The action of phenyl-thiourea on melanogenesis in *Xenopus laevis*

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With one plate (fig. 1)

Summary

From *Xenopus laevis* tadpoles which possessed conical hind-limb buds with pigmented melanophores the caudal two-thirds of the tail was amputated. One group of these animals was kept in tap-water and another group in 0·01% phenyl-thiourea in tap-water while regeneration of the tails took place. After one month the tails of the operated animals together with tails from a control group of normal animals at the same stage of development were examined histologically. In the normal tail melanin was only absent from the region of the ventral fin immediately behind the anus: in the untreated regenerate melanin was only absent from the major part of the ventral fin: in the treated regenerate melanin was absent from the whole regenerate. Branched DOPA-positive cells situated beneath the epidermis and each accommodated by a circle 0·05 mm in diameter were present in the normal tails, untreated regenerates, and treated regenerates. They were present only in the regions lacking melanophores in the normal tail and untreated regenerate, but in the animals treated with phenyl-thiourea they were present in the unpigmented regenerate and in the pigmented part of the tail-stump. The pigmented melanophores were DOPA-negative. Cells which were stained by the hexamine-silver technique and Schmorl's reaction were found only in the regenerate of the treated tadpoles. It is suggested that the DOPA-positive cells are potential melanophores. In the normal tail and untreated regenerate tyrosinase activity was present without promelanin granules and in the treated regenerate tyrosinase activity was present with promelanin granules. The negative response of pigmented melanophores could reflect a loss of tyrosinase activity as pigmentation of the granules is completed. Phenyl-thiourea does not inhibit melanogenesis *in vivo* by acting on a metabolic process essential for the normal activity of the cell, or by inhibiting the production and action of tyrosinase, or by preventing formation of promelanin granules, as these were all present in the treated animals. A test-tube experiment showed that phenyl-thiourea inhibits the autoxidation of DOPA. It is suggested that the drug acts by combining with an intermediate produced during the conversion of tyrosine to melanin.

Introduction

THE study of inhibitors of melanogenesis is conducted in the hope that a method will be found to exploit the process for the treatment of malignant melanoma. Phenyl-thiourea is a powerful inhibitor of melanogenesis worthy of further study. Its mode of action has been investigated *in vitro* on tyrosinase prepared from plant and animal sources, but with inconsistent results. Bernheim and Bernheim (1942) stated that phenyl-thiourea had no effect on the autoxidation of 3, 4-dihydroxyphenylalanine (DOPA) and concluded from their experiments with plant tyrosinase that the drug becomes attached to the

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surface of the enzyme. Paschkis, Cantorow, Hart, and Rakoff (1944) suggested that phenyl-thiourea combined with the copper of tyrosinase, and they showed that copper sulphate decreased the ability of phenyl-thiourea to inhibit melanogenesis by plant tyrosinase. This effect of copper sulphate was confirmed by Dubois and Erway (1946). Experiments with mammalian tyrosinase were performed by Lerner, Fitzpatrick, Calkins, and Summerson (1950). They found that the curve obtained for phenyl-thiourea by plotting the relative enzyme activity against the logarithm of the inhibitor concentration did not resemble that of any simple enzyme-inhibitor reaction. Also copper sulphate did not reverse the inhibition of tyrosinase by phenyl-thiourea, although it did reverse the inhibition produced by other copper-combining substances. They concluded that phenyl-thiourea inhibits tyrosinase in a way which does not involve combination with the copper in the enzyme.

The experiments reported here represent an attempt to obtain information about melanogenesis *in vivo* and the inhibitory action of phