15% concentration dissolves this complex). It also dissociates the protamines and histones to yield free sodium deoxyribonucleate. The proteins formed a fine precipitate which was centrifuged out at a speed of 20,000 rpm's for twenty minutes at -2 degrees centigrade leaving the DNA dissolved in supernatant fraction.

Once the DNA solution was separated from the proteins formerly bound to it, the DNA was selectively

precipitated by the addition of two volumes of 95% ethyl alcohol. The resulting precipitate was a mass of white fibrous material that was recovered by wrapping it around a stirring rod. The precipitated DNA was then re-dissolved in water forming a viscous colorless solution. The high viscosity is a function of the long narrow shape of the molecule (15 A° by 2000-3000 A°), and is a characteristic that can be accurately measured by appropriate apparatus.

3. Method of Detection and Quantitation

The Dische diphenylamine method for detection of nucleic acids was used. Six test tubes were placed in a test tube rack and labeled. Five milligrams of lyophillized DNA were dissolved in 5 milliliters of distilled water. This became the stock solution of DNA (1 mg./ml). Two milliliters of stock DNA solution were pipetted into tube one, and 2 milliliters of distilled water were pipetted into tubes two, three, four, and five, respectively. Tube two was mixed well, then 2 milliliters from it transferred to tube three. The contents of tube three were mixed well, then 2 milliliters transferred to tube

four. Two milliliters of the extracted DNA were pipetted into tube six. A 1000 milliliter beaker containing about 700 milliliters of tap water was placed on a ring stand and brought to a boil with a bunsen burner. Four milliliters of Dische diphenylamine reagent were pipetted into each of the six test tubes, and placed into a boiling water bath for ten minutes. After the ten minute heating period all six test tubes were transferred to an ice bath for a period of five minutes. This was the final step in preparing the solution to be read on the spectrophotometer.

The spectrophotometer was turned on and adjusted for reading purposes. After a period of about five minutes (time alloted for the spectrophotometer to warm up) tubes one through four were read and a concentration curve was prepared on graph paper. From the graph the amount of DNA in each of the unknown samples was determined.

RESULTS

A. Metamorphic Changes

The animals used in this investigation were stage IV Rana clamitans larvae, with an average body length of 39 mm. Experimental animals were immersed in 200 ug/l of thyroxine, while the control larvae were immersed in tap water.

The experimental larvae showed numerous external responses to thyroxine which were manifested by abrupt changes in development. Changes noticed on the third day were; narrowing of the body, resorption of the tail, emergence of the fore limbs, development of numerous blood vessels around the hind limb buds, and widening of the mouth. Changes on the fourth day were more pronounced than on the third, and many animals were sluggish with abnormal swimming habits. Numerous disharmonies in development occurred, and by the fifth day many animals had reached a state of immotility with a very high mortality rate. Many of the metamorphic changes cited above do not occur until metamorphic climax, about larval stage XX (Taylor and Kollros, 1946). Animals changing at this rate

usually do not live beyond the fifth day. The control tadpoles showed an average increase in length of about 3 mm, and advanced from stage IV to larval stage V or VI during the five day period.

B. Spectrophotometric Measurement of Deoxyribonucleic Acid

The amount of DNA extracted from the brain of each group of Rana clamitans tadpoles was determined by the Dische diphenylamine method of detection on a spectrophotometer at 100% transmittance and zero absorbance. The results are summarized in Table I.

Figures I through V show a comparison of DNA content extracted from the brains of the control and experimental tadpoles with respect to the percent transmittance and absorbance.

The control animals showed a reading of 92% transmittance and o.04 absorbance. The readings obtained for each experimental group from one through five days are presented on page 14.

TABLE I

A COMPARISON OF TADPOLES OF THE
EXPERIMENTAL GROUPS WITH THE CONTROL GROUP

Group	% Trans-mittance	% Difference from control	Absorbance	Absorbance difference
Control	92	------	0.04	------
Experimental First Day	93.5	-1.5	0.025	-0.015
Second Day	93	-1	0.03	-.01
Third Day	91.5	+ .5	0.039	+0.001
Fourth Day	90	+2	0.05	+0.01
Fifth Day	87	+5	0.06	+0.02

One Day

Tadpoles immersed in thyroxine for one day showed a transmittance of 93.5% and an absorbance of 0.025. Figure I shows a comparison of the control and experimental animals during the first day.

Two Days

This group showed a slight increase over the first day with 93% transmittance and 0.03 absorbance (See Figure II).

Three Days

The experimental animals showed a reading of 91.5% transmittance and 0.039 absorbance after three days of immersion in thyroxine (See Figure III).

Four Days

DNA extracted from brains of tadpoles after four days immersion in thyroxine, showed a reading of 90% transmittance and 0.05 absorbance (See Figure IV).

Five Days

The highest degree of absorbance was seen in animals immersed in thyroxine for five days. Transmittance was 87% and absorbance was 0.064 (See Figure V).

Although spectrophotometric measurements revealed gradual increase in DNA content in the experimental animals between one and five days, statistical analysis of the data reveals that the increase in DNA content in thyroxine-treated animals over that in the control animals is not significant.

DISCUSSION

The results of studies on DNA content in the brain of Rana clamitans larvae following thyroxine treatment have been presented.

The exact moment in which DNA synthesis occurs remains a matter of argument. The earliest observations come from Caspersson's ultraviolet work (1939) on the spermatogenesis of the grasshopper. He suggested that the synthesis of DNA occurs during the first phases of the division and reaches its maximum at metaphase. Ris, (1947) on the other hand, studied mitosis in terminal meristems of onion root tips and spermatogenesis of Chorthophaga by the photometric technique in visible light and obtained results in agreement with Caspersson's. The synthesis of DNA occurred at prophase and was complete at metaphase when the total amount of DNA of the chromosomes was twice that of a normal nucleus. But more recent work indicates that the synthesis of DNA must occur earlier. It should be pointed out that a number of authors (Lison and Pasteels, 1949; Schrader, 1950; and Swift, 1950), has shown that in meiosis, the first spermatocyte already contains twice the amount of DNA of the

diploid nuclei; all the DNA necessary for the four spermatids which will be derived from it. On the other hand, Alfert (1950) has found in primary oocytes before meiosis four times as much DNA in the pronuclei resulting from the two meiotic divisions. Swift (1950) studied the behavior of DNA in the nuclei of developing liver tissue of the 11-day mouse embryo and in the pronephros and erythrocyte nuclei of the recently hatched Amblystoma larva. He concluded that synthesis of DNA occurs during interphase before the visible stages of mitosis. Seshachar (1949) found a similar process in the division of the ciliate micronucleus.

Walker and Yates (1952) have studied by ultraviolet absorption and by the Feulgen method the DNA content of nuclei in tissue culture. They found that posttelophase nuclei contain the same amount of DNA as erythrocyte nuclei. This amount is doubled during interphase and reaches its maximum before prophase. This study upon living cells, using phase contrast film, seems to be more reliable than results from fixed and stained material. It, therefore, seems well established that the synthesis of DNA occurs during late interphase, before

prophase. Nevertheless, Pasteels and Lison (1950) working upon erythroblasts of the rat embryo, Lieberkuhn gland of the adult rat, and fibroblasts of the chick heart embryo in tissue culture have found that in these rapidly growing cells the DNA content of the nucleus does not change during prophase. At anaphase the two daughter nuclei contain half this value, but the initial value is reached again at telophase, so that the synthesis of the DNA would appear to occur in telophase and would be completed during the reconstruction of the daughter nuclei.

Although statistical analysis reveals that the increase in DNA content after five days of thyroxine treatment, was not significant, there was a gradual increase in DNA noted in the brains of the animals studied. On the basis of previous work done on the nervous system of Rana pipiens dealing with mitotic activity under the influence of thyroid hormone, it was found that there is an initial increase in mitotic rate followed by a decline after prolonged treatment (Weiss and Rossetti, 1951; Martin, 1962; Reynolds, 1966). These investigators suggest that thyroxine brings about a precocious maturation of cells resulting from mitosis, thus, there are more

interphase cells present in animals treated for five days than in those subjected to thyroid hormone for a fewer number of days. Therefore, from the results obtained from this investigation, the interphase theory on DNA synthesis seems probable.

Some workers have suggested that DNA is not constant even within the nuclei of one cell type but, rather, varies with the metabolic conditions of the cell. During development of the sea-urchin egg, Brachet (1944) reported that DNA content of nuclei, as determined by the Feulgen reaction, was low per nucleus in the early cleavage stages, becoming much higher in blastula and pluteus nuclei. Vickers (1966) found that during the development of the boll weevil the larva showed a greater amount of DNA and RNA due to the increased mitotic rate during the larva stage. The next instar, that is the pupal stage, showed a decline in the DNA and RNA content. The results of this investigation on DNA content do not concur with the findings of Vickers (1966).

Wessel (1964) reports actual division of the nucleus occurs in a zone removed from the epithelial surface, usually in cells associated with a luminal cavity.

Whether the shift is due to nuclear migration within cells or actual movement of whole cells is not certain. There are indications that at least some of the deeper lying cells retain connection with the surface via long, fiber-like processes. In any event, the products of division appear either to return to the peripheral layer for DNA synthesis or to remain centrally located where DNA synthesis is at least much less likely. It has been found by Weiss and Rossetti (1951), Martin, (1962), and Reynolds, (1966) that cell division in the tadpole brain occurs in the ependymal region of the brain. This region is very compact with numerous nuclei. If newly formed cells have to migrate to the epithelial surface to synthesize DNA, this would be a contributing factor to the results obtained in this study. Should there be variations in the DNA content of the nuclei of cells of the same type, the nuclei within the ependymal would contain smaller amounts of DNA than the cells in the peripheral area.

The treatment of tissues with various hormones has been found to have varying effects on the DNA content. Leslie (1952) added cortisone to the growth-promoting

medium of cultures of embryonic chick heart explants and found slight increases in the total DNA synthesis and in the PNA/DNA ratio. When cortisone and growth hormone were combined, the total synthesis of PNA and DNA rose as much as 75% above normal over 6 days. Growth hormones applied alone slightly inhibited cell multiplication. Mandel (1951, 1952) and Baxi (1951) reported an increase in the DNA content after administering growth hormone. The present findings support the conclusion of those authors reporting an increase in DNA content.

It is suggested that further investigations using different concentrations of thyroxine over a longer period of time be done to ascertain the effects of different concentrations of thyroxine on DNA content of the tadpole brain. It is further suggested that a concomitant study be done on the ribonucleic acid content of the tadpole brain as well.

SUMMARY

1. *Rana clamitans* tadpoles from select stages were used in order to determine the effects of thyroxine on DNA content of the tadpole brain.

2. It was found that the brain in tadpoles treated with thyroxine showed a slight increase in DNA content.

3. The increase in DNA content of the tadpole brain in experimental animals was not significant according to statistical data.

4. It was concluded that synthesis of DNA in the nucleus of dividing cells probably occurs during the interphase stage.

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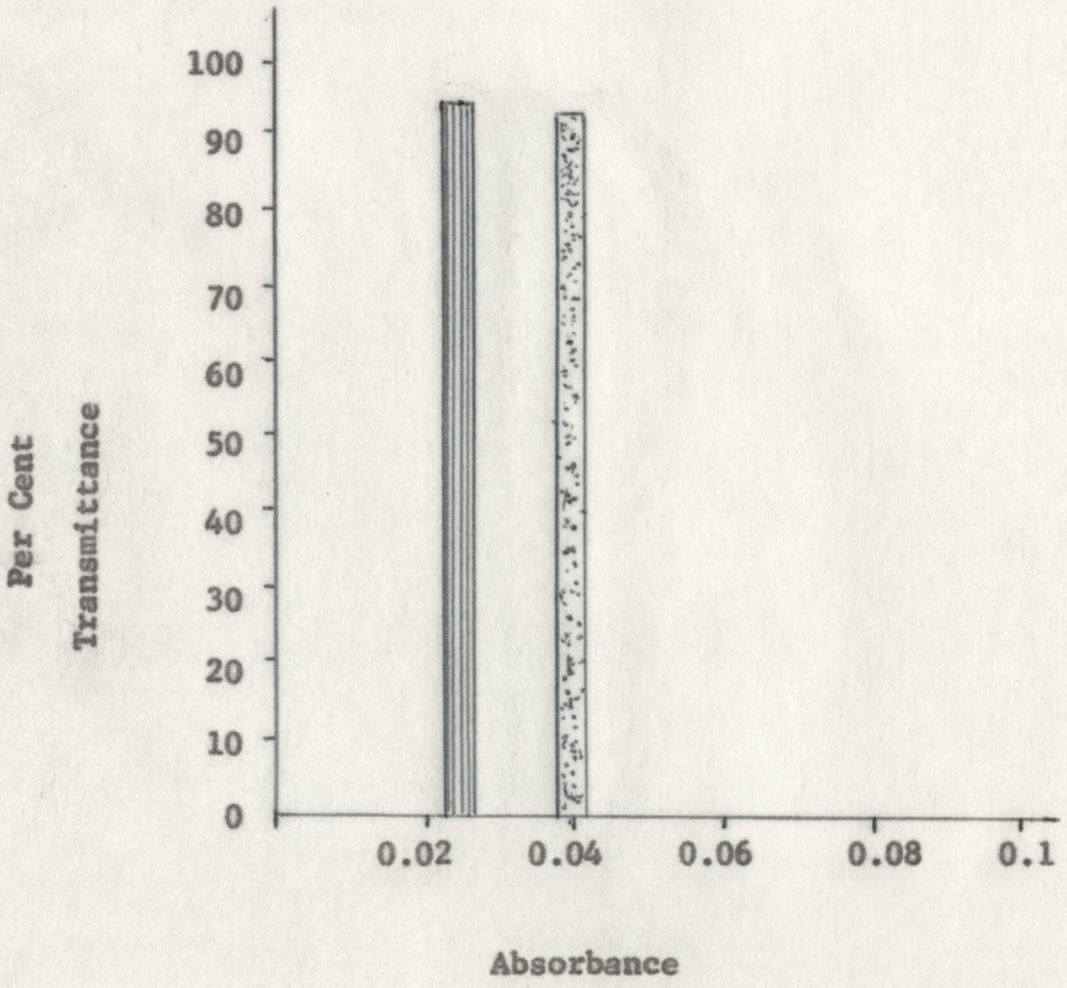
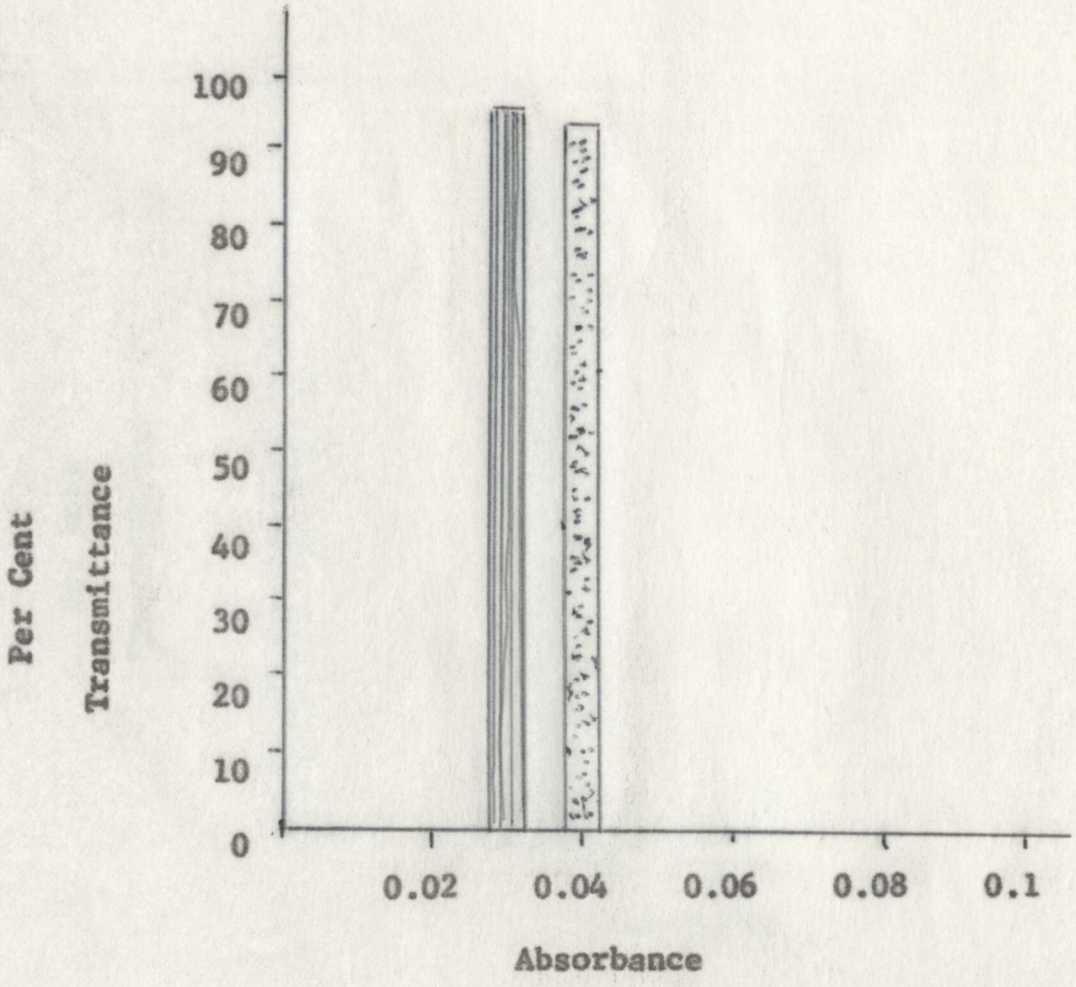
A P P E N D I X

FIGURE I



A comparison of DNA between the control and experimental animals following immersion of animals in thyroxine for one day.

FIGURE II

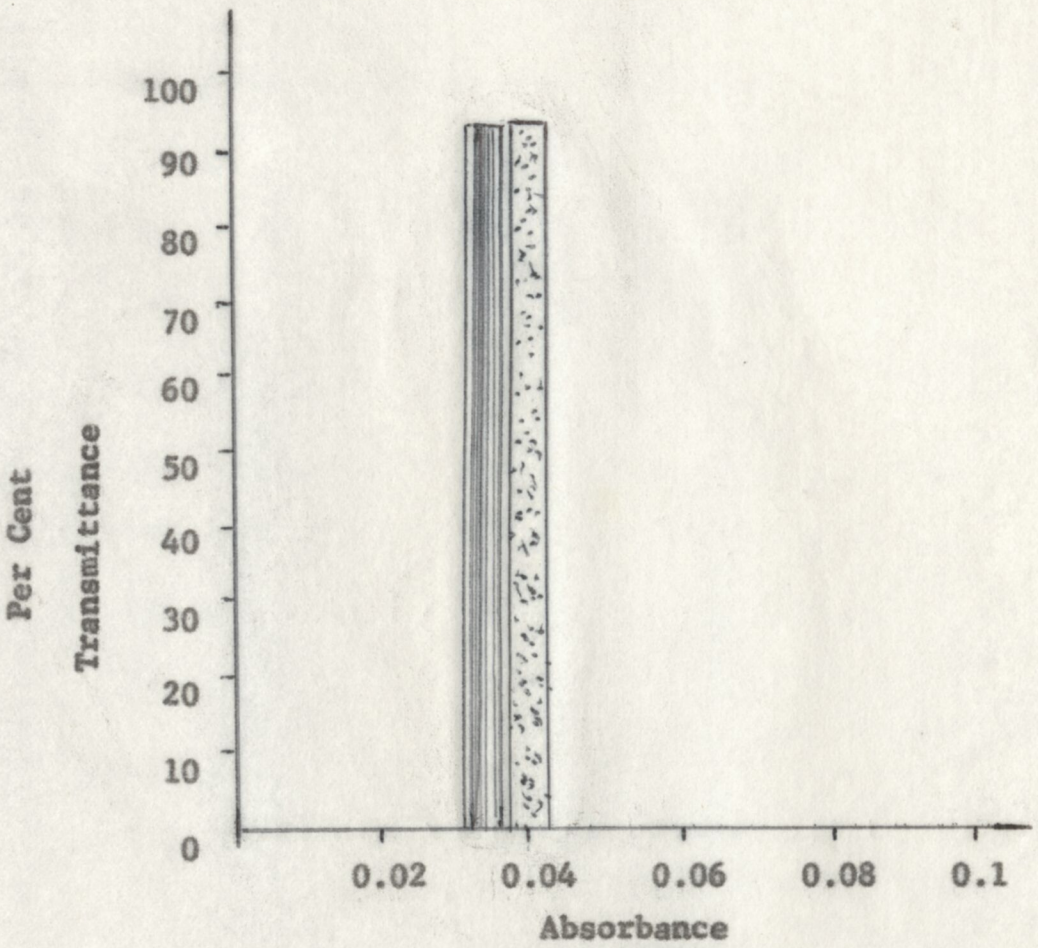


A comparison of DNA between the control and the thyroxine treated tadpoles for two days.

