The Turnover of Melanin in *Xenopus laevis* treated with Phenylthiourea

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With one plate (fig. 2)

SUMMARY

Four sets of experiments were performed in which developing *Xenopus laevis* were treated with 0·1% phenylthiourea. The animals used in experiments 1 and 2 were taken from a single batch of eggs, those in experiment 3 from another batch of eggs, and those in experiment 4 from a third batch. A suitable control group of untreated tadpoles was arranged for each experiment. In experiment 1 gastrulae were reared in a solution of phenylthiourea, which inhibited melanogenesis. In experiment 2 tadpoles, which possessed melanophores containing melanin, were kept in a solution of phenylthiourea for 6 weeks. No turnover of melanin was detected in these animals. Experiment 3 was carried out on tail regenerates. It showed that melanogenesis is inhibited by phenylthiourea and that no turnover of melanin occurs during treatment with this drug. In experiment 4 animals which had almost completed metamorphosis showed no evidence of turnover of melanin when treated with phenylthiourea.

The arrest of development of the tadpoles and the histological changes in the thyroid glands which occurred during treatment were used as evidence that phenylthiourea reached the tissues of the experimental animals.

INTRODUCTION

MELANOPHORES of vertebrates effect colour changes of the integument in two ways, by dispersion and concentration of the pigment granules within the melanophores and by alteration of the amount of pigment formed and released into the integument. The ability of melanophores in Anura to disperse and concentrate their pigment is well established, but I have been unable to find any description of the release of pigment into the anuran integument later than that of Ehrmann (1885). Experiments with inhibitors of melanogenesis indicate that there is a turnover of melanin during the development of anurans. Lewis (1932) found that tadpoles of *Rana sylvatica* reared in solutions of indophenol dyes lost the pigment of their melanophores. Richter and Clisby (1941) reported that black rats treated with phenylthiourea became grey after 27 to 58 days, and Lynn (1948) showed that embryos of *Eleutherodactylus ricordii* raised in solutions of phenylthiourea lost the melanin of the skin and pigmented coat of the eyes in 5 or 6 days. In the same paper reference was made to unpublished experiments in which tadpoles of *R. pipiens* reared in a solution of phenylthiourea lost melanin from the skin but not from the eyes. Millott and Lynn (1954) reported experiments with embryos of *E. martinicensis*, in which the melanin of the skin, iris, and retina disappeared after 5 days' treatment in solutions of phenylthiourea.

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The mode of action of phenylthiourea is uncertain but it is generally agreed that it inhibits melanogenesis by a direct effect on the melanophore. If it has no effect on the pigment itself, loss of melanin from melanophores after 5 days' treatment represents a rapid turnover for a substance which chemically is only affected by strong oxidizing agents. The experiments reported here were undertaken to investigate this paradox. It was hoped to estimate the rate of turnover of melanin and the fate of the granules which disappear in an anuran tadpole readily available in the laboratory, and to observe the effect of phenylthiourea on development of the tadpole and the histology of the thyroid gland.

Material and Methods

Chorionic gonadotrophin was used to induce amplexus and ovulation in adult *Xenopus laevis*. The animals were reared and all experiments carried out in glass vessels kept on a matt black surface in a cupboard, the temperature of which was maintained at 20° C by a thermostat. Environmental factors that affect development, such as temperature, light, degree of crowding, and amount of food given per animal, were all standardized. The tadpoles were fed with a suspension of nettle-powder in water and when necessary their water was aerated. The methods of rearing and staging the animals were those recommended by Nieuwkoop and Faber (1956).

Preliminary experiments showed that phenylthiourea at a concentration of 0·01% was required in the water to prevent the formation of melanin in the tadpoles and that fatalities occurred at intervals during treatment at this dose. A concentration of phenylthiourea low enough to avoid fatalities did not completely inhibit melanogenesis. The water and treatment-solutions were changed daily.

The course of the experiments was followed by anaesthetizing tadpoles with a 1:5000 solution of M.S. 222 and observing pigmentation both with the naked eye and with a dissecting microscope; they were then returned to their respective vessels to recover. For more detailed examination specimens were fixed in this solution:

formalin (commercial solution of formaldehyde, about 40%) 5 ml
acetic acid (glacial) 0·5 ml
distilled water up to 100 ml

The specimens were dehydrated in ascending grades of alcohol and passed through xylene into Canada balsam. When tails were mounted whole, they were stained in 1% aqueous neutral red, washed in distilled water, transferred on to a microscope slide, dehydrated in an oven at 39° C, and then cleared in xylene before being mounted in balsam. This method of dehydration greatly reduces the thickness of the tail and allows a search for melanin granules to be made with a 3·6-mm oil-immersion objective. Serial paraffin sections 10 μ thick were prepared and stained with Weigert's haematoxylin and eosin, but it was found that the best method for demonstrating melanin was to stain in bulk with borax carmine.

Four sets of experiments were conducted and will be outlined here; details will be presented with the observations. The animals were 'staged' at the beginning and end of the experiments; that is to say, the stage of development was determined and noted down as a number, in accordance with the scheme published by Niewkoop and Faber (1956). At the same time observations were made on the histology of their thyroid glands. The animals used in experiments 1 and 2 were taken from a single batch of eggs, those in experiment 3 from another batch of eggs, and those in experiment 4 from a third batch of eggs.

Experiment 1 was designed to demonstrate the inhibition of melanogenesis by 0·01% phenylthiourea. Eggs were reared in tap-water and in tap-water containing 0·01% phenylthiourea.

Experiment 2 was designed to demonstrate the turnover of melanin by treating one set of tadpoles with phenylthiourea to inhibit melanogenesis while another set was kept in tap-water as controls.

Experiment 3 was designed to demonstrate both the inhibition of melanogenesis and the rate of turnover of melanin of known age in tadpoles at the same stage (later than that of experiment 2).

Experiment 4 was designed to demonstrate the turnover of melanin in animals which had almost completed metamorphosis.

Observations

Experiment 1. Early in the morning 150 eggs were taken from a batch laid overnight and divided into two groups. 50 were reared in tap-water while the other 100 were reared in a solution of phenylthiourea in tap-water. At this time the eggs were undergoing gastrulation: formation of the neural plate and its derivatives had not begun.

During the following days a difference in colour between the control and treated animals became progressively more marked. The control group developed deeply pigmented melanophores around the nasal pits, on the dorsum of the trunk, over the tail musculature, and in the meninges, parietal peritoneum, and pronephros. The pigmented coat of the eye was a dense black. The treated animals were almost transparent, except the coiled intestine which was dark brown and opaque even before feeding had started. No melanophores containing black pigment could be found when the animals were examined alive and the pigmented layer of the eye had a faint yellow appearance.

In order to confirm that the distribution of melanophores was similar in the treated animals and in the controls, several of the treated animals were transferred to tap-water alone on the 9th day of the experiment. Melanin began to appear after 2 days. Its distribution was the same as that in the controls and it did not appear in any one region before another.

The experiment was terminated on the 20th day. All the animals were staged. The results are presented in a histogram (fig. 1), from which it can be seen that the number of survivors in each group was about the same, and

the majority of the treated group were one stage behind the control group in development.

In the sectioned material the thyroid glands of the control group consisted of cell-nests showing the beginning of follicle formation, while in the treated group the cell-nests were smaller and no evidence of follicle formation was found.

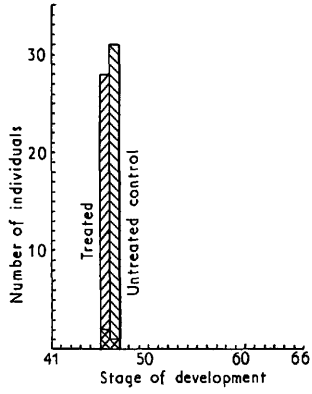

FIG. 1. *Experiment 1.* Histogram showing the number of *Xenopus* tadpoles at each stage of development, 20 days after laying. ▨ treated with phenylthiourea; ▧ untreated control group.

The melanophores of the control group contained dark black melanin. In the whole mounts melanophores were seen in the region of the nasal pits, on the medial wall of the otic capsule, on the superficial surface of the axial muscles of the trunk and tail, on the pronephros, over the parietal peritoneum, and scattered over the head immediately beneath the epithelium and in the dorsal and ventral fins. The melanin of the eye rendered it pitch-black (fig. 2, A). These observations were readily confirmed on the sectioned specimens, in which an abundance of melanophores was found also on the lungs, in the mesentery of the intestine, and among the muscle-fibres of the axial muscles. Cells packed full of melanin granules were scattered throughout the

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FIG. 2 (plate). A, *Experiment 1.* Oblique section through the pigmented coat of the eye of a tadpole of *X. laevis* at stage 47, reared in tap-water for 20 days after the eggs were laid. An abundance of melanin is present.

B, *Experiment 1.* Oblique section through the pigmented coat of the eye of a tadpole of *X. laevis* at stage 46, reared in a solution of phenylthiourea for 20 days after the eggs were laid. Melanin is lacking.

C, *Experiment 2.* Oblique section through the pigmented coat of the eye of a tadpole of *X. laevis* at stage 47, after it had been kept in a solution of phenylthiourea for 6 weeks from the time it reached stage 46 or 47 in development. No reduction in the amount of melanin is apparent. Compare A.

D, *Experiment 3.* Area of tail-regenerate of group A kept in tap-water for 8 weeks after amputation of the tail. There is an abundance of melanin in the melanophores.

E, *Experiment 3.* Area of tail-regenerate of group C which was allowed to develop a little melanin before being treated with phenylthiourea for 6 weeks. No loss of melanin was detected.

F, *Experiment 3.* Area of tail-regenerate of Group B which was treated with phenylthiourea continuously for 8 weeks after amputation of the tail. Melanophores cannot be seen owing to the absence of melanin.

G, *Experiment 4.* Part of a section of the hind limb of an untreated control animal, showing melanophores among the cells of the epidermis and beneath it.

H, *Experiment 4.* Part of a section of the hind limb of an animal treated with phenylthiourea for 6 weeks, showing melanophores among the cells of the epidermis and beneath it containing the same amount of melanin as those in the control group.

