# QUANTITATIVE OBSERVATIONS ON THYROXINE AND ALLIED SUBSTANCES. I. The use of tadpoles.

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In 1912 Gudernatsch(1) discovered a remarkable effect of thyroid preparations on tadpoles. They shrink rapidly in size and their metamorphosis is hastened. The phenomenon has been described in great detail by Romeis(2), Kahn(3), and others. Various workers have suggested that thyroid preparations should be assayed by methods depending on these phenomena. Swingle(4) discusses these papers and criticises them on the ground that the results are not a specific effect of the active principle itself, but may be produced in varying degree by almost any iodine compound. In most of these experiments the tadpoles were given repeated doses of the substance to be tested throughout the period of the experiment. Romeis(5), however, has shown that if tadpoles are immersed for a short time in a dilute solution of thyroxine the characteristic effects develop several days later. He found that the concentration of diiodotyrosine necessary to produce the same effects by such a method was hundreds of times larger than that of thyroxine. It seemed probable that under these conditions the test would be more specific. If the tadpoles were almost continuously in the presence of high concentrations of iodine, their own thyroid glands might be expected to be maintained in a perpetual state of hyperactivity, but phenomena which occur some days after a short immersion in a toxic concentration would not be so readily produced by substances which had no specific connection with the active principle of the gland.

Dr C. R. Harington kindly offered me the opportunity of testing the biological effects of the synthetic and natural thyroxines. At the same time he submitted to me a number of intermediate and related compounds which he has prepared and identified. I took the opportunity in the early summer of this year to examine the effects of this interesting series on tadpoles. Unfortunately the work was still incomplete when tadpoles became no longer available. Pending the examination of these compounds by other biological methods, and the resumption of the tadpole tests next spring, it appears worth while to put on record the results already obtained.

## METHODS.

There is some doubt as to the best criterion to take of the effect on the tadpoles. Romeis suggested that the results should depend on the date of the appearance of the hind legs or front legs. This is in many ways a convenient end-point, though the observation is not so simple as might be supposed. Every tadpole must be examined separately, as the leg buds are difficult to see on their first appearance. This examination is conveniently achieved by placing the tadpoles on a piece of looking-glass in a bright light. Other changes of a less definite nature, such as the change in shape of the body, will often suffice to place different substances in the order of their activities in producing metamorphosis. Lenhart (6) laid stress on the interval which elapsed before all the tadpoles were dead. Such a method could only be satisfactory if it were possible to exclude death from causes other than the specific effect of the substance under test.

It was thought probable that some more definitely quantitative measurement would give a more trustworthy indication of the effects of different doses. One of the most marked changes is a great diminution of the length of the gut, and measurements of this have been made by Swingle (7), but this method was not adopted because it was wished to follow the course of development of the effect, and a measurement of the length of the gut can only be made once on each tadpole.

The most obvious change which occurs is a rapid diminution in size. This appears to be due partly to the hastening of the normal processes of metamorphosis and partly to the wasting which is associated with the increase in metabolic rate. The simplest measurement to make, and the one most usually made, is that of the total length, but a measurement of volume or weight would, of course, show much larger differences, and might thus be thought likely to prove the most sensitive index of the effect.

The volume may be determined by drying the tadpoles and adding them to water in a measuring cylinder (2), but it is difficult to remove water completely from the surface without injuring them. If small tadpoles are strained on a coffee strainer the volume of the water which fills up the spaces between them may be almost equal to that of the tadpoles themselves. Volumes have, therefore, been determined less directly by taking 12 tadpoles and adding water to make a total volume of 2 c.c. in a small cylinder, and then adding 1 c.c. of a dilution of cochineal (liq.

cocci about one drop to 10 c.c.). The mixture is then shaken and filtered, and the filtrate compared with the original dilution in a colorimeter. A simple calculation gives the volume of water by which the 1 c.c. of cochineal solution has been diluted, and so, by difference from 2 c.c., the volume of the tadpoles. Various other pigments have been tried, but have been rejected, either because they were toxic (methylene blue) or because they were absorbed (Indian ink). Tadpoles were kept for one hour in diluted cochineal without producing any change in the depth or quality of its colour. They were also kept for 24 hours in the solution without showing immediate signs of toxic effects. The determination of volumes was, however, eventually abandoned owing to the fact that the body cavities of the tadpoles occasionally became distended with watery effusion. Though this distension did not appear to be caused by the short immersions in cochineal or by the iodine preparations, it obviously interfered with the accuracy of the measurement of volume as an index of growth or general shrinkage. The same objections would apply to the measurement of the weight of tadpoles, which was adopted by McCarrison (8) as an index of their growth.

