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THE EFFECT OF NA-L-THYROXINE ON VIABILITY AND REGENERATION IN DUGESIA TIGRINA

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The effect of vertebrate hormones on invertebrate function has always been open to question. For every experiment purporting positive results with the use of hormonal compounds derived from vertebrate endocrine glands, several may be found in which the original interpretations are either questioned or denied. An excellent review of these contradictory results is given by Hanstrom (1939).

Recently, however, at least one paper has appeared in which concrete proof of the effect of a vertebrate endocrine derivative on invertebrate function has been claimed. Gilbert and Schneiderman (1958) have published results in which the "mimicry" of the juvenile growth hormone activity of insects was obtained by extracts of beef adrenal cortex.

With these results in mind, and since among invertebrates the planarians lend themselves admirably to growth studies, a series of experiments was undertaken to ascertain whether the rate of regenerative growth of specific segments of the animals could be modified by addition of certain vertebrate hormones or their derivatives.

Wulzen (1916) and Wulzen and Bahrs (1928) reported that the rates of both fission and growth in planarian worms could be modified by the feeding of vertebrate glandular material such as liver and pituitary. The authors attributed their results to the absorption of certain specific growth-promoting materials by the worms rather than a simple food effect. Contemporary work in this field has yielded similar results. Jenkins (1959), utilizing various goitrogens, and Agrell and Wiman (1959), using oestradiol, have both clearly shown that compounds affecting vertebrates can to a significant degree influence the rate of growth of planarians.

In all regenerative studies performed on planarians, however, there seem to be two main obstacles that to some extent affect interpretation of results. The first of these is the well known axial gradient (Childs, 1911, nos. 10 and 11). To measure accurately growth rates under the conditions imposed by the gradient theory requires the use of identical segments of the experimental animals Unfortunately, variance in size and basic structure of the worms makes any duplication of a point of transection extremely difficult. In this series of experiments, it was decided to cut each worm midway through the pharyngeal region. Although this type of section results in high mortality of the segments (Childs, 1911, no. 10), the ease by which duplication of sectioning can be accomplished regardless of an animal's size, and the fact that the pharyngeal area is considered to be a neutral point insofar as the gradient is concerned, seemed to warrant this type of operation.

The second problem common to any experiment involving the effects of organic compounds on planaria, is whether the material is simply being utilized by the animals as food or whether it is exerting regulatory effect. In an attempt to reduce this problem, it was decided to run two sets of experiments. The first of these would simply determine the general effect of Na-L-Thyroxine on planaria segments; the second would compare these results, if any, to those obtained by treatment with another hormonal derivative, cortisone acetate, and an amino acid similar in basic structure to thyroxine, phenyl alanine. Since phenyl alanine may be considered as a basic nutritive substance with no regulatory characteristics, and since cortisone derivatives have been shown to inhibit mitotic activity in vertebrate forms (Tipton et al., 1959), a valid basis for comparative examination seemed to exist.

METHODS AND MATERIALS

The species of Turbellaria utilized was *Dugesia tigrina.* All animals were obtained from the Carolina Biological Supply Company. This source of material proved extremely satisfactory since each group of worms consisted of individuals all within a given size range and could be accepted as having arrived in the laboratory in a similar physiological state insofar as nourishment was concerned. Each group was kept in a glass tank filled with distilled water to which Elodea had been added for cover. All worms were kept without food for forty-eight hours prior to experimentation.

The choice of distilled water for both experimental and control solutions was made because of the variety and fluctuation of food material present in pond water. It was felt that although distilled water is not a normal medium for the animals, the lack of a natural food source or mineral component would offer a more stringent control insofar as the "food effect" of experimental compounds was concerned.

The worms were immobilized by cooling, and sectioned transversly through the middle of the pharyngeal region. In those experiments in which only the effect of Na-L-Thyroxine was being studied, anterior and posterior segments of consecutive worms were alternated so that one section of each worm was used as a control and the other half subjected to experimental conditions. Random sampling in those experiments involving several substances was attempted by placing all segments in a dish and selecting individual segments by chance.