Experimental

Control

FIGURE III

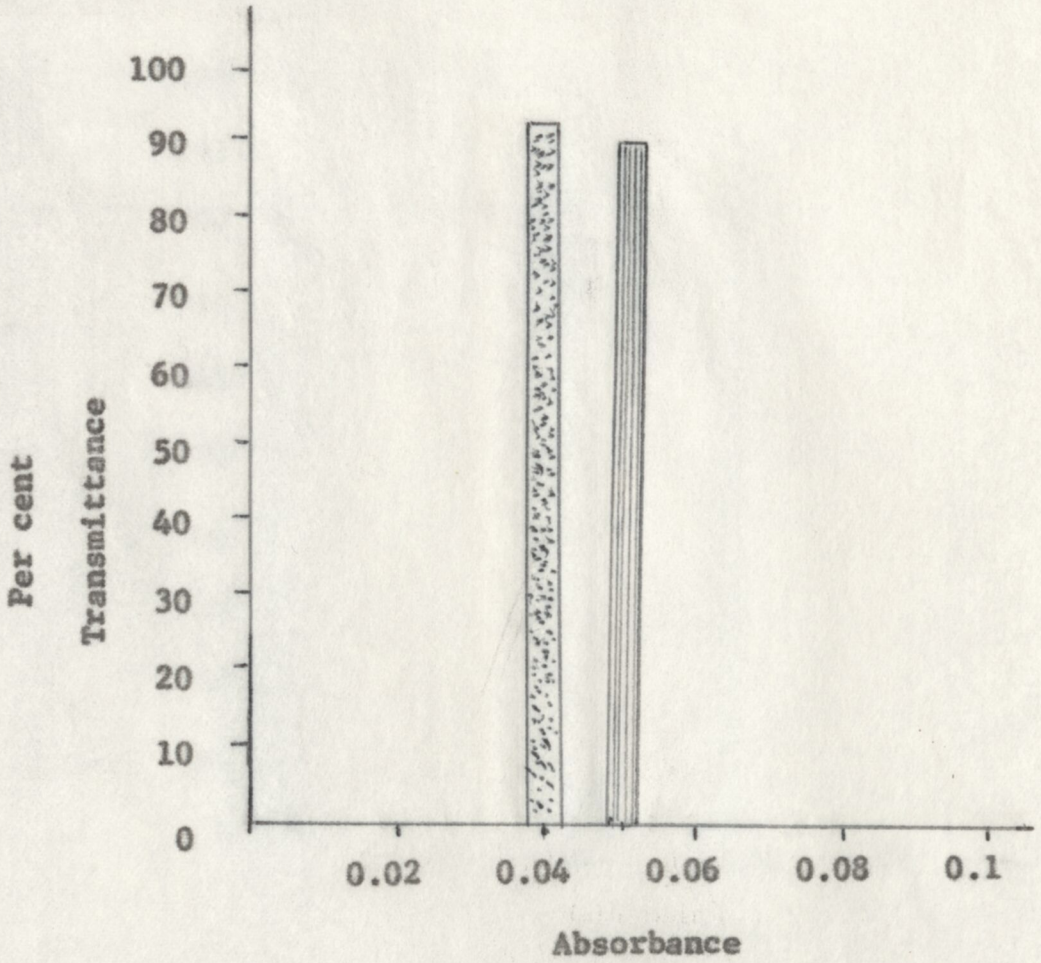


A comparison of DNA between the thyroxine treated and the control group for three days.

Experimental

Control

FIGURE IV

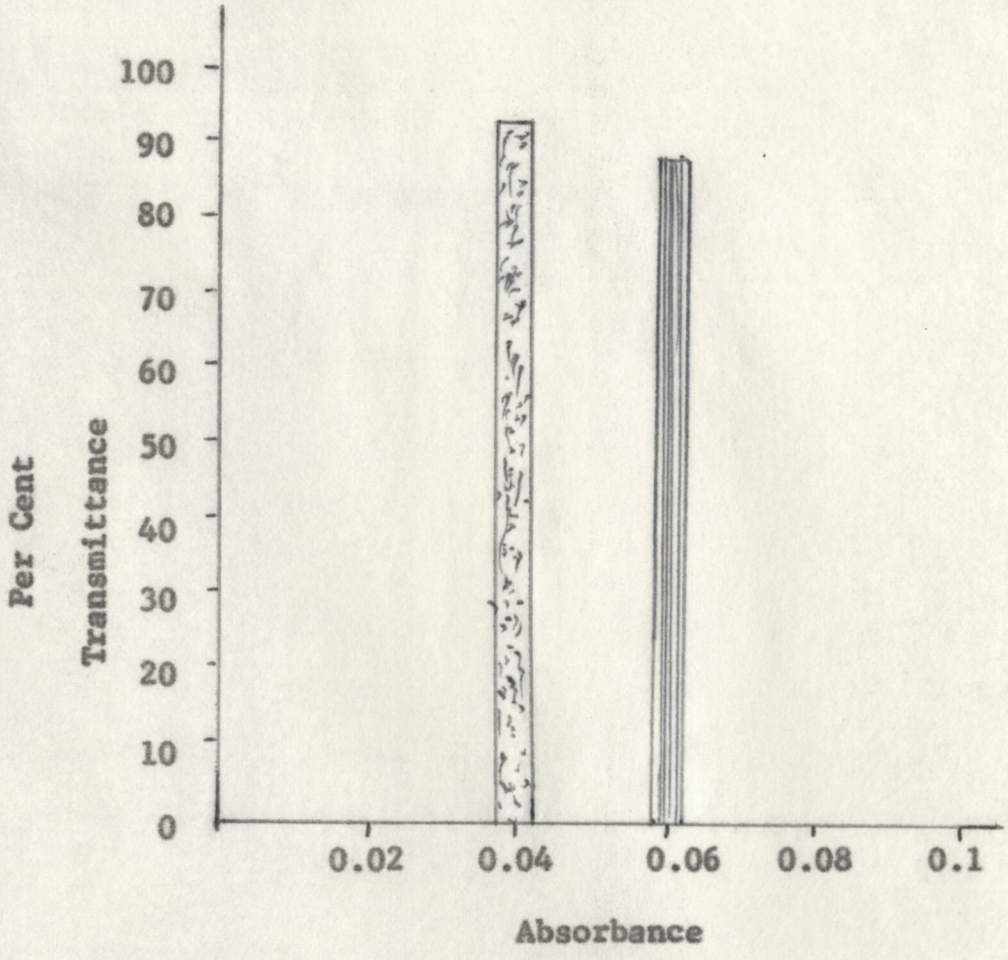


A comparison of DNA between the thyroxine treated group and control tadpoles at the end of four days.

Experimental

Control

FIGURE V



A comparison of DNA between the control and the experimental group of animals following five days of thyroxine treatment.

Experimental

Control