It was, therefore, decided to measure the over-all length of each tadpole. They were sucked up one by one into a piece of glass tubing and the water was run out. When they became immobile in extension, they were held against a scale and measured to the nearest millimetre. Tadpoles taken from stock were thus measured and placed in four or six jars so that all the tadpoles in one jar were equal in length. A number of similar batches of twelve tadpoles was then prepared by taking in each batch an equal number of tadpoles from each jar. It is difficult to interpret the results when some of the tadpoles die during the period of observation and all such results have been neglected. The mean daily length of a batch of twelve controls showed steady growth as is shown in Figs. 1 and 2.

In order to obtain an idea of the errors introduced by individual variations among the tadpoles, the figures obtained for the lengths of the tadpoles in the same batch were arranged in order of size, and the amount that each tadpole had grown was calculated on the assumption that this procedure would arrange them in the same order at any stage of the experiment. It was found that the administration of thyroxine greatly increased the variability among the tadpoles, the small tadpoles showing larger absolute decreases in length than the large ones. The difference between the means of the lengths of two batches, however, one of which had received twice as much thyroxine as the other, was greater than three times the standard deviation of the differences of the means for batches of twelve, as calculated by the mean squares method. The fact

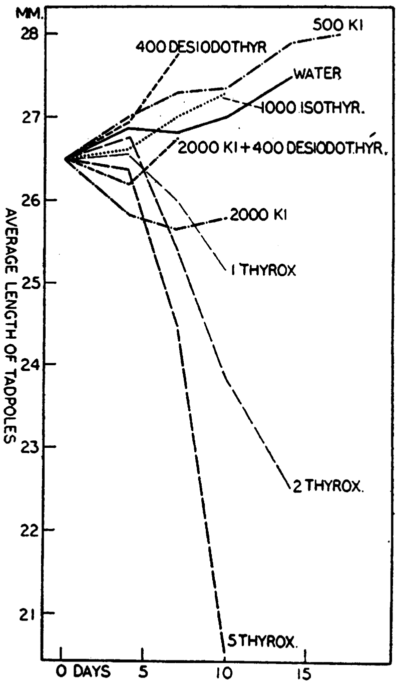



Fig. 1.

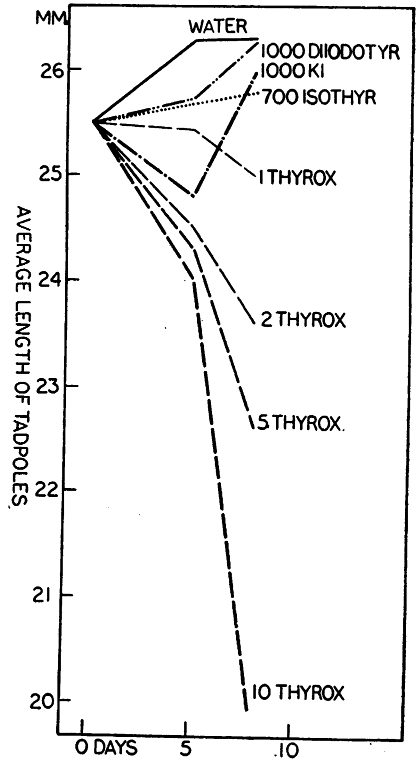



Fig. 2.

Fig. 1. The effect on the average length of batches of 12 tadpoles, hatched 65 days before, of an immersion for 24 hours in solutions whose concentrations are given in parts per ten millions. Isothyr represents a substance whose name and formula are given in the text which is isomeric with thyroxine. No measurements are recorded after the first tadpole in a batch died.

Fig. 2. The effect on the average length of batches of 12 tadpoles, hatched 59 days before, of an immersion for 24 hours in various solutions. The notation is as in Fig. 1. Diiodotyr denotes a solution of 3, 5 diiodotyrosine.

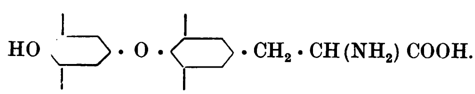
that a given batch of tadpoles shows a diminution of average length is of course not in itself sufficient evidence of occurrence of the phenomenon described by Gudernatsch, but in the presence of other evidence of the nature of the effect it gives a good indication of its magnitude.