All experiments were carried out at room temperature, 24°±1° C. Individual segments were placed in finger bowls containing 100 ml. distilled water or 100 ml. of the experimental solution. The final concentrations of experimental substances were made up from 1% solutions. They were as follows: Na-L-Thyroxine–3.13 x 10^{-5}M, cortisone acetate–3.73 x 10^{-5}M, and phenyl alanine–1.44 x 10^{-4}M. No significant changes in results were obtained by using lower concentrations of phenyl alanine.

Each bowl was examined daily and the solutions changed. Measurements were made with a standard micrometer and growth rates plotted according to the method of Wulzen (1927) as modified by Jenkins (1959). Photographs were taken with a Leica camera with a Leitz Micro-Ibso attachment.

RESULTS

Viability studies

The effect of Na-L-Thyroxine on the survival of anterior and posterior segments of *Dugesia tigrina* sectioned transversely through the pharynx is shown in Table I. Animals living after the fifth day completed the normal regeneration process.

Table 1. Fragments surviving after sectioning and treatment with Na-L-Thyroxine.

	No. of Segments	Days Post-Operative 1	2	3	4	5
Control						
Heads	26	12	7	4	2	2
Tails	26	19	13	7	7	6
Thyroxine						
Heads	26	21	19	19	19	18
Tails	26	23	21	19	19	19

Table 2. Fragments surviving after sectioning and treatment with Na-L-Thyroxine, cortisone acetate, and phenyl alanine.

	No. of Segments	Days Post-Operative 1	2	3	4	5
Control						
Heads	15	11	5	3	1	1
Tails	15	12	8	4	2	2
Thyroxine						
Heads	15	13	13	11	11	9
Tails	15	13	12	10	10	10
Cortisone						
Heads	15	8	5	0	0	0
Tails	15	11	9	5	0	0
Phenyl Alanine						
Heads	15	10	6	2	1	1
Tails	15	14	10	8	3	2

In an attempt to clarify the role played by the thyroxine, a second experiment utilizing phenyl alanine and cortisone acetate as well as Na-L-Thyroxine was performed. These results are given in Table II.

A study of the two tables clearly shows that a cut made in the mid pharyngeal region of *Dugesia tigrina* results in a high mortality of the control animals. It should be pointed out that not only does the addition of thyroxine significantly reduce this mortality rate, but that the amount of reduction as well as the mortality rates of the controls are consistent for both experiments. The probability that the mortality rate is independent of treatment is less than 0.01 as calculated by the method of Brandt and Snedecor.

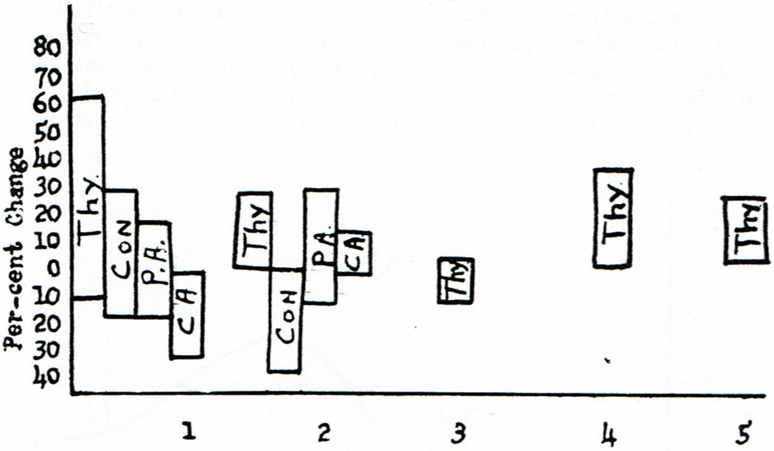
The critical period for both control segments and those placed in phenyl alanine appears to be during the third 24 hour interval. In direct contrast to this, the action of cortisone acetate is evident during the first 24 hour interval and finally results in disintegration of all segments by the fourth day.