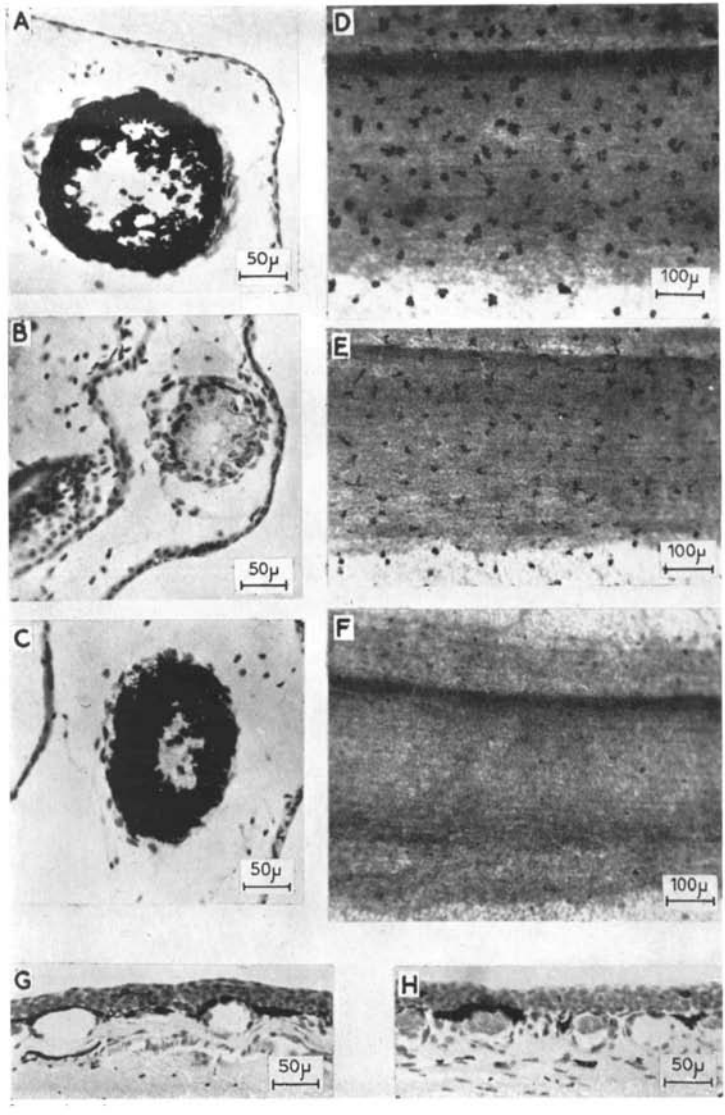

FIG. 2

R. T. SIMS

liver, along the walls of the sinuses. Occasional melanophores were insinuated about the bases of the epithelial cells of the intestine.

The pigment within the melanophores of the treated animals was a very light yellow in the whole-mount specimens. It was visible only in the meninges and on the superficial surface of the axial musculature; the pigmented coat of the eye was light yellow (fig. 2, B). Scattered throughout the liver were small areas of pigment varying in colour from light yellow to black. The coiled intestine was uniformly dark brown, with pitch-black areas distributed in its walls. Where it was present, the pigment in the melanophores of the sectioned material of the treated animals was of the same yellow colour as that seen in the whole mounts. The number of pigment granules in each cell was judged to be less than in the control series, as each granule was distinct from the others in its cell, while in the control series individual granules could not be discerned as a result of overlapping and super-positioning. The amount of pigment in each section was markedly less in the treated group than in the controls. This was especially noticeable in the meninges, axial musculature, and pronephros. The greatest amount of melanin present in the treated animals was in the liver and coiled intestine. As in the control series the pigmented cells were on the walls of the liver sinuses and even here the granules were separate in the treated animals in contrast to the controls. The coiled intestine was the only site where melanin was more abundant in the treated animals than in the controls. The melanophores were insinuated about the bases of cells of the intestinal epithelium and were full of black granules. No melanin was found in the region of the nasal pits, the otic capsules, the parietal peritoneum, scattered over the head immediately beneath the epidermis, in the lungs, or in the mesentery of the intestine. These were sites in which it developed in those animals that had their treatment stopped on the 9th day of the experiment. Apart from the marked difference in pigmentation, the structure of the eyes in the treated group differed in no respect from those of the controls.

Experiment 2. A large batch of tadpoles reared from the same spawn as those used in the first experiment was taken when they had reached stage 46 or 47, as at this point they had well-developed melanophores, feeding had begun, and the tadpoles were breathing air. A number were fixed and the rest were divided into two groups, one of which was reared in tap-water while the other was reared in a solution of phenylthiourea in tap-water. Six weeks later all the animals were staged and fixed.

The animals fixed at the beginning of the experiment were at stage 46 or 47. In sections their thyroid glands were seen to consist of large groups of cells; no follicles were present. The distribution of melanophores, which contained large amounts of black pigment, was identical with that found in the control group at the end of experiment 1.

The stages that the animals had reached by the end of the experiment are presented in the histogram (fig. 3). Almost nine-tenths of the control population were at stages 55, 56, or 57, whereas the treated group were still at stage

46 or 47. The thyroid glands of the control group were made up of large follicles with deeply pigmented melanophores in the connective tissue between them. The epithelial cells lining the follicles were about twice as high as they were broad and contained chromophobe vacuoles: the colloid also contained many chromophobe vacuoles. In the treated group the thyroid glands were made up of large follicles, surrounded by a cuboidal epithelium with scanty cytoplasm and no chromophobe vacuoles in the cells or the colloid. No melanin was present in the connective tissue.

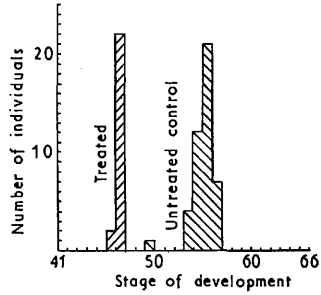

FIG. 3. *Experiment 2.* Histogram showing the number of *Xenopus* tadpoles at each stage of development. ▨ treated with phenylthiourea for 6 weeks; ▧ untreated control group.

The melanin of the experimental animals (fig. 2, c) was pitch-black and had exactly the same distribution as that of the normal animals fixed 6 weeks earlier. As the stages of the experimental animals were so different from those of the control animals at the end of the experiment, it was felt that a comparison of melanin in these two groups was unwarranted. A more reliable comparison was obtained by comparing the experimental animals at the end of the experiment with those fixed at the beginning, as they were at the same stage of development.

Experiment 3. 132 tadpoles, all at stage 49, were taken from a batch obtained from two different adults from those used in the first two experiments. They were kept in phenylthiourea solution for 12 days in order to overcome any latent period between the beginning of administration of the drug and the full effect of its action: during this time 7 of the tadpoles died. They were then all staged again, and without exception found to be still at stage 49. The last 5 mm was cut off their tails while they were lightly anaesthetized and a number of the amputated segments were fixed. Over the next 15 days the amputated portion of the tails regenerated and this was allowed to occur in tap-water with 40 of them and in phenylthiourea solution with the remainder. When the tails had regenerated, the two groups were examined for the presence of melanin in the regenerate of the living animals and a small number were fixed. No melanin could be seen in the treated group, while an abundance was present in the control group kept in tap-water. At this point half of the treated group were transferred to tap-water for 4 days to allow melanogenesis to begin. At the end of this time black melanin granules had just begun to appear and the treatment with phenylthiourea was started again. In this way 3 groups of experimental animals were obtained and designated A, B, and C. Group A had been kept continuously in tap-water since the tails had been amputated, group B had been kept continuously in phenylthiourea solution since the beginning of the experiment, and

group C had been kept under the same conditions as group B except for 4 days, when it was allowed to form a small quantity of melanin. From the time of appearance of this melanin the experiment was allowed to run for 6 weeks; then all the surviving animals were staged, all the members of groups A and C and half the members of group B were fixed. The rest of group B had their treatment stopped and were kept in tap-water until melanin could be seen in the regenerates, when they were fixed.

The melanophores in the amputated segments of the tails were packed so full of black melanin that individual granules could not be distinguished. They were distributed in the dorsal and ventral fins, over the tail-muscles, and in the meninges. After regeneration had taken place the regenerates of those kept in tap-water contained melanophores in the same sites as the amputated segments, and again their colour was pitch-black and the individual granules could not be distinguished. On the contrary, in the treated group the number of melanophores containing melanin decreased rapidly at the base of the regenerate and so did the number of granules within them, until no melanin could be seen in the distal three-quarters of the regenerate.

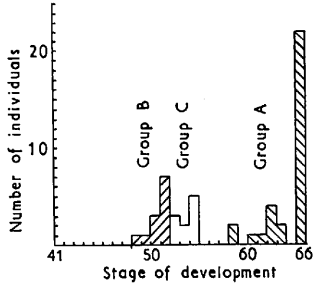

FIG. 4. *Experiment 3.* Histogram showing the number of *Xenopus* tadpoles at each stage of development. All the tadpoles were at stage 49 at the beginning of treatment and all had the last 5 mm of their tails amputated. ▨ group A treated for 12 days with phenylthiourea and then kept in tap-water for 8 weeks; ▨ group B treated continuously for 10 weeks with phenylthiourea; □ group C treated for 10 weeks with phenylthiourea, except during 4 days towards the beginning of the experiment.

It was at this point that the three groups were set up and group C was allowed to begin melanogenesis. The animals were examined every day for the appearance of melanin in the regenerate and it was noticed that it formed at the same time throughout this region and not in any one area before another. The distribution of the melanophores was the same as that in group A, kept in tap-water, but treatment was started again while the individual black melanin granules could still be distinguished and the melanophores were not packed full of them. There was no change in the regenerates of group B during this time.

The stages of development that the 3 groups had reached by the end of the experiment are presented as the histogram (fig. 4). It can be seen that group A were about 10 stages in advance of groups B and C, and group C was slightly ahead of group B. The thyroid glands of groups B and C consisted of large follicles, surrounded by cuboidal epithelial cells that were strongly basiphil. No chromophobe vacuoles were present in the colloid or in the cells. In group A the thyroid glands consisted of large follicles in which the epithelial cells formed a larger part of the follicle than did the colloid. The

epithelial cells were columnar and had a long axis about 10 times greater than their transverse axis, and both cells and colloid contained numerous chromophobe vacuoles that were confluent in the latter.

At the end of the experiment the tails of group A were in various stages of regression. However, in the less mature members of the group the melanophores of the regenerates were distributed in the same manner as before and contained too much melanin for the individual granules to be discerned (fig. 2, D). In group B the melanin in the regenerates showed no change. The melanophores containing melanin decreased rapidly in number at the base of the regenerate as did the amount of melanin within them, and in the distal three-quarters of the regenerate melanin was absent (fig. 2, F). In the part of the tail not removed by the operation the melanophores were still so full of melanin that the individual granules could not be discerned. The regenerates in group C contained melanophores in which melanin was still present as before (fig. 2, E) except at the very tips where it was absent or present in only one or two cells.

Although the mortality was high, the deaths occurred uniformly throughout the experiment and did not warrant reducing the concentration of phenylthiourea or shortening the duration of the experiment. When dead animals were found they were fixed immediately and the regenerates examined for melanin. In all cases it was found that their appearance at death corresponded to that of the survivors in the group to which they belonged.

The members of group B that were kept in tap-water until they developed melanin all did so within 14 days, and it appeared at the same time throughout the regenerate and not in one place before another.

Experiment 4. Twenty-six tadpoles all at stage 66 were taken from a batch obtained from two different adults from those used in the first 3 experiments. At this stage only a small stump of a tail remains to be absorbed before metamorphosis is completed. Eighteen were kept in a solution of phenylthiourea in tap-water and 8 kept in tap-water as controls. The experiment was run for 6 weeks and then all the surviving animals were fixed: during this time 7 of the treated animals died. When the experiment was terminated all the animals in both control and treated groups had completed metamorphosis.

Macroscopic examination revealed only one difference between the two groups of animals. The claws of the 3 pre-axial digits of the foot were black in the control group and brown in the treated group. The thyroid gland with the larynx, the liver, and the right hind-limb were dissected out of each animal for sectioning.

The thyroid glands of the control group consisted of follicles that were only half full of stainable granular colloid, in which chromophobe vacuoles were infrequent. The epithelium was columnar, the long axis of the cells being twice as long as the width, and the nuclei were densely stained and compact. The thyroid glands of the treated group consisted of follicles that contained scanty stainable, homogeneous, hyaline colloid which contained numerous chromophobe vacuoles. The epithelium was columnar, the long

axis of the cells being 5 to 10 times greater than their width, and the basal region of the cell was basiphil while the follicular region was eosinophil and granular. The nuclei were placed towards the free margin of the cells and were vesicular in appearance with prominent basiphil nucleoli.

In the livers and hind limbs of the control animals were many melanophores which contained an abundance of black melanin granules. As in the earlier stages those in the liver lined the sinuses. The melanophores of the hind limbs were distributed among the cells of the epidermis, immediately beneath the epidermis, along the blood-vessels, and in the marrow cavity of some of the bones, notably the phalanges. No difference in appearance or distribution was found in the treated animals (fig. 2, G–H).

The cornified claws of the control animals were pitch-black in sections whereas those of the treated group were light brown with a granular appearance, but neither group had any melanophores situated near this region of the epidermis or dermis.

Discussion

In the first experiment the formation of melanin was prevented by treating eggs with phenylthiourea. At the same time development of the treated group was slightly retarded but the histology of their thyroid glands was usual for the stage of development that they had reached. It is concluded that phenylthiourea at a concentration of 0·01% inhibits melanogenesis in tadpoles of *X. laevis* as it does in other Anura (Lynn, 1948; Millott and Lynn, 1954). Unlike the indophenol dyes used by Lewis (1932), no abnormalities of the optic cup were produced apart from the absence of melanin. This is evidence against her suggestion that melanin must be present in the pigmented layer if normal development of the eye is to occur. When tadpoles reared in the solution of phenylthiourea were transferred to water, melanin appeared simultaneously in the melanophores of all regions. This resembles the observations of Lehmann (1957) on *Amblystoma mexicanum* and supports the conclusion that lack of pigment does not influence the distribution of melanophores.

When it had been shown that phenylthiourea inhibited melanogenesis in eggs taken from one batch, the second experiment was undertaken to demonstrate the turnover of melanin. Tadpoles taken from the same batch were used. Thus genetic variations in the effect of phenylthiourea were minimized. Although the tadpoles were treated for 6 weeks, no loss of melanin was detected. This is in marked contrast to the disappearance of pigment in 5 days in *Eleutherodactylus* reported by Lynn (1948) and Millott and Lynn (1954). The check in development and the changes in the histology of the thyroid glands resemble those found by Gordon, Goldsmith, and Charipper (1945) in tadpoles of *R. pipiens* after prolonged administration of thiourea. This was regarded as evidence that phenylthiourea was reaching the tissues of the treated animals. This raised the possibility that phenylthiourea only prevented melanin formation if treatment was started immediately after the

eggs were laid and not if treatment was started at later stages. The third experiment was designed to cover this eventuality.

The basis of the third experiment was that 3 groups of experimental animals, designated A, B, and C, were treated in different ways to influence the melanophores of tail-regenerates. They were all reared from a single batch of eggs and at the beginning of the experiment were all selected at the same stage of development. Group A were controls kept in tap-water. They showed the formation of melanin in the melanophores of the regenerate, the development of untreated tadpoles, and the histology of the thyroid gland. Group B were treated with phenylthiourea continuously for a period of 10 weeks. They showed the inhibition of melanogenesis in the regenerate and lack of turnover in the rest of the animal. The treatment of group C with phenylthiourea was suspended for 4 days to allow a small amount of melanin of known age to appear in the regenerate. As in the tadpoles of the first experiment which had their treatment stopped, melanin formed simultaneously in the melanophores throughout the regenerate, confirming again the conclusion of Lehmann (1957) that lack of pigment does not affect the distribution of melanophores. No detectable loss of this new melanin occurred during the following 6 weeks' treatment. The absence of melanin at the tip of the tail at the end of the experiment was regarded as evidence that inhibition of melanin formation occurred. The development of group C was arrested at a slightly later stage than that of group B, as a result of the short suspension of treatment. The histology of the thyroid glands at the end of this experiment was similar in groups B and C. The changes resembled those found in the second experiment, and indicated that phenylthiourea reached the tissues of the animals.

In view of the fact that *Eleutherodactylus* has no tadpole stage in its development, the possibility that the turnover of melanin was faster in the adult form than in the tadpole form remained to be excluded. A fourth experiment was performed with animals possessing only a small stump of tail at stage 66. Again no diminution of melanin was detected after a period of 6 weeks' treatment. The changes in the histology of the thyroid glands, which resembled those described by Joel, D'Angelo, and Charipper (1949) in adult *R. pipiens* treated with thiourea, indicated that phenylthiourea reached the tissues of the treated animals.

These experiments show that in *X. laevis* tadpoles treated with phenylthiourea there is no significant turnover of melanin, melanin formed before the beginning of treatment is not destroyed, development is arrested, and changes are produced in the histology of the thyroid gland resembling those produced by thiourea. Re-examination of all the sections prepared from treated and untreated animals used in this investigation revealed no instance in which melanin granules were situated within epithelial cells of the skin, as described and illustrated by Ehrmann (1885) for an unspecified frog. This is consistent with the lack of turnover of melanin in the treated animals. Once melanin has appeared it remains in the melanophores of this animal. They

would be a sensitive indicator of the efficacy of inhibitors of melanogenesis during life, and make it easy to compare the action of these agents *in vitro* and *in vivo*.

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