Each batch of twelve tadpoles (*Rana temporaria*) was immersed for 24 or 48 hours in 500 c.c. of a clear solution, in tap-water, of one of the compounds under test, a small quantity of sodium carbonate being added when necessary to secure complete solution. An experiment in which the tadpoles were exposed for a like period to carbonate alone in the same dilution showed no difference from the control with pure tap-water, so that a batch in tap-water was considered to give an adequate control in each experiment. After this preliminary treatment each batch was kept in 500 c.c. of tap-water in a two-pound jam-jar. Each day they were washed in a coffee-strainer, and the water was changed.

The diet which the tadpoles receive is not without importance. Jarisch (9) found that tadpoles were most sensitive to thyroid when they were starved or fed on protein, and least sensitive when their diet was complete. Lenhart (6) found that the addition of carbohydrate to their diet decreased their sensitivity. In the present experiments two small pieces of hard-boiled white of egg were placed in each jar daily, and partially eaten by the tadpoles. The main stock was kept in running water with pond-weeds, and also given boiled white of egg.

## Results.

*Thyroxine.*



Figs. 1 and 2 show the effect of various dilutions of synthetic thyroxin on the average lengths of different batches of tadpoles. It will be seen that after a latent period of three or four days they showed definite decreases in length, and that the effect increased with increasing concentration. Fig. 3 shows the result of a comparison of natural thyroxine with synthetic thyroxine, both in the racemic condition. The natural thyroxine appears in this test to have an equal potency with the synthetic thyroxine. The counting of legs gave similar but less definite results.

*Potassium iodide.* $KI$.

It will be seen in Figs. 1 and 2 that high concentrations of potassium iodide produced a smaller effect on the length, which developed and passed away more rapidly. There was no other evidence of changes in the tadpoles such as are produced by thyroxine.

Swingle (7) has produced the characteristic thyroid effect on normal tadpoles and on tadpoles whose development had been arrested by thyroidectomy by mixing various inorganic iodine preparations with their food. In these experiments, however, potassium iodide differed from the other substances tested in that it increased the rate of growth of the

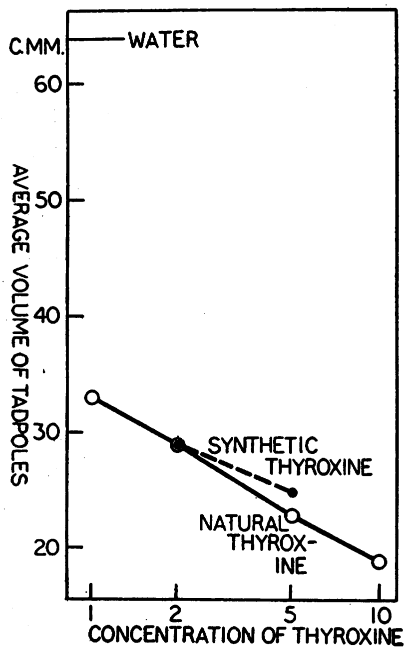



Fig. 3.

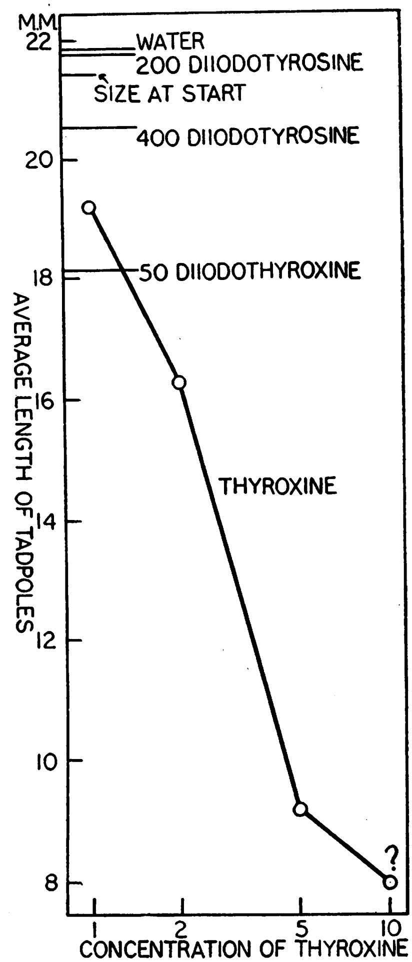
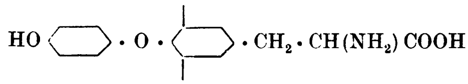



Fig. 4.

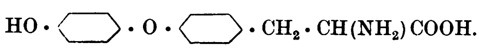
Fig. 3. Measurements taken on May 5th of the volume of tadpoles which had hatched on April 12th, and been placed in solutions of thyroxine for 48 hours on April 27–29th. The volume of the control batch is given at the top of the diagram. The concentration is given in parts per ten million. It was plotted on logarithmic paper.