Healing and formation of the blastema

The regenerative process in planarian worms consists of three phases. Immediately after sectioning, the wound is closed presumably by the action of the musculature (Bardeen, 1901), with the resultant formation of a dark brown or black indentation outlining the boundary of the cut. This stage is followed by the formation of a translucent mass of material which will give rise to the lost parts. This mass will be referred to as the blastema (Agrell and Wiman, 1958), since it originally consists of undifferentiated embryonic tissue. The final step is differentiation of the blastema, marked by an increased pigmentation which starts at the point of sectioning and progresses into the differentiated tissue.

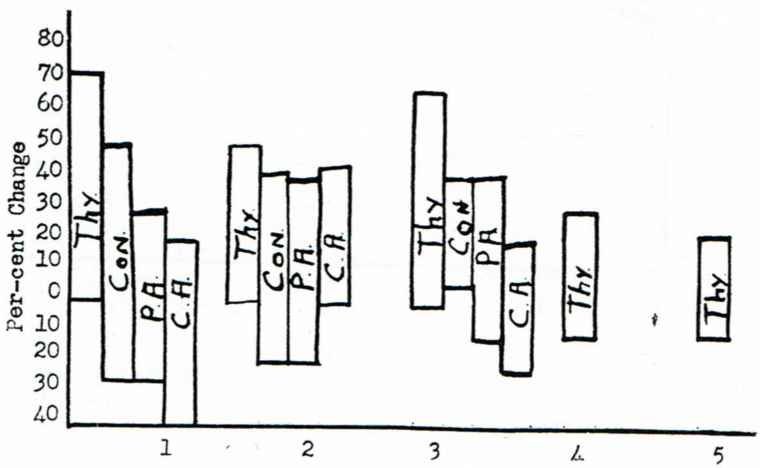
All the segments involved in these experiments exhibited the first step of the regenerative process; the pigmented edges of all cuts were sharply defined. In the cortisone treated segments, the formation of a blastema was either retarded or absent. This was especially true of the "head" segments in which regeneration stopped with the closure of the wound. Tail segments treated with cortisone did show the formation of a blastema after 48 hours, but the mass was small when compared with both the control and other experimental groups.

The most rapid formation of the blastema was found in those segments treated with thyroxine. During the first 24 hour interval, a recognizable embryonic mass could be differentiated in each segment and within 72 hours the original point of sectioning was difficult, and in some cases impossible, to distinguish from surrounding tissue. Regenerating control fragments fol-



DAYS POST OPERATIVE

Figure 1. Percentage changes in lengths of head segments of *Dugesia tigrina* treated with Na-L-Thyroxine (Thy.), phenyl alanine (P.A.), and corisone acetate (C.A.) as compared to controls (Con.).



DAYS POST OPERATIVE

Figure 2. Percentage changes in lengths of tail segments of *Dugesia tigrina* treated with Na-L-Thyroxine (Thy.), phenyl alanine (P.A.), and cortisone acetate (C.A.) as compared to controls (Con.).

lowed the same pattern, but the formation of a clearly recognizable blastema generally occurred after 48 hours.

Segments treated with phenyl alanine reacted essentially as did the control groups. There was a 24 hour lag in the formation of the blastema as compared with the thyroxine treated animals with the exception of two tail segments; these fragments exhibited a rate of regeneration much like that of the thyroxine group.

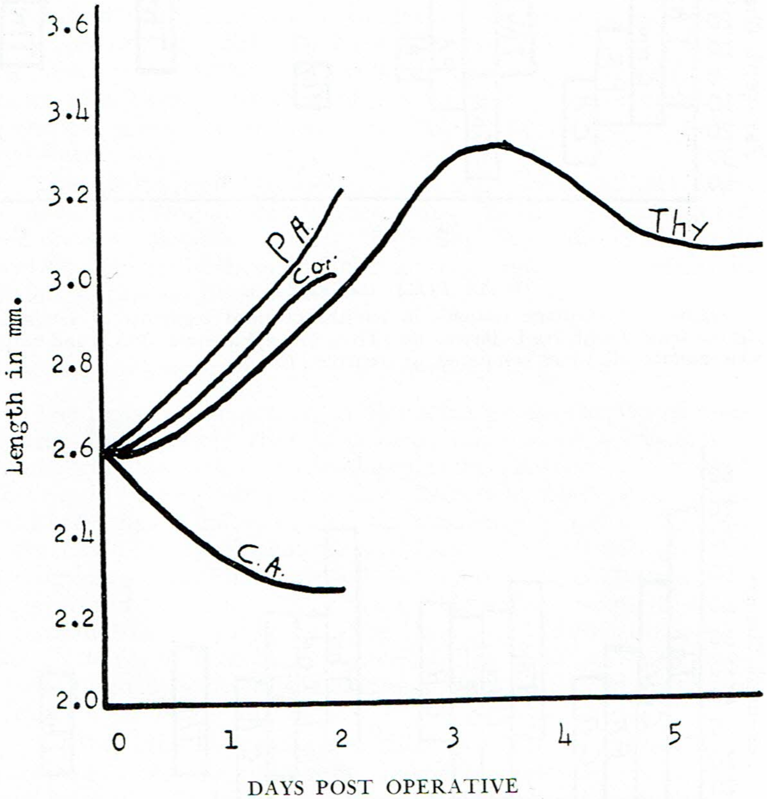


Figure 3. Growth rates of head segments treated with Na-L-Thyroxine (Thy.), cortisone acetate (C.A.), and phenyl alanine (P.A.) as compared with control segments (Con.).

Rates of Regeneration

The variance in rates of growth exhibited within each group, and the degree of mortality produced by the pharyngeal section, makes a comparative representation of regeneration difficult. In Figures 1 and 2, a summary of the percentage change in size for anterior and posterior segments over a five day period is given. Only those groups in which five or more segments were living (Table 2) are included. These bar graphs, in addition to showing the variance involved, emphasize the overall rate of increase in growth during the first two days exhibited by the thyroxine treated animals as compared to the other groups. This increase continues throughout the third day for the tail segments, but shows a decrease for the head fragments.

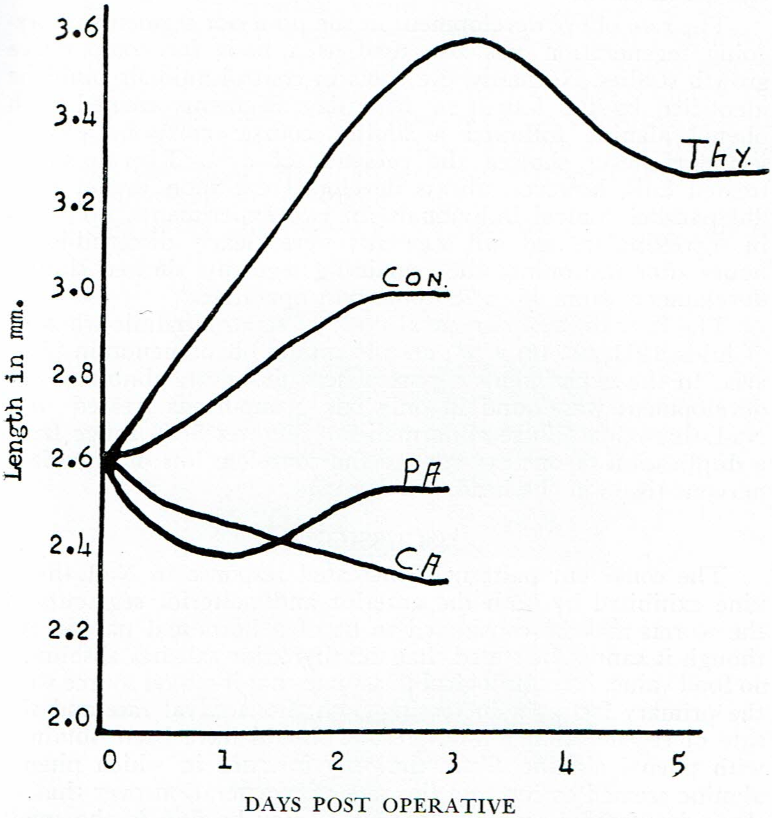


Figure 4. Growth rates of tail segments treated with Na-L-Thyroxine (Thy.), cortisone acetate (C.A.), and phenyl alanine (P.A.) as compared with control segments (Con.).

Unit growth rates are given in Figures 3 and 4. Points on the curve represent only those cases in which five or more segments were living. The general increase in length for the control segments during the first two days for the anterior portions and the the first three days for the posterior fragments is in general agreement with the results shown by Jenkins (1959). The thyroxine treated group also shows an increase during the first two days, but a sharp decrease in length is evident by the fourth day for both the heads and tails. Treatment with cortisone brings about an immediate decrease in both segments, but is much greater in the case of the anterior fragments. The curves for phenyl alanine are difficult to interpret. Although an increase is evident in both graphs, the rate of increase for the head sections is much greater.

Eye development

The rate of eye development in the posterior segments undergoing regeneration was also used as a basis for comparative growth studies. Normally, eye spots in control animals could be identified by the fourth or fifth day. Segments treated with phenyl alanine followed a similar course; cortisone treated segments never showed the presence of eyes. Thyroxine treated tails, however, always developed eye spots earlier than did parallel control individuals. In two experiments, eye spots in thyroxine treated tail segments were clearly discernible 24 hours after sectioning; the remaining segments showed similar development from 48 to 72 hours post operative.

The formation of abnormal eyes or "teratopthalmic wholes," (Childs, 1911, no. 10) is not an uncommon phenomenon in planaria. In the experiments reported here, however, abnormal eye development was found in only one group-heads treated with Na-L-thyroxine. These abnormalities (Figures 5-10) range from a duplication of one eye spot to the complete loss of organized nervous tissue in the head.

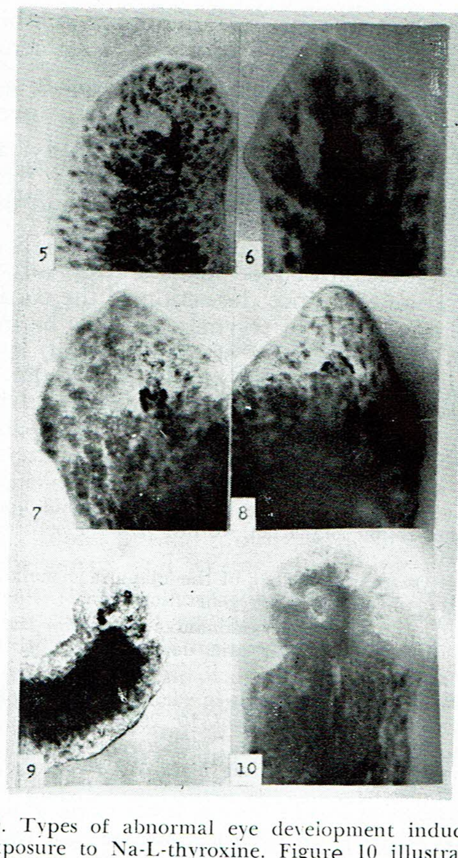
DISCUSSION

The consistent pattern of increased response to Na-L-thyroxine exhibited by both the anterior and posterior segments of the worms may be considered to be of a hormonal nature. Although it cannot be stated that the thyroxine salt has absolutely no food value, it seems logical to assume that if a food source were the primary factor in increasing both the survival rate and the rate of regeneration, similar results should have been obtained with phenyl alanine. Since the only instance in which phenyl alanine seemed to increase the rate of regeneration over that of thyroxine treated animals (Figure 1) may be due to the small sample size, and since this same group showed no evidence of increased viability, the effect, if any, cannot be compared to that of thyroxine.

Further evidence favoring a metabolic control effect is found by comparing eye formation in posterior segments treated with goitrogen as reported by Jenkins (1959) and the results given in this paper. Whereas treatment with thiourea is reported to result in animals with smaller eyes and a decrease in pigmentation, treatment with thryoxine results in a more rapid formation of eye spots, all of which are darkly pigmented, and in the case of treated heads brings about an increase in the size and number of eyes. Since the activities of thyroxine and thiourea may be said to be antagonistic, the comparative results shown in these experiments point to responses in an invertebrate form similar to the type expected in a vertebrate species. In regard to the formation of abnormal eyes, it is of interest to note that as early as 1913, Lloyd postulated that abnormal eye development in planaria was chiefly a function of metabolic change.

Segments of planarian worms continue to grow in size for five to six days after sectioning; a decrease in total length will then occur if nourishment is not available. Since an approximate developmental stage in thyroxine-treated worms is found at the third day, the decrease in size apparent in the following days for both anterior and posterior segments (Figures 1 and 2) may be interpreted as being due to an increased metabolic rate induced in the animals by the thyroxine.

Regeneration in planaria is accompanied by a high mitotic



Figures 5-10. Types of abnormal eye development induced in anterior segments by exposure to Na-L-thyroxine. Figure 10 illustrates a complete degeneration of organized eye spots and nervous tissue. Magnification 30 X.

rate in the blastema (Agrell and Wiman, 1958). As noted previously, the rate of blastema formation in pharyngeally sectioned fragments is enhanced by thyroxine and inhibited by cortisone. Although a definite relationship between the mitotic rates and treatment with either of the endocrine derivatives cannot be established without mitotic indices, the fact that both thyroxine and cortisone induce similar results in vertebrate material lends credence to the supposition that throughout these experiments, we are dealing with a direct hormonal effect on invertebrate tissue.

SUMMARY

1. The high rate of mortality of anterior and posterior segments of *Dugesia tigrina,* formed by sectioning the animals in the mid-pharyngeal region, was reduced significantly by the addition of Na-L-thyroxine.

2. Segments treated with Na-L-thyroxine exhibited a more rapid rate of regeneration than did control segments.

3. Abnormal eye development was present in a number of head segments treated with the thyroxine derivative.

4. Treatment with a basic amino acid, phenyl alanine, had no effect on the rate of mortality or regeneration; treatment with cortisone acetate increased the mortality rate and decreased the rate of regeneration.

5. Since the addition of food had no effect on viability and rate of regeneration and since the action of the cortisone derivative in decreasing the rate of regeneration may be interpreted as a reduction in mitotic activity of the blastema, it is suggested that the positive action of Na-L-thyroxine on the invertebrate form is essentially a hormonal stimulation.

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BOOK REVIEW

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Hesler, L. R. *Mushrooms of the Great Smokies.* xii plus 289 pp., University of Tennessee Press, Knoxville. 1960. $5.50

Readers of this journal will recall through the years a long series of papers by L. R. Hesler dealing with fungi of the Southern Appalachian region. The present book, part of the publication costs of which were borne by the L. R. Hesler Research Fund, contributed by faculty colleagues, former students, and friends of the author, represent a product of 39 years of experience with the subject.

The book is directed to the amateur, and deals with the mushrooms and related fleshy fungi of the Great Smoky Mountains National Park, an area with an unusually rich fungus flora. Part One, entitled *Mushrooms in Nature,* includes a general introductory account of the structure and development of mushrooms, a discussion of their edibility and poisonous qualities, and kindred topics. Part Two is taxonomic, dealing wth some thirty genera of mushrooms most apt to be met with in the Park. There are keys to the genera and species, numbering 117. Each species listed is described, and has a photograph. The quality of the photographs is uniformly excellent, and represents to this reviewer the most attractive single feature of the book. Part Three includes a similar treatment of 66 species of mushroom relatives, such as polypores, boletes, stinkhorns, morels, puffballs, etc. There is a brief glossary and an index. The attractive end papers portray a map of the park, and a mushroom spore print. This volume represents a significant contribution to the flora of the state and region, and should be a stimulus to interest on the part of others. Both author and publisher are to be commended on its appearance.