A STUDY OF THE EFFECTS OF LEVOTHYROXINE AND

L-TRIIODOTHYRONINE ON THE EARLY EMBRYONIC

DEVELOPMENT OF <u>BRACHYDANIO</u> <u>RERIO</u>

(HAMILTON)

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BRACHYDANIO RERIO   (HAMILTON)

By

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CHAPTER I

INTRODUCTION

Much study in the last half century has been concentrated on the
endocrine glands and their hormone secretions. Not the least of the
glands studied has been the thyroid, which plays a vital part in body
metabolism.

Through the efforts of scientists studying the chemistry of the
thyroid, (Harington and Barger, 1927; Gross and Pitt-Rivers, 1952) two
new thyroid substances have been prepared synthetically. Sodium levo-
thyroxine, an active principle of the thyroid gland, is now being pro-
duced commercially for use as a therapeutic agent for the treatment of
thyroid deficiencies. Triiodothyronine in its levorotatory form is now
being synthesized as a hormonal substance directed at thyroid activity
at the cellular level.

It was the intended purpose of the study herein reported to show
some of the effects of these drugs on developing embryos of the Zebra
fish, Brachydanio rerio (Hamilton) and to compare these effects with
those found by Gibson (1954) in a similar work with thyroxine.

Embryos of the Zebra fish were exposed to varying concentrations
(0.0001 ppm. to 10 ppm.) of levothyroxine and triiodothyronine to
determine the effects of these substances in vivo. Tests were made to
determine the effects of exposure for a limited time period versus

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continuous exposure during certain stages of development. Some 2,500

embryos were studied during the experiments.

CHAPTER II

REVIEW OF LITERATURE

Since this work is essentially a continuation of that submitted by Gibson (1950) much of the history and review of the literature on the thyroid up to that time, which is so adequately covered by Gibson, will be deleted herein in order to avoid repetition.

As early as 1882 Kocher, a German scientist, recognized that the thyroid secretes an active principle which is necessary for the maintenance of normal metabolic functions. This opened the gateway for the study of the thyroid by other scientists, and only nine years later, the first successful treatment (Murray, 1891) of hypothyroidism by use of subcutaneous injections of sheep thyroid was accomplished.

Early attempts to isolate the active principle of the thyroid were unsuccessful, as the methods used destroyed the physiologic activity of the substance. However, Baumann (1896) discovered that the thyroid gland contained iodine.

Kendall (1915) successfully isolated the hormone by alkaline hydrolysis and called it thyroxine. The method of extraction was later improved by Harington (1926) who hypothesized that the empirical formula of thyroxine was $C_{15}H_{11}O_4NI_4$. The following year, Harington and Barger, (1927), described the synthesis of thyroxine.

Harington and Randall (1929) in an attempt to account for all of the iodine in the thyroid gland, succeeded in isolating crystalline

thyroxine and diiodotyrosine from dessicated thyroid. They concluded that all of the organic iodine in the thyroid could be accounted for by the above two compounds, both of which occur naturally as L isomers. Fink and Fink (1948) isolated monoiodotyrosine and this compound was added to the list of normal thyroid components.

Recent studies indicate that these three amino acids account for more than 90 percent of the iodine in the thyroid.

Taurog and co-workers (1950) established that monoiodotyrosine is actually a normal component of the thyroid of the rat, chicken and sheep and were able to establish the percentage of monoiodotyrosine and diiodotyrosine found in the gland.

It has been indicated through radioactive studies of the specific activities of iodine, that monoiodotyrosine is the precursor of diiodotryrosine which is the precursor of thyroxine. (Council on Pharmacy and Chemistry of the American Medical Association 1942.)

In 1952, Gross and Pitt-Rivers reported the isolation of another iodine-containing compound from human plasma, which they were later able to synthesize. They called this compound $3,5,3'$-L-triiodothyronine and found it to be a normal constituent of the organic iodine fraction of the plasma. These findings were later supported by Kennard (1953). Investigations by these workers showed that this compound has a similar but more profound action than L-thyroxine, which most investigators consider to be the physiologically active form of thyroxine. L-Triiodothyronine differs from L-thyroxine in the absence of one iodine atom at the $5'$ position.

In preliminary experiments, involving thiouracil-treated mice, Gross and Pitt-Rivers (1953) obtained results which indicated that

L-triiodothyronine is three to four times as potent as L-thyroxine in goiter prevention assay. This supported previous results which were obtained by these same investigators (1952) in experiments involving human patients suffering from myxedema.

Lerman (1953) further substantiated the findings of Gross and Pitt-Rivers on the high degree of physiologic activity of L-triiodothyronine. He found the compound to be four to five times as active as L-thyroxine in human myxedemic subjects. In giving a single large dose of L-triiodothyronine, he was able to produce a rise in metabolism and reach the peak of metabolism sooner than with an equivalent amount of L-thyroxine. These results indicated that the former may be formed by deiodination of the latter and would add a further step in the biological synthesis of the thyroid hormone.

Up to this time, the stages in the formation of the thyroid hormone were believed to be:

1. Oxidation of iodide to iodine.
2. Iodination of tyrosine to diiodotyrosine.
3. Coupling of two molecules of diiodotyrosine to form thyroxine.

Lerman put forth the premise that, since L-triiodothyronine has been shown to be more active than L-thyroxine, it is only natural to assume that L-triiodothyronine is the active hormone.

Support is lent to the argument by the fact that L-triiodothyronine is rapidly cleared from the blood after prolonged administration and that single doses fail to raise the level of serum-protein bound-iodine appreciably. This suggests that triiodothyronine leaves the extra-cellular spaces rapidly to enter the cells, which accounts for the rapid rise in metabolism encountered. The rapid drop in basal metabolism after withdrawal of injections of the substance suggests that it disappears rapidly

from the intracellular fluids also. The added fact that blood and tissue

normally contain only small amounts of this material seems to further

support the concept that L-triiodothyronine is the active thyroid hormone.

Lerman's theory, therefore, on the normal thyroid hormone equilibrium

may be stated thusly. Thyroglobulin is stored in the thyroid gland and

serves as a source of thyroxine. As the need for thyroxine arises, thyro-

globulin is hydrolyzed to thyroxine and passed through the thyroid folli-

cle into the bloodstream where it constitutes the major portion of the

organic iodine of the blood plasma. It is important to point out here

that the protein-bound iodine content of the blood is due to thyroxine

and not to triiodothyronine, the former merely serving as a reservoir

for the latter. When thyroxine penetrates the cells of the end organ,

it is converted to triiodothyronine by deiodination at the 5' position

and utilized by the cells.

According to Lerman's theory, the rate of conversion of thyroxine

to triiodothyronine depends on two variables. The first of these is that

the permeability of the cell membrane to thyroxine may be low. The sec-

ond is that the speed of degradation of thyroxine to triiodothyronine

may be slow. These variations would tend to explain the difference in

the speed of action and decay between the two substances.

Rawson and co-workers (1954) were able to show a prompt but short

lived increase in basal metabolism and in the excretion of nitrogen,

phosphorous and creatinine following a 1 mg. dose of triiodothyronine.

An identical amount of thyroxine produced similar, although slower and

more prolonged effects. These investigators found that similar quanti-

ties of diiodotyrosine had no effect on B.M.R. or on total metabolism,

but that (disregarding the time of action) there were no qualitative,

and on an equimolecular basis, no quantitative differences between L-thyroxine and L-triiodothyronine.

Another possible explanation for the more rapid action of triiodothyronine was advanced by Deiss and Albright (1953) in their study on the serum-protein binding power of thyroxine and triiodothyronine. In in vitro studies, radioactive amino acids were used to measure the attraction of both these substances to serum proteins. Their findings indicated that, although both were shown to be bound by alpha globulin, thyroxine is more firmly bound and is not as easily displaced as triiodothyronine and that thyroxine will displace triiodothyronine from the linkage. Therefore, it is possible that because thyroxine is present in greater amounts in the serum than is triiodothyronine, thyroxine blocks the binding of triiodothyronine with this protein or replaces it once it is bound. This could account for the greater diffusibility of triiodothyronine and its more rapid disappearance from the serum.

Gemmill (1953) in comparing the activity of thyroxine and 3,5,3' triiodothyronine in rat heart homogenates found that both compounds had a comparable activity on a molar basis in inhibiting the cupric ion-catalyzed oxidation of ascorbic acid and in stimulating the oxidation of succinate. Studies by Newcomer (1957) on the effects of the same two compounds on thiouracil-treated chickens resulted in finding that both had similar effects on feather length, heart rate, suffocation time, weight of the thyroid, rectal temperature and oxygen consumption, but that thyroxine had a more potent anti-goitrogenic effect and triiodothyronine appeared to be more potent in elevating rectal temperature. Weiss (1956) however, using liver slices from the frog, guinea pig, turtle and man, did not produce an increased oxygen consumption with either L-thyroxine or triiodothyronine. Sholem (1957) tried to treat induced

fetal goiters in normal, pregnant guinea pigs by parenteral administration of triiodothyronine in graded doses, but was unable to detect any response as it was rapidly degraded and the placenta restricted its entry into the fetus.

Heming, et al. (1953) found L-triiodothyronine to be about 3.5 times as active as L-thyroxine on a molar basis, in anti-goitrogenic tests on thyroidectomized rats. The results obtained by Heming are consistent with those obtained by Gross and Pitt-Rivers (1952, 1953) which indicated that L-triiodothyronine is three to five times as active as L-thyroxine in the goiter-prevention assay on rats. Gemmill and Chalmers (1956) were also able to show a greater rise in metabolism among thyroidectomized rats by using an equal amount of thyroxine.

Gross, Pitt-Rivers, and Trotter (1952) and, later, Selenkow and Asper (1955) in treating myxedema in human patients, found L-triiodothyronine to be at least twice as potent calorigenically as L-thyroxine. The above results, combined with the fact that triiodothyronine is present in normal plasma, would suggest that this may be the compound directly responsible for the peripheral action of the thyroid gland.

The concentration and binding power of both named thyroid compounds in muscle tissue, was demonstrated by Hogness and co-workers (1957) by the use of rat hemidiaphragms immersed in I^{131}-labeled compounds. Their results showed that the amount of the hormone bound by the muscle was linearly related to the time of incubation of all concentrations. There was also a direct linear relationship between the amount of thyroxine available and the amount bound to the tissue. It was further shown that triiodothyronine is specifically bound to rat muscle and that the binding takes place at a much more rapid rate than that in the case of thyroxine.

Weinstein, Edwin and Allen (1957) collaborating in a similar work sug-
gested that thyroxine is not the active form of the thyroid hormone,
that it competitively inhibits the active form, and is itself converted
to the active form by peripheral tissues.

The distribution and metabolism of thyroxine and triiodothyronine
was studied by Keating and Albert (1953) and Van Arsdel and co-workers
(1954). The former reported that in rats treated with radioactive thy-
roid compounds and killed at intervals of fifteen days, thyroxine was
concentrated more, and retained more selectively by the liver. They
also reported gastric mucosal secretion and urinary excretion of la-
beled I^{131}. Thyrodial uptake of nonhormonal iodine was increased when
triiodothyronine was administered, suggesting that it was more rapidly
deiodinated, thereby concurring with Gross and Pitt-Rivers that tri-
iodothyronine is perhaps the tissue form of the thyroid hormone.

Van Arsdel's group sacrificed their experimental animals at shorter
intervals and stated that "at each interval studied, the concentration of
triiodothyronine in plasma was much lower than that of thyroxine and some-
what lower in the liver, except for an unexplained, but significantly
higher concentration five minutes following administration." They were
also able to present evidence that triiodothyronine, for the first twelve
hours, was present in higher concentration in the kidneys. It then drop-
ped to a level below that of thyroxine. The concentration of both hor-
mones was relatively low in skeletal muscles, but the level of triiodo-
thyronine again was significantly higher than that of thyroxine during
the first twelve hours.

Studies on the effects of the two thyroid substances in question,
on euthyroid human subjects, were conducted by Starr and Liebhold-Schueck

(1953) and Sterling, Lashof and Man (1954). The former authors were able to produce a decrease of 77 percent in I^{131} uptake by the thyroid with as little as eight micrograms of L-triiodothyronine and in general, found this compound to be from 5 to 60 times more active than L-thyroxine in depressing I^{131} uptake. The latter authors disclosed that the mean half-time of thyroxine turnover in a small group of subjects was 6.7 days in contrast to a 2.7-day mean half-time in L-triiodothyronine turnover.

The interesting problem of the in-vitro conversion of thyroxine to triiodothyronine has been attacked by several investigators. Albright, Larsen and Tust (1954) investigated the phenomenon through the use of kidney slices immersed in I^{131} labeled thyroxine. Their results support-ed the concept that 3,5,3' L-triiodothyronine is derived from thyroxine by deiodination at the 5' position in extrathyroidal tissues and has led to the theory that injected triiodothyronine is absorbed directly by the target cells, doing away with the latent period required for the body to remove the 5' atom. They also believed that the reservoir of unmetaboli-zed exogenous thyroid hormone should also be diminished thereby providing a more rapid cut off of effects where the drug is withdrawn.

Findings published in 1955 by Pitt-Rivers and Rapp, and Larsen and co-workers also indicated that it was possible for extrathyroidal deiodi-nation of thyroxine to occur in the formation of triiodothyronine. Larsen et al. further pointed out that "thyroxine, unlike triiodothyronine, is bound to a serum alpha-globulin which probably alters its diffusibility. This may in part account for the observed differences in the speed of action." Tata and co-workers (1957) were able to form small amounts of triiodothyronine in brain tissues as a product of the incubation of thy-roxine.

Roche and collaborators (1956) stated that both thyroid compounds are metabolized in two principal ways, dehalogenation and oxidative deamination. This in turn leads to the iodothyropyruvic acid, present in bile and urine in humans. The authors were able to detect the presence of 3,5,3' triiodothyracetic acid (TRITA) and L-3,3' diiodothyronine in the kidney of rats injected with labeled L-3,5,3' triiodothyronine. The presence of L-3,3' diiodothyronine shows the partial deiodination of the triiodinated hormone and that of 3,5,3' TRITA proves the oxidative degradation of the latter (oxidative deaminzation followed by decarboxylation of the iodothyropyruvic acid formed). Two aerobic, partially heat-resistant systems capable of deiodinating L-thyroxine and triiodo-L-thyronine were earlier reported by Spratt and Maclagan (1955) in homogenates of rat tissues to further support the contention that 3,5,3' triiodothyronine is the active form of the thyroid hormone.

In 1958, Frieden and Mathews found that triiodothyronine caused no marked effect on body weight or liver protein in tadpoles under metamorphosis but did cause a significant increase in the proportion of the liver weight in relation to the total body weight. They stated that this represents a triiodothyronine induced synthesis of liver protein. Shellbarger and Godwin had found earlier (1954) that tadpoles immersed continually for a period of 10 days with either 40 micrograms of thyroxine or 10 micrograms of triiodothyronine per 500 cc solution showed a decreased body length. However, no marked decrease in body length was observed in tadpoles exposed to lower concentrations until after twenty days of continuous exposure. The authors considered these results to be evidence that both compounds hasten metamorphosis. Triiodothyronine was found to be approximately 3.8 times more potent than thyroxine in

these experiments which, stated the authors, added merit to Göss and Pitt-Rivers suggestion that "triiodothyronine must receive further consideration in the question of what is the thyroid hormone, not only in mammals, but in the lower vertebrates as well."

CHAPTER III

MATERIALS AND METHODS

The techniques used in the experimental work were basically those
used by Gibson (1954) and subsequently described by Jones and Huffman
(1959).

Embryos of the Zebra fish, _Brachydanio rerio_, were used as these
fish are easily raised, lay eggs in large numbers, and the eggs can be
easily treated with chemicals by dissolving the chemicals in the water
in which the eggs are kept.

Zebra fish are stimulated to spawn by light following a period of
darkness. Therefore, the batteries of tanks containing adult male and
female fish were kept in darkness until the appointed time for egg col-
lections each day. At this time, the fish were exposed to light and
spawning and fertilization of the eggs occurred. In order to prevent
adult fish from eating the eggs, marbles were kept in the bottom of the
tank to provide a refuge until the eggs were collected.

Collection of the eggs was conducted by siphoning the debris from
the bottom of the fish tanks and catching the eggs and other materials
in a tea strainer. The eggs were then cleaned and sorted for use. For
the sake of uniformity, only normal, healthy eggs of four to sixty-four
cell stages were selected. These eggs were separated into groups of
twenty-five each, which constituted an experimental culture unit.

The drugs used in the experiments were diluted in various concen-

trations in aged, aerated, tap water. Each culture of embryos was kept in 50 cc. of culture media. The treated and control specimens were incubated at 80° F. $\pm$ 1°. Treated specimens were either constantly exposed for 144 hours to the materials being tested or were exposed for 30 minutes during the very early cleavage stages of development, washed, and then allowed to continue development in untreated, aged, aerated, tap water.

The two drugs used were sodium levothyroxine obtained from Travenol Laboratories and L-triiodothyronine obtained from Smith, Kline and French Laboratories. The concentrations of levothyroxine used were from 20 to 0.0001 ppm. Triiodothyronine was used in concentrations of 1 to 0.0001 ppm.

Levothyroxine easily dissolved in water but triiodothyronine proved to be quite insoluble. However, H. A. Klusek of Smith, Kline and French Laboratories informed me of a method which rendered triiodothyronine soluble for my purposes.

Four mg. of triiodothyronine powder was weighed on an analytical balance and dissolved in 1.3 cc. of 0.01N sodium hydroxide. Ninety mg. of sodium chloride was then added and the final volume brought up to 10 cc. by the addition of cold, boiled, freshly distilled water. The solution was then autoclaved at ten pounds pressure for thirty minutes. At this point the solution was clear, without any undissolved particles, and had a pH of 8.0 - 8.5. Each 1/10 cc. solution contained 40 micrograms of triiodothyronine.

In order to determine the effects on embryos of the substances added to triiodothyronine, these chemicals were tested on four to sixty-four cell embryos. It was found that concentrations contained in one

ppm. and less of the triiodothyronine mixture had no obvious effects on the embryos. Therefore, only these concentrations were used in the experiments.

Each culture was examined at twenty-four hour intervals by use of a wide-field dissecting microscope. As an aid to distinguishing differences in pigmentation, each culture was viewed against a white background. In addition to pigmentation, the following were recorded for each culture:

1. The number of embryos that had died during each twenty-four hour period of exposure.

2. The number of embryos that had hatched.

3. The number of abnormalities that appeared among the embryos. (Each abnormal embryo was carefully examined and a record of the type of abnormality was made.)

4. Development of pigmentation.

5. General activity of the embryos in each culture.

At the end of each 144 hours of observation, the remaining embryos were killed and pickled in Bouin's solution.

A gross comparison of pigmentation was made by examining and comparing the overall amount of pigmentation in a treated culture with that of a control culture. A numerical designation of 0 was given those cultures in which pigmentation was similar to that in a control culture at an identical stage of development. Treated cultures which showed half the pigmentation of the controls at a similar stage were designated as -1, and those in which pigmentation was completely absent were designated as -2 with other differences being given corresponding values.

In order to obtain a more accurate picture of the effects of levo-

thyroxine and triiodothyronine on developing melanophores, direct micro-
scopic counts were made on preserved specimens. Since a total melanophore
count on each specimen would prove cumbersome, it was decided to count
the melanophores on two separated areas on the dorsal surface of the head.
These areas are designated as regions I and II in Figure 1.

Region I extended from the lips caudally to a region midway between
the eyes, on the top center of the head. Region II extended from the at-
tachment of the rudimentary fins, cephalically to where it approached
Region I. Between the two regions was a distinct line of demarcation
which clearly separated them. This natural separation which delimited
Regions I and II appeared as a narrow tract at the top center of the head
which fanned out on either side of the head as it approached the eyes.
Melanophores which fell within this area were excluded from our counts.
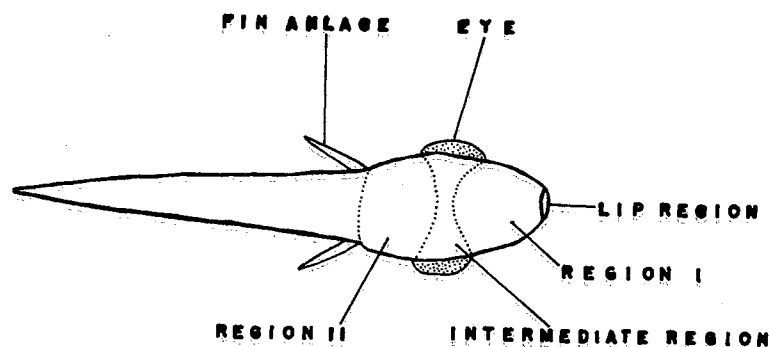
Figure 1. Schematic drawing showing where
          melanophore counts were made in
          the cephalic region.

CHAPTER IV

EXPERIMENTAL DATA

Effects of Constant Exposure

Levothyroxine

Pigmentation

One of the most striking phenomena that could be linked to the effects of levothyroxine in this study was its effect on pigmentation. Because this effect was so noticeable, especially in the most minute dilutions where there is apparently no other effect of the drug, much time was devoted to the study of this factor.

Melanophores were found to be sufficiently well developed in embryos of approximately 48 hours of age that it was possible to recognize differences in control and treated specimens. At this stage, on gross comparison of embryos, it was seen that dilutions as low as 0.0001 ppm. of levothyroxine had caused an inhibitive effect on pigmentation of the embryos. Zebra fish eggs constantly exposed to this dilution of levothyroxine began to show a diminished pigmentation at 72 hours, only 48 hours after the melanophores first began to visibly appear. Observations at this stage of development indicated that pigmentation had been decreased approximately only 1/8 as compared to normal pigmentation at this stage, but considering the minuteness of the levothyroxine to which the embryos were being exposed, it was felt that this was of great

significance. As can be seen in Table I below, further observations confirmed these findings.

TABLE I

EFFECTS ON GROSS PIGMENTATION PER 24 HOURS
UNDER CONDITIONS OF CONSTANT EXPOSURE

Concentration of Levothyroxine ppm.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	144 Hrs.
Controls	0.	0.	0.	0.	0.
0.0001	0.	-0.25	-0.50	-0.50	-0.50
0.001	0.	-0.75	-0.75	-1.25	-1.25
0.01	-0.25	-1.0	-1.0	-1.25	-1.25
0.1	-1.0	-1.5	-1.0	-1.0	-1.0
1.0	-1.0	-1.5	-1.5	-1.5	-1.75
5.0	-0.75	-1.5	-1.75	-1.75	
10.0	-0.75	-1.5	-1.75		
20.0	-0.75	-1.5	All died		

  0 = normal
-1.0 = ½ normal density
-2.0 = No pigmentation
Values given are visual estimates of entire cultures, not individuals

Individual melanophore counts were made as an aid in establishing whether decreased pigmentation was caused by decreased melanin production or failure of melanophores to develop or both. Upon examining and comparing the size of individual melanophores it was discovered that embryos which had been constantly exposed to 1 ppm. and more of levo-

thyroxine exhibited melanophores which were very much contracted. In some cases the melanophores were contracted to such an extent that it was difficult to distinguish them as melanophores without careful study.

Further observations showed that levothyroxine had an effect not only on the size of melanophores, but also on their number. Individual melanophore counts made in Regions I and II showed a decreased average number of melanophores in all concentrations tested. This was particularly pronounced in Region I. In this area, the lowest concentration of the drug used (0.0001 ppm.) showed approximately only one-half the number of melanophores in comparison to controls. In one of the highest concentration tested (10 ppm.) the number of melanophores was decreased to one-sixth the normal number.

In Region II, the decrease in the number of melanophores was not as great although they were distinctly fewer in number. Melanophores of embryos immersed in concentrations of 0.0001 ppm. of levothyroxine showed only a slight decrease in number while those embryos kept in solutions of 10 ppm. developed slightly more than half the normal number of melanophores. The tables below illustrate the results obtained in making individual melanophore counts in Regions I and II.

In cultures containing higher concentrations of levothyroxine, where the melanophores were greatly contracted, it is possible that some may have been contracted to such an extent that they were overlooked in the counts. On the other hand, in lower concentrations, melanophores were well expanded and sometimes merged with each other. This merging sometimes caused difficulties in getting an accurate count as it was not always possible to determine the exact number of melanophores which may have merged in each instance. However, great care was taken in making

TABLE II

MELANOPHORE COUNTS ON EMBRYOS CONSTANTLY EXPOSED TO LEVOTHYROXINE
REGION I

Concentration of Levothyroxine ppm.	Number of Melanophores								Average Number	Melanophores
Control	11,	14,	12,	18,	10,	9,	9,	11	11.8	Well Expanded
0.0001	2,	5,	4,	12,	5,	8,	5,	3	5.5	Moderately Expanded
0.001	3,	1,	3,	0,	0,	3,	4,	2	2.0	Well Defined
0.01	3,	2,	4,	0,	1,	4,	6,	2	2.8	Moderately Expanded
1.0	3,	1,	5,	5,	3,	2			3.3	Very Much Contracted
10.0	1,	0,	4,	3					2.0	Very Much contracted

REGION II

Concentration of Levothyroxine ppm.	Number of Melanophores								Average Number	Melanophores
Control	38,	46,	47,	44,	45,	48,	38,	35	42.6	Well Expanded
0.0001	44,	29,	33,	32,	47,	38,	25,	48	37.0	Moderately Expanded
0.001	44,	48,	24,	32,	27,	39,	22,	41	34.6	Moderately Expanded
0.01	33,	32,	30,	35,	24,	27,	43,	30	31.8	Moderately Expanded
1.0	25,	17,	26,	28,	24,	24			24.0	Very Much Contracted
10.0	24,	23,	34,	27					27.0	Very Much Contracted

counts and the writer feels that the figures given are a very close representation of the actual number.

Effects on Development

Many of the levothyroxine-treated specimens exhibited abnormalities in both posture and morphology. A morphological characteristic regularly encountered was lateral curvature of the spine resulting in the affected individuals assuming a crescent-like posture which they seemed incapable of correcting. Even when stimulated to movement, these individuals retained this shape upon actively moving away from the source of stimulus. Although this phenomenon was found quite commonly among levothyroxine-treated specimens, none of the control embryos in any of the experiments demonstrated it. (See figures in Plate I.)

Acephaly, tail deformities and cyclops were also observed. However, these types of abnormalities occurred rather infrequently so that it would be difficult to pin-point their exact cause. The number and types of abnormalities encountered with constant exposures of levothyroxine are shown in Table III.

It can be seen from this table that levothyroxine in dilutions as low as 1 ppm. caused very definite reactions. It either killed the exposed embryos or had caused abnormalities in their development during 144 hours of treatment. As concentrations were increased over this amount, a larger number of abnormalities occurred during the earlier periods of observation.

Fatalities also increased during the early stages so that all embryos exposed to levothyroxine concentrations of 10 and 20 ppm. died within 96 hours.

Although daily routine observations were made on the effects of levothyroxine on the hatching of the treated eggs, it was not possible to arrive at any specificity attributable to the drug. In several cases, it

TABLE III

DATA RELATING TO EMBRYOS CONSTANTLY EXPOSED TO LEVOTHYROXINE

Concentration of Levothyroxine ppm.	No. of Embryos Treated	No. of Embryos Dead by 144 Hrs.	No. of Abnormal Embryos by 144 Hrs.	Types of Abnormalities Produced	No. of Live Embryos Within the Chorion at 144 Hrs. (Unhatched)
Controls	350	21 (6%)	5 (1.4%)	3 club shaped tails 1 milky protoplasm 1 pigment retardation	6 (1.7%)
0.0001	50	4 (8%)	1 (2%)	Twisted body	0
0.001	50	6 (12%)	0		1 (2%)
0.01	75	6 (8%)	5 (6.6%)	Twisted bodies 1 acephalic	1 (1.3%)
0.1	50	9 (18%)	9 (18%)	No record	1 (2%)
1.0 (a)	50	31 (62%)	19 (38%) All dead or abnormal at 144 hrs.	Twisted bodies Some tail deformities	5 (10%)
1.0 (b)	50	8 (16%) by 96 hrs.	0		23 by 96 hrs.
1.0 (c)	25	2 (8%) by 120 hrs.	All dead or abnormal by 120 hrs.	Twisted bodies	0
5.0	50	5 (10%) by 96 hrs.	35 (70%) by 96 hrs.	Twisted bodies	24 by 96 hrs.
10.0	47	47 (100%) by 96 hrs.	24 (5%) by 72 hrs.	1 was abnormal mass growth of cells 1 cyclops, twisted bodies	0 by 96 hrs.
20.0	50	All dead by 96. hrs	All abnormal by 72 hrs.	Twisted bodies	

was shown that some treated specimens failed to emerge from their chorions by the end of 144 hours, although in most cases hatching had been completed by 96 - 120 hours. Owing to the fact that individual control-embryos sometimes exhibited the same delay in hatching, it cannot be said that delayed hatching was caused by levothyroxine. However, it is interesting to note that levothyronine at 1 ppm. showed almost a 10 percent unhatched embryo rate. This might possibly be attributed to a toxic effect of the drug on the embryos. The embryos which remained in the chorion at the end of 144 hours were, in each case, lethargic and apparently unable to rupture the membranes to free themselves.

L-Triiodothyronine

Pigmentation

Studies concerning the effects on embryos of constant exposure to various levels of L-Triiodothyronine showed somewhat similar results to those obtained in the work with levothyroxine. Dilutions of 0.0001 ppm. of triiodothyronine also affected pigmentation. However, pigmentation did not seem to be affected as much in the lowest ranges of the dilutions when a gross comparison of pigmentation was made. Embryos which were exposed to 0.0001 and 0.001 ppm. showed a mild effect on the inhibition of pigmentation. Although pigmentation was definitely reduced at these levels, the reduction was not as pronounced as that found in specimens exposed to similar dilutions of levothyroxine.

In the solutions containing higher amounts of the drug, in most cases, both levothyroxine and triiodothyronine held similar control over pigmentation. Table IV below indicates results obtained in making a gross comparison of the pigmentation of constantly exposed L-triiodothyronine-treated specimens with the controls.

TABLE IV

EFFECTS ON GROSS PIGMENTATION PER 24 HOURS
UNDER CONDITIONS OF CONSTANT EXPOSURE

Concentration of L-triiodothy- ronine ppm.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	144 Hrs.
Controls	0	0	0	0	0
0.0001	0	0	0	-0.25	-0.25
0.001	0	-0.25	-0.50	-0.50	-0.50
0.01	-0.25	-1.0	-1.0	-1.25	-1.25
0.1	-0.75	-1.25	-1.50	-1.50	-1.50
1.0	-0.75	-1.25	-1.25	-1.50	-1.50

0 = Normal
-1.0 = ½ normal density
-2.0 = No pigmentation
Values given are visual estimates of entire cultures, not individuals

When individual melanophore counts were made in Regions I and II the
results again indicated that reduction in pigmentation was caused mainly
by a decreased melanophore number accompanied by contraction of the mel-
anophores which had developed. The results of the melanophore counts
may be seen by consulting Table V.

Effects on Development

The number and types of abnormalities encountered in the constant
exposure experiments with triiodothyronine are recorded in Table VI.
Here again it can be seen that the greatest abnormality in development
which occurred was that of the embryos assuming a crescent-like pos-
ture. See Figure 5 Plate I. This reaction was identical to that pro-
duced by embryos in response to levothyroxine. The individuals

TABLE V

MELANOPHORE COUNTS ON EMBRYOS CONSTANTLY EXPOSED TO L-TRIIODOTHYRONINE
REGION I

Concentration of L-triiodothy-ronine ppm.	Number of Melanophores								Average Number	Melanophores
Control	11,	14,	12,	18,	10,	9,	9,	11	11.8	Well Expanded
0.0001	2,	2,	4,	3,	7,	5,	3,	1	3.4	Moderately Expanded
0.001	3,	4,	0,	3,	4,	0,	2,	3	2.4	Moderately Expanded
0.01	2,	3,	1,	4,	3,	11,	5,	3	4.0	Moderately Expanded
0.1	0,	0,	4,	2,	1,	1,	4,	3	1.9	Very Much Contracted
1.0	3,	5,	3,	7,	1,	6,	0,	8	4.1	Completely Contracted

REGION II

Concentration of L-triiodothy-ronine ppm.	Number Melanophores								Average Number	Melanophores
Control	38,	46,	47,	44,	45,	48,	38,	35	42.6	Well Expanded
0.0001	29,	26,	19,	25,	23,	20,	27,	15	23.0	Moderately Expanded
0.001	17,	14,	26,	23,	26,	20,	22,	20	21.0	Moderately Expanded
0.01	34,	36,	37,	46,	33,	34,	42,	44	38.2	Moderately Expanded
0.1	26,	6,	32,	30,	25,	27,	28,	25	24.9	Much Contracted
1.0	32,	43,	23,	23,	15,	31,	21,	22	28.3	Completely Contracted

TABLE VI

DATA RELATING TO EMBRYOS CONSTANTLY EXPOSED TO L-TRIIODOTHYRONINE

Concentration of L-Triiodo-thyronine ppm.	No. of Embryos Treated	No. of Embryos Dead by 144 Hrs.	No. of Abnormal Embryos by 144 Hrs.	Types of Abnormalities Produced	No. of Live Embryos Within the Chorion at 144 Hrs.
Controls	350	21 (6%)	5 (1.4%)	3 club shaped tails 1 milky protoplasm 1 pigment retardation	6 (1.7%)
0.0001	50	3 (6%)	0		0
0.001	50	4 (8%)	5 (10%)	Twisted bodies	0
0.01	75	25 (33 1/3%)	31 (41%)	Twisted bodies	4 (5.3%)
0.1	50	7 (14%) all dead or ab-normal	43 (86%)	1 acephalic all have twisted bodies	6 (12%)
1.0	50	27 (54%)	28 (56%)	Twisted bodies	2 (4%)

demonstrating this abnormal posture, as before, were incapable of correcting it, even upon stimulation.

The occurrence of an acephalic freak is interesting to note here, in view of the fact that we also encountered a similar case in our work with levothyroxine.

Effects of 30-Minute Exposure

Levothyroxine

Pigmentation

The work on limited exposure with levothyroxine presented some challenging results. To begin with, a noticeable decrease in pigmentation was encountered in concentrations as low as 0.1 ppm. even though the young eggs (8 - 64 cells) were exposed to this dilution for only this short period of time so early in their development. As soon as melanophores began to make their appearance (approximately 48 hours of development) a light decrease in pigmentation was noted at this dilution on gross comparison. This decrease amounted to only about 1/8 less than normal pigmentation at 48 hours, but at a stage 24 hours later, had decreased to 1/4 less than normal with no further lessening in pigmentation occurring for the remainder of the 144-hour observation period.

Concentrations greater than 0.1 ppm. showed, as would be expected, a further decrease, which is depicted in Table VII.

Individual melanophore counts indicated, as before, that levothyroxine caused a decrease in the normal amount of pigment by inhibiting melanophore genesis and by causing those melanophores which had developed, to contract. Melanophore counts revealed that in both Regions I and II, the decrease in melanophore numbers is, in itself, not enough to

to cause a noticeable differentiation when compared to untreated specimens. However, the fact that the melanophores were in a semi-contracted state makes it possible for the differentiation to be made.

TABLE VII

EFFECTS ON GROSS PIGMENTATION PER 24 HOURS
UNDER CONDITIONS OF 30-MINUTE EXPOSURE

Concentration of Levothyroxine ppm.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	144 Hrs.
0.0001	0	0	0	0	0
0.001	0	0	0	0	0
0.01	0	0	0	0	0
0.1	-0.25	-0.50	-0.50	-0.50	-0.50
1.0	-0.5	-1.0	-1.0	-1.0	-1.0
10.0	-0.75	-1.25	-1.25	-1.25	

0 = Normal
-1.0 = ½ normal density
-2.0 = No pigmentation
Values given are visual estimates of entire cultures, not individuals

In concentrations greater than 0.1 ppm. a very definite decrease in melanophore numbers was seen, especially in Region I, accompanied by an even greater contraction. This presents a clearer picture of levothyroxine's pigmentation inhibiting powers. The results of the melanophore counts which we made are shown in Table VIII.

The above results were impressive in that, in Region I nonproduction and contraction of melanophores seemed to be affected equally.

TABLE VIII

MELANOPHORE COUNTS ON EMBRYOS EXPOSED TO LEVOTHYROXINE FOR 30 MINUTES
REGION I

Concentration of Levothyroxine ppm.	Number of Melanophores								Average Number	Melanophores
Control	14,	17,	11,	12,	11,	13,	12,	12	12.6	Well Expanded
0.0001	11,	14,	15,	11,	12,	9,	10,	11	11.6	Well Expanded
0.001	9,	9,	12,	16,	8,	9,	10,	10	10.4	Well Expanded
0.01	5,	14,	9,	10,	10,	11,	9,	12	10.0	Well Expanded
0.1	5,	7,	12,	9,	10,	9,	10,	9	8.9	Moderately Expanded
1.0	0,	7,	3,	3,	4,	2,	2,	2	2.9	Much Contracted
10.0	0,	5,	4,	0,	2,	2,	3,	3	2.4	Much Contracted

REGION II

Concentration of Levothyroxine ppm.	Number Melanophores								Average Number	Melanophores
Control	35,	54,	38,	43,	35,	60,	51,	43	45.1	Well Expanded
0.0001	51,	46,	43,	44,	39,	50,	46,	42	45.1	Well Expanded
0.001	52,	42,	48,	34,	43,	53,	42,	58	46.5	Well Expanded
0.01	39,	38,	27,	38,	36,	34,	44,	39	36.9	Well Expanded
0.1	63,	49,	43,	35,	30,	34,	49,	28	41.4	Moderately Expanded
1.0	45,	40,	39,	53,	38,	45,	65,	33	44.8	Much Contracted
10.0	39,	46,	35,	26,	25,	45,	23,	36	34.4	Much contracted

In Region II, contraction of melanophores seemed to be greatly affected but the formation of malanophores was affected to a much lesser extent. This phenomenon was not as noticeable in the work involving constant exposure, so it is felt that it deserves special mention here.

Effects on Development

There was no significant evidence that a 30-minute exposure to levothyroxine affected mortality, abnormality or hatchability of individuals until a strength of 1 ppm. was reached (see Table IX). At this level, there was an abrupt rise in abnormally developing embryos. By approximately 144 hours of age, 80 percent of the embryos exposed to this concentration showed the now familiar crescent posture encountered in constant exposure experiments. (See Plate I). However, the mortality rate at this level was very low with 96 percent of the abnormally postured specimens having survived to 144 hours. All of the embryos had hatched by this time (144 hours).

Triiodothyronine

Pigmentation

The effects of 30-minute exposures of various concentrations of L-triiodothyronine on the pigmentation of developing embryos are recorded in Tables X and XI. In Region I, it was found again that decreased pigmentation was the product of both contraction and nondevelopment of melanophores, while in Region II this seems to have been caused almost entirely by contraction alone.

In the lighter concentrations and in control-embryos, only approximations of melanophore numbers could be made as melanophores had enlarged and merged to such an extent that there was much difficulty in determining their actual numbers.

TABLE IX

DATA RELATING TO EMBRYOS EXPOSED TO LEVOTHYROXINE FOR 30 MINUTES

Concentration of Levothyroxine ppm.	No. of Embryos Treated	No. of Embryos Dead by 144 Hrs.	No. of Abnormal Embryos by 144 Hrs.	Types of Abnormalities Produced	No. of Live Embryos Within the Chorion at 144 Hrs. (Unhatched)
Controls	350	21 (6%)	5 (1.4%)	3 club-shaped tails 1 milky protoplasm 1 pigment retardation	6 (1.7%)
0.0001	50	2 (4%)	1 (2%)	Twisted body	0
0.001	50	2 (4%)	1 (2%)	Acephalic	0
0.01	50	3 (6%)	*1 (2%)	Slightly twisted body	0
0.1	50	2 (4%)	*0		0
1.0	50	2 (4%)	40 (80%)	Slightly twisted bodies	0

* At 0.01 ppm. one juvenile appeared abnormally twisted at 72 hours through 96 hours but apparently recovered from this effect as we were unable to pick it out from normal embryos after 96 hours. At 0.1 ppm. this same type of abnormality occurred in five juveniles and within 12 hours they were indistinguishable from the rest of the normal individuals.

TABLE X

EFFECTS ON GROSS PIGMENTATION PER 24 HOURS
UNDER CONDITIONS OF 30-MINUTE EXPOSURE

Concentration of L-triiodothy-ronine ppm.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	144 Hrs.
0.0001	0	0	0	0	0
0.01	0	0	0	0	0
0.1	0	-0.50	-0.75	-0.75	-0.75
1.0	-0.25	-0.50	-0.50	-0.75	-1

  0 = Normal
-1.0 = ½ normal density
-2.0 = No pigmentation
Values given are visual estimates of entire cultures, not individuals

Effects on Development

The number and types of abnormalities encountered when developing

cultures were exposed to L-triiodothyronine for thirty minutes may be seen

by referring to Table XII.

Comparison of Levothyroxine with Triiodothyronine

Constant Exposure

Pigmentation

A striking difference in effects between levothytoxine and triiodo-

thyronine that was noted was that pigmentation at 0.0001 ppm. was only

slightly affected by the latter and did not begin to show an effect until

after approximately 120 hours of development. Levothyroxine at this lev-

el, however, showed an effect at a stage forty-eight hours earlier and

at the end of 144 hours showed that pigmentation was retarded approximately

TABLE XI

MELANOPHORE COUNTS ON EMBRYOS EXPOSED TO L-TRIIODOTHYRONINE FOR 30 MINUTES
REGION I

Concentration of L-triiodothy-ronine ppm.	Number of Melanophores								Average Number	Remarks
Control	14,	17,	12,	12,	11,	13,	11,	12	12.6	Well Expanded
0.001	15,	11,	14,	10,	10,	15,	11,	11	12.1	Well Expanded
0.01	9,	13,	10,	11,	12,	14,	12,	10	11.4	Well Expanded
0.1	8,	8,	8,	10,	7,	11,	8,	11	8.9	Moderately Expanded
1.0	2,	3,	2,	1,	3,	3,	4,	3	2.6	Moderately Expanded

REGION II

Concentration of L-triiodothy-ronine ppm.	Number of Melanophores								Average Number	Remarks
Control	36,	35,	34,	34,	35,	34,	37,	34	34.9	Well Expanded
0.001	34,	31,	30,	29,	34,	34,	36,	33	32.6	Well Expanded
0.01	29,	29,	33,	28,	28,	32,	29,	34	30.3	Well Expanded
0.1	31,	30,	32,	32,	29,	33,	35,	33	31.9	Moderately Expanded
1.0	37,	20,	33,	16,	25,	28,	24,	33	27.0	Moderately Expanded

TABLE XII

DATA RELATING TO EMBRYOS EXPOSED TO L-TRIIODOTHYRONINE FOR 30 MINUTES

Concentration of L-Triiodo- thyronine ppm.	No. of Embryos Treated	No. of Embryos Dead by 144 Hrs.	No. of Abnormal Embryos by 144 Hrs.	Types of Abnormalities Produced	No. of Live Embryos Within the Chorion at 144 Hrs.
Controls	350	21 (6%)	5 (1.4%)	3 club-shaped 1 milky protoplasm 1 pigment retardation	6 (1.7%)
0.001	50	6 (12%)	0		2 (4%)
0.01	50	1 (2%)	7 (14%)	3 acephalic 4 twisted bodies	0
0.1	50	5 (10%)	2 (4%)	2 twisted bodies	0
1.0	75	11 (14.7%)	22 (29.3%)	1 acephalic 22 twisted bodies	0

twice as much as triiodothyronine-treated specimens at a similar stage.

In comparing Table V to Table II it can be seen that although embryos exposed to triiodothyronine showed a lesser effect on pigmentation at the most dilute concentrations used, they also showed a greater decrease in melanophore numbers at these concentrations (comparing 0.0001 and 0.001 ppm.). Therefore, it would seem that by expansion of melanophores, although fewer in number, the amount of pigmentation in triiodothyronine-treated individuals was greater, even though levothyroxine-treated individuals possessed a greater number of melanophores at these concentrations.

In the higher concentrations of both drugs these differences in melanophore counts and gross comparison of pigmentation did not seem to be of comparable significance, because, in the higher concentrations there was a close similarity in the effects produced.

Effects on Development

A truer picture of triiodothyronine's greater potential was unmasked as a comparison of its effects on mortality and normal development was made with those of levothyroxine. Here was found an unmistakable indication that given concentrations of triiodothyronine were capable of producing a more powerful reaction than identical concentrations of levothyroxine. This became evident in the lower concentrations and became even more evident as the concentrations were increased.

At 0.001 ppm., a larger number of abnormalities occurred while at 0.01 ppm. the number of these cases increased to such a great extent that there was no doubt as to triiodothyronine's greater potency. A comparison of Table VI with Table III will help to point this out.

The increased mortality rate encountered at 0.01 ppm. seems to further

point up the greater potency of triiodothyronine. At this concentration the number of fatalities was approximately three times greater in comparison to the number produced by the same concentration of levothyroxine. Where solutions were more dilute both drugs seemed to have fairly similar effects, although triiodothyronine-treated specimens began to show a slightly greater abnormality rate at 0.001 ppm. At strengths above 0.01 ppm. none of the individuals were able to survive without showing signs of abnormal development. Many died.

Thirty Minute Exposures

Pigmentation

The differences between the reactions of levothyroxine and triiodo-thyronine at 30-minute exposures were not as sharply defined as those found in the studies with constant exposures. When pigmentation of developing embryos exposed to triiodothyronine for 30 minutes was grossly examined, the writer was, as with levothyroxine, unable to detect any effects on pigmentation until the strength of 0.1 ppm. was reached. The only appreciable difference in pigmentation effects between the two drugs at this concentration was that levothyroxine cultures showed less pigmentation during the first 24 hours.

At 1 ppm. pigmentation of triiodothyronine-exposed individuals lagged slightly behind levothyroxine-exposed embryos through most of the observation period, but by 144 hours we could find no distinguishable difference.

Individual melanophore counts revealed that both chemicals affected pigmentation by contraction and inhibition of development of melanophores to a similar degree.

Effects on Development

Although both drugs seemed to have a similar effect on pigmentation in this limited exposure work, triiodothyronine appeared to have a lesser effect in other areas under investigation. This was in direct contrast to the results obtained under constant exposure conditions. At 30-minute exposures of 0.001 through 0.1 ppm. of triiodothyronine, the number of fatalities and abnormal and unhatched embryos encountered were fairly similar to reactions of identical dilutions of levothyroxine. However, at 1.0 ppm. less than half the number of abnormalities which occurred at 1 ppm. of levothyroxine were produced.

Statistical Analysis

Using Duncan's (1953) multiple range test, a statistical analysis conducted on a study of the effects of both drugs on melanophore counts gave the results shown in Tables XIII through XVI. Any two means not underlined by the same line are statistically significantly different at the 5 percent level of confidence (possibility that we say they are different when no difference exists is 5 percent). The tables listed refer to those tables previously mentioned.

As the foregoing results indicate, on the basis of melanophore counts alone, there is a definite linear response in Region I to both drugs, but particularly so in the 30-minute exposure tests. Although this response is apparently not as clearly defined in Region II in the statistical analysis of melanophore numbers, it should be remembered that both substances had an effect on size and number of melanophores and the size of melanophores was not taken into consideration in the analysis.

TABLE XIII

A STATISTICAL ANALYSIS OF MELANOPHORE NUMBERS OF EMBRYOS CONSTANTLY EXPOSED TO VARIOUS CONCENTRATIONS OF LEVOTHYRONINE
REGION I

* S.E.M. = 0.251		Error d.F. = 36					Means	I.D.
Pi		(2)	(3)	(4)	(5)	(6)	3.4	Control
RP		0.72	0.76	0.78	0.80	0.81	2.25	0.0001 ppm.
I.D.	10.0	0.001	0.01	1.0	0.0001	Control	1.19	0.001 ppm.
R.M.	1.18	1.19	1.49	1.70	2.25	3.40	1.49	0.01 ppm.
							1.70	1.0 ppm.
			II	II			1.18	10.0 ppm.

REGION II

* S.E.M. = 2.63		Error d.F. = 36					Means	I.D.
Pi		(2)	(3)	(4)	(5)	(6)	42.63	Control
RP		7.57	7.97	8.18	8.39	8.52	37.00	0.0001 ppm.
I.D.	1.0	10.0	0.01	0.001	0.0001	Control	34.63	0.001 ppm.
R.M.	24.00	27.00	31.75	34.63	37.00	42.63	31.75	0.01 ppm.
							24.00	1.0 ppm.
	II						27.00	10.0 ppm.

* = Standard error of a treatment mean   II = Not in normal sequence of dilutions

TABLE XIV

A STATISTICAL ANALYSIS OF MELANOPHORE NUMBERS OF EMBRYOS CONSTANTLY EXPOSED
TO VARIOUS CONCENTRATIONS OF L-TRIIODOTHYRONINE
REGION I

* S.E.M. = 0.254			Error d.F. = 42			Means	I.D.
Pi		(2)	(3)	(4)	(5)	(6)	3.40	Control
RP		0.73	0.76	0.79	0.81	0.82	1.75	0.0001 ppm
I.D.	0.1	0.001	0.0001	1.0	0.01	Control	1.31	0.001 ppm
R.M.	1.14	1.31	1.75	1.80	1.88	3.40	1.88	0.01 ppm
							1.14	0.1 ppm
				II	II		1.80	1.0 ppm

REGION II

* S.E.M. = 2.18			Error d.F. = 42			Means	I.D.
Pi		(2)	(3)	(4)	(5)	(6)	42.63	Control
RP		6.23	6.56	6.76	6.91	7.02	23.00	0.0001 ppm
I.D.	0.001	0.0001	0.1	1.0	0.01	Control	21.00	0.001 ppm
R.M.	21.00	23.00	24.88	26.25	38.25	42.63	38.25	0.01 ppm
							24.88	0.1 ppm
II							26.25	1.0 ppm

* = Standard error of the treatment mean
II = Not in normal sequence of dilutions

TABLE XV

A STATISTICAL ANALYSIS OF MELANOPHORE NUMBERS OF EMBRYOS EXPOSED FOR
30 MINUTES TO VARIOUS CONCENTRATIONS OF LEVOTHYRONINE
REGION I

* S.E.M. = 0.1853			Error d.F. = 49				Means	I.D.
Pis		(2)	(3)	(4)	(5)	(6)	(7)	3.54 Control
RP		0.53	0.56	0.57	0.59	0.59	0.60	3.40 0.0001 ppm.
I.D.	10.0	1.0	0.1	0.01	0.001	0.0001	Control	3.21 0.001 ppm.
R.M.	1.30	1.53	2.96	3.14	3.21	3.40	3.54	3.14 0.01 ppm.
								2.96 0.1 ppm.
								1.53 1.0 ppm.
								1.30 10.0 ppm.

REGION II

* S.E.M. = 3.01			Error d.F. = 49				Means	I.D.
Pi		(2)	(3)	(4)	(5)	(6)	(7)	44.88 Control
RP		8.58	9.03	9.30	9.51	9.66	9.81	45.13 0.0001 ppm.
I.D.	10.0	0.01	0.1	1.0	Control	0.0001	0.001	46.50 0.001 ppm.
R.M.	34.38	36.88	41.38	44.75	44.88	45.13	45.50	36.88 0.01 ppm.
								41.38 0.1 ppm.
								44.75 1.0 ppm.
II								34.38 10.0 ppm.

* = Standard error of a treatment mean   II = Not arranged by normal sequence of dilutions

TABLE XVI

A STATISTICAL ANALYSIS OF MELANOPHORE NUMBERS OF EMBRYOS EXPOSED FOR 30 MINUTES TO VARIOUS CONCENTRATIONS OF L-TRIIODOTHYRONINE

REGION I

* S.E.M. = 0.095		Error d.F. = 35				Means	I.D.
Pi		(2)	(3)	(4)	(5)	3.56	Control
RP		0.274	0.289	0.295	0.303	3.45	0.001 ppm.
I.D.	1.0	0.1	0.01	0.001	Control	3.38	0.01 ppm.
R.M.	1.58	2.95	3.38	3.45	3.56	2.95	0.1 ppm.
						1.58	1.0 ppm.

REGION II

* S.E.M. = 1.29		Error d.F. = 35				Means	I.D.
Pi		(2)	(3)	(4)	(5)	34.88	Control
RP		3.72	3.91	4.01	4.12	32.63	0.001 ppm.
I.D.	1.0	0.01	0.1	0.001	Control	30.25	0.01 ppm.
R.M.	27.00	30.25	31.88	32.63	34.88	31.88	0.1 ppm.
			II			27.00	1.0 ppm.

* = Standard error of a treatment mean

II = Not in normal sequence of dilutions

The total effects of levothyroxine and triiodothyronine on pigmentation are adequately seen on visual inspection of treated cultures and the statistical treatment of melanophore numbers supports the visual observations recorded by the author.

Statistical results also indicate that one drug or concentration cannot be distinguished from another on the basis of melanophore counts. This is in agreement with results reported herein. However, the writer feels justified in stating that, when a comparison of gross pigmentation is made, it is possible to distinguish a culture which has been exposed to a very low concentration (e. g. 0.0001 ppm.) of one of the drugs from a culture that has been exposed to a higher concentration (e. g. 1 ppm.) providing of course, that both cultures have been treated under the same conditions and are compared at similar stages of development.

It has not been possible to distinguish one drug from the other on statistical evaluation of melanophore numbers, but here again we must take into consideration expansion and contraction of melanophores which account for the visual observations presented herein.

CHAPTER V

DISCUSSION

The role of the thyroid hormones in fishes has been the subject of studies by many workers. Drexler and Issekutz (1934), Root and Etkin (1937), and Etkin et al. (1940) reported negative findings to increased oxygen consumption when injecting or feeding thyroid to fish. Hasler et al. (1942) reviewed and re-examined the work of these authors and corroborated their findings. Smith and Everett (1943) using the fish _Lebistes reticulatus_ also found no significant rise in oxygen consumption or increased rate of sexual development in response to thyroxine. Lynn and Wachowski (1951) were also doubtful that the thyroid played any part in the metamorphosis of fishes.

Contrary to the above authors' findings, Smith and Matthews (1948) were able to produce a significant rise in the oxygen consumption of fish injected with thyroid extract and Hopper (1952) was able to show a growth increase in fish in response to thyroid powder. Frieders (1954) raised various species of fishes in either 0.002 percent phenylthiourea or 0.025 percent thiourea and produced significant inhibition of growth in comparison to controls, indicating that the thyroid does play an important part in fish metabolism. Gibson (1954) reported a retardation of growth of fish embryos subjected to thyroxine as did Jones, Gibson and Nickolls (1951).

Color changes in fishes have stimulated the curiosity of scientific investigators for many hundreds of years. Pliny wrote in

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book IX of his "Natural History" of the chromatic activities of the red
mullet fish. The color changes of this same fish also aroused the in-
terest of Seneca as evidenced by his awed descriptions in book III of
his "Physical Investigations."

However, it remained for later workers, those of the nineteenth
and twentieth centuries to ferret out the cause of these color changes.
The number of men who have contributed to the present day knowledge of
color changes in fishes is vast indeed, as is the number of substances
which are now known to be capable of producing color changes in fishes.

Sparth (1913) showed beyond doubt that the malanophores of fishes
were readily influenced by hormones rather than by nerves per se,
through the use of adrenaline on isolated scales of Fundulus. This
initiated the neurohumoral study of color changes of bony fishes.
These findings were substantiated in Wyman's work (1924). Using drugs
which stimulate the central or sympathetic nervous system to release
epinephrine, he was able to cause a contraction of melanophores through
stimulation of their pigment motor mechanism. He was also able to
cause an expansion of melanophores by administering drugs which have
a depressant effect on these parts of the nervous system. Through his
experimental work, this worker surmised that other substances, such as
the secretions of the endocrine glands, do not act through the nervous
system when introduced into the body, but are carried to the melanophores
in the blood stream. These above concepts were supported in a great
part many years later by Parker (1940) who recognized three substances
which act to control the pigmentary effector system of fish. Adrenalin
and acetylocholine were named as concentrating and dispersing agents
respectively, being produced by the sympathetic and parasympathetic

nerves supplying the pigment cells. Intermedin, secreted by the intermediate lobe of the pituitary was named as a third factor. This agent was found to cause a dispersion of melanin granules, but acted more slowly than the other two named agents.

Perhaps the most extensive work conducted to date in the study of fish melanophores has been that of Robertson. Employing the Rainbow trout in his experiments, he reported (1948) that the thyroid gland remained in a quiescent state in immature fish until transformation from parr to silvery smolt stage occurred (at about two years of age) when it becomes active. The silvery color was observed to be caused principally by the deposition of a thick layer of guanine crystals on the internal surface of the scales. Contraction and disintegration of the dermal melanophores were also found to play important roles on the ventral surface and opercula.

This work was followed by further experiments (1949, 1951) in which Robertson wished to see if a change in color identical with that occurring spontaneously in nature could be produced by increasing the thyroid hormone concentration in the body. In the earlier cited work, an immediate contraction of dermal melanophores was produced at the site of injection of a 2 percent suspension of thyroid powder. At the end of a six-to seven-week period of tri-weekly injections, trout closely resembling naturally occurring silvery smolts were produced, thus substantiating earlier reports by Landgrebe (1941). Injections of thyrotropic hormone brought about the same changes, but did not cause local contraction of melanophores at the site of injection. An examination of scales following injections of these substances revealed depositions of guanine crystals, and melanophores of the ventral surface were found to be in a state of contraction as found

in naturally transforming fish. Through these results, Robertson concluded that the transformation from parr to silvery smolt in Rainbow trout was brought about by an increased concentration of thyroid hormone in the body.

Isolated skin strips from Rainbow trout were also used by Robertson (1951) to further study the effect of the thyroid hormone on melanophores. He was able to produce a "prompt and marked contraction" of melanophores by immersing the skin strips in thyroid extract and stated:

It is of interest that extracts of the whole thyroid gland were much more potent in their effect on melanophores than were solutions of compounds isolated from the thyroid, namely thyroglobulin, diiodotyrosine and thyronine.

He further stated,

This suggests either the presence of an unknown substance which is the principle agent in causing melanophore concentration or the loss of some fraction from or change in the constitution of thyroglobulin or diiodotyrosine during their isolation from the thyroid or synthesis in vitro.

Robertson also found evidence for metabolic and growth promoting functions of dessicated thyroid gland and thyroxine in fishes to be largely negative in nature.

In a collaborated work with Chaney (1953) Robertson related that the highest concentration of iodine, in a study of the distribution of this element in various tissues of sexually mature Michigan trout, was found in the eggs. The egg mass was reported to have contained more iodine than the combined total of all other tissues including the thyroid. Similar results were revealed on examination of sea-run trout.

La Roche, et al. (1952) employing the Atlantic salmon Salmo salar at the fry, parr and smolt stages and the brook trout Salvelinus frontinalis at the parr stage were able to induce a pallor and thickening of

the integument, slightly in the fry, moderately in the parr and intensly in the smolt stage upon administration of thyroid preparations. As Gibson (1954) stated ,

It is unknown whether or not thyroxine inhibits melanoblast production in the fish, alters the membrane permeability of potential melanophores, inhibits synthesis of tyrosine, inhibits the action of tyrosinase or other intermediate metabolic steps in melanin formation.

The results obtained in our experiments with levothyroxine and triiodothyronine would lead us to believe that several other factors could be responsible for the decreased pigmentation encountered. These factors are:

1. Contraction of melanophores

2. Blanching of melanin

3. Orton's theory (1953) on the neural crest origin of vertebrate pigment cells could come into play here. It is possible that in some way, migration of developing melanophores from the neural crest ectodermal cells to their definitive sites in the body may have been interfered with.

The melanin content in the melanophores of the treated specimens observed in our work apparently was equal to that of the controls in most cases so that it would appear that blanching of melanin did not occur to any great extent. Contraction of melanophores was very definitely observed and Orton's theory of neural crest origin and migration of melanophores must be taken into consideration. Jones (personal communication) encountered similar, but not as pronounced effects upon using hormones and other substances and suggested, as does Gibson, that metabolism must also be considered.

In comparing our experimental data with those obtained by Gibson, several similarities are noted:

1. Development of pigmentation seems to be inversely proportional to the concentration of thyroid substances used.

2. Melanophores appeared at the same time in both treated and control specimens.

3. A decreased number of melanophores was observed in the treated specimens.

However, several differences are also noted in comparing both works:

1. In Gibson's work, pigment retardation was produced by concentrations of thyroxine as low as 0.1 ppm. The writer was able to produce a noticeable reduction in pigmentation with both levothyroxine and triiodothyronine at concentrations as low as 0.0001 ppm.

2. The length of time of exposure of embryos in early cleavage stages to thyroxine in Gibson's work produced little variation in the effects on pigmentation, whether exposed for 3 minutes or 72 hours.

   In contrast to this, this writer observed a definitely more decreased pigmentation in those embryos constantly exposed to levothyroxine and triiodothyronine than in those embryos exposed for a thirty-minute period.

3. Gibson reported a nonspecific growth retardation with thyroxine. The present writer noted no effects on growth with levothyroxine or triiodothyronine.

4. The percentage of kill in Gibson's work was reported to be 25 percent for both control and treated embryos, death being attributed to a fungus.

   The percentage of kill in control embryos encountered in the

present work was approximately 6 percent. In experiments involving triiodothyronine and levothyroxine at constant exposures, the percentage of kill in all cases was equal to or greater than that in the controls. In limited (30-minute) exposure experiments, the percentage of kill in levothyroxine treated embryos was equal to or less than that of the controls while embryos exposed to triiodothyronine, in all but one dilution (0.01 ppm.) showed a greater kill than that of the controls.

Of particular interest to note is the fact that developing eggs which were exposed to varying concentrations of levothyroxine and triiodothyronine for 30 minutes during early cleavage stages showed definite effects during the later stages of development. This phenomenon was also witnessed by Gibson after exposing danio eggs to thyroxine during early developmental stages.

The first question that enters one's mind is how these thyroid compounds affect development of the embryos when they are exposed to these compounds for only a limited period of time and at a time when the thyroid has not yet begun to develop. It has previously been pointed out that eggs exposed for 30 minutes to levothyroxine and triiodothyronine were washed before being transferred to aged tap water to continue development and it is assumed that Gibson also followed this procedure although he mentions it for only one culture. Therefore, it would seem that little or none of the compounds would be adhered to the eggs when they were removed from the treated solutions and placed in untreated water. If some small amount had been transferred with the egg, the dilution factor would be such that the effects

should be negligible.

How then is it possible for these substances to produce their apparent effects? The only explanation that the writer can advance is that these compounds were absorbed and retained by either the yolk mass or the developing cells or both and manifested their presence at a later stage when the cells became responsive to them. Whether this hypothesis is correct or not remains to be seen. It is hoped that some future worker will find the solution to this interesting problem.

CHAPTER VI

SUMMARY AND CONCLUSIONS

This study involving developing embryos of the Zebra fish, Brach-
ydanio rerio, exposed to varying concentrations of levothyroxine and
triiodothyronine indicates that these embryos are sensitive to both
substances in concentrations as low as 0.0001 ppm. when constantly ex-
posed. Exposure for 30 minutes to higher concentrations of either sub-
stance during early developmental stages was observed to produce simi-
lar but less profound effects. There was no apparent acceleration or
retardation of morphogenesis noted which could be attributed to either
compound. Both levothyroxine and triiodothyronine appeared to cause a
measurable retardation in the production of pigmentation which could be
partially accounted for by a decreased number and/or contraction of
melanophores. Various concentrations of both substances were apparent-
ly capable of causing morphological change in the developing specimens,
manifested by lateral curvature of the body so that the affected in-
dividuals assumed a characteristic shape.

The data presented lead to the following conclusions:

1. Levothyroxine and triiodothyronine in solutions as low as

    0.0001 ppm. retard the production of melanophores and pigmen-

    tation in Brachydanio rerio.

2. There is an apparent difference in the quantitative effects

    of both levothyroxine and triiodothyronine in "constant"

versus "limited" exposure of Zebra fish embryos.

3. There is apparently no acceleration or retardation in morpho-
genesis of danio embryos which can be specifically attributed
to either substance studied.

4. There is a definite linear response in melanophore numbers to
both compounds in Region I (as reported herein), particularly
when embryos are exposed to the compounds for only 30 minutes.

5. Triiodothyronine cannot be distinguished from levothyroxine,
nor can one concentration be distinguished from another on the
basis of effect on melanophore numbers.

6. There is apparently no significant difference in mortality or
morphological effects produced by similar concentrations of
both substances used in this work.

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APPENDIX

PLATE I

Photographs of specimens selected at random to illustrate experimental findings. (All were fixed at 144 hours of development).

1. Control embryos.

2. Specimens exposed to 1.0 ppm. of L-triiodothyronine for 30 minutes.

3. Specimens constantly exposed to 0.01 ppm. of L-thyroxine.

4. Specimens constantly exposed to 0.1 ppm. of L-triiodothyronine.

5. Specimens constantly exposed to 1.0 ppm. of L-triiodo- showing typical lateral curvature of the body encountered throughout the experimental work.

6. Two unhatched specimens from an experimental unit of embryos constantly exposed to 0.1 ppm. of L-triiodothyonine.
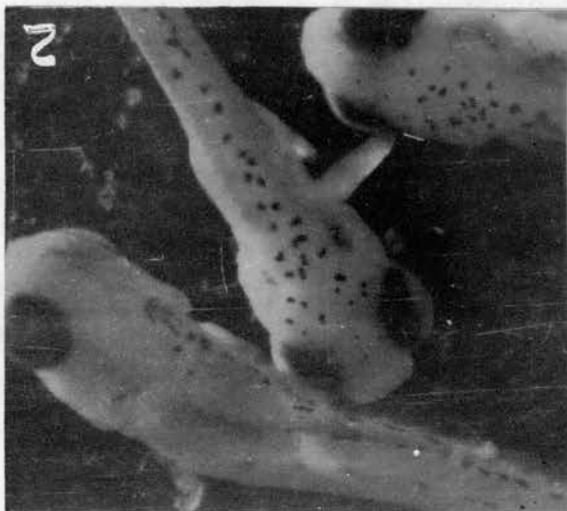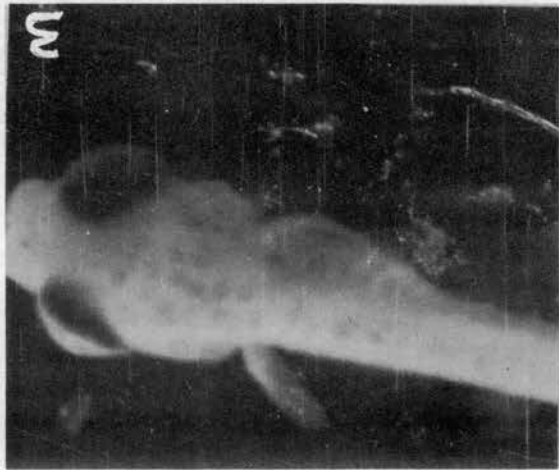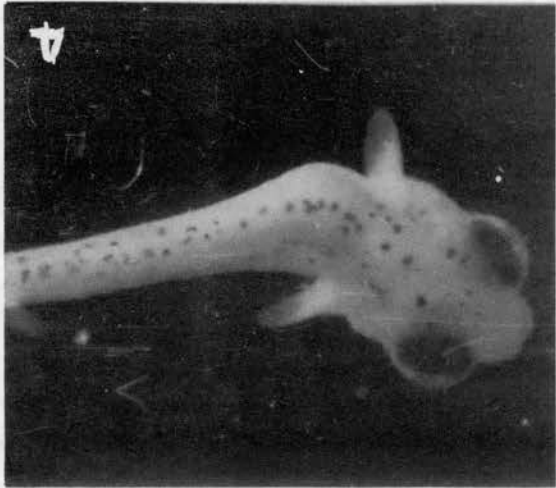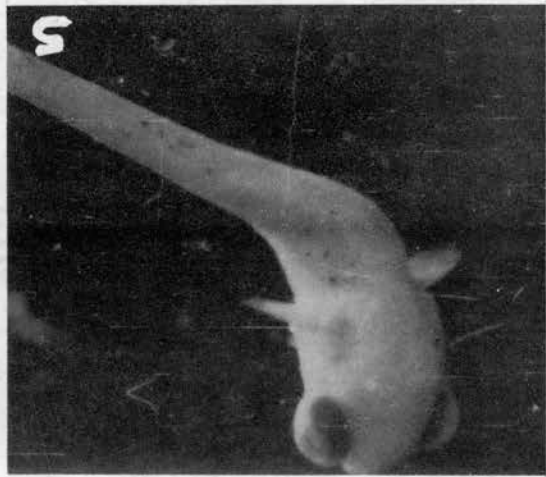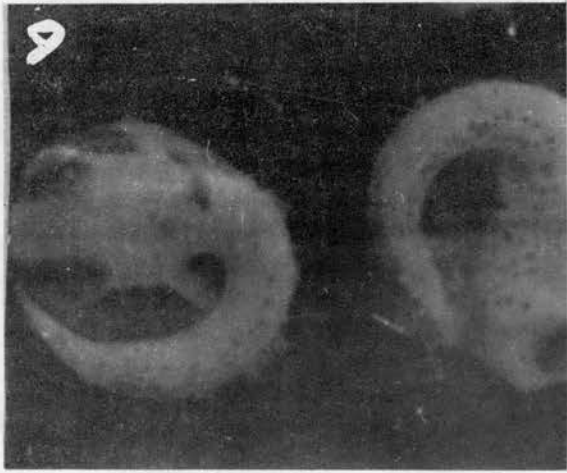
VITA

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Thesis: A STUDY OF THE EFFECTS OF LEVOTHYROXINE AND L-TRIIODOTHYRONINE
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