Fig. 4. Measurements of the average length of batches of 12 tadpoles 46 days old, 7 days after an immersion in solutions of synthetic thyroxine, diiodotyrosine and "diiodothyroxine." The reading for the highest concentration of thyroxine should be neglected because many of the tadpoles were dead. The concentration is given in parts per ten million.

tadpoles. He also produced the characteristic effect by immersing the tadpoles in dilute solutions ($4 \times 10^{-7}$ for normal and $1{\cdot}1 \times 10^{-6}$ for thyroidectomised tadpoles) of the element iodine. He found that these solutions produced the effect more rapidly than thyroid preparations themselves, and he was led to the conclusion that the absorption of iodine was the essential factor in the development of metamorphosis. Other workers have failed to obtain these effects (see Abelin (11)), but they have been confirmed by Huxley (10), and it is probable that the differences in the results are to be attributed to differences in technique.

"*Diiodothyroxine.*" 

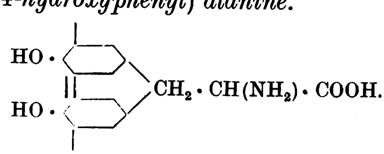
This substance, which has only two of the four iodine atoms of thyroxine, showed definite thyroid activity under the conditions of these tests. In Fig. 4 the average length of batches of 12 tadpoles seven days after an immersion for 24 hours in different concentrations of thyroxine is plotted against the concentration. The average length of a batch similarly treated with "diiodothyroxine" is also shown. It will be seen that "diiodothyroxine" appeared to be active in about 40 times the concentration of thyroxine. In other experiments it showed rather more activity. The counting of legs gave similar results.

*Desiodothyroxine.* 

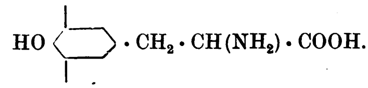
This substance, representing thyroxine deprived of all its iodine atoms, appeared to be quite devoid of the specific thyroxine action. In Fig. 1 the tadpoles which had been immersed in this substance are seen to have grown quicker than the controls. Those which had been exposed to a mixture of this substance and potassium iodide also grew quicker than those which had been exposed to the same concentration of potassium iodide alone. It appears, therefore, that tadpoles do not synthesise thyroxine under these circumstances.

In another experiment this substance had no effect on the rate of appearance of the legs.

*ββdi* (3, 5 *diiodo-4-hydroxyphenyl*) *alanine.*



This isomer of thyroxine showed no activity, as can be seen in Figs. 1 and 2.

3, 5 *Diiodotyrosine.* 

(I am indebted to Mr Thorpe for a sample of this substance.)

Under the conditions of these tests this substance produced little if any effect in high concentrations. In Fig. 4 it is seen to have less than one-eighth of the activity of "diiodothyroxine." In Fig. 1 it has less activity than the same concentration of potassium iodide and less than one-thousandth of that of thyroxine.

This result does not conflict with the results of previous workers. It is generally agreed that diiodotyrosine affects tadpoles like thyroxine when it is given over long periods and in high concentrations (see (5) p. 159 and (12)), but it has not been found to produce the effects after a single short immersion (compare the paper by Zawadowsky and his co-workers (13)).

*Amines.*

Experiments were done with the amines (amino-acid *minus* $CO_2$) corresponding to thyroxine and "diiodothyroxine" which seemed to indicate that they had a definitely specific effect similar to that of the same concentration of the corresponding amino acids; but it was necessary to add a small amount of alcohol and acid in order to get these preparations into solution, and it was found that the concentrations of these additions required were by themselves not without effect on the growth of the tadpoles, so that the results do not afford a very accurate comparison. In this connection it is interesting to note that Abelin (14) found that diiodotyramine had an effect similar to that of diiodotyrosine.

## Discussion.

It is thought that these experiments indicate that the decrease in length which occurs in tadpoles some days after an immersion in a solution of thyroid preparations is a specific quantitative test for thyroxine. The only other substance tested which definitely showed activity comparable with that of thyroxine, though much smaller, was "diiodothyroxine," and it is possible that this is iodised directly by the tadpoles. Swingle's results seem to point to the conclusion that this specificity depends on the fact that iodine can be more rapidly absorbed and stored in a stable form when it is present as thyroxine than when it is present in other forms. Much of the iodine absorbed from solutions of potassium iodide was presumably excreted rapidly when the tadpoles were placed in tap-water again, so that the effect was only temporary.

I wish to thank Dr H. H. Dale for his help and advice, and also my assistant F. F. McDonald, who has made several thousand measurements of tadpoles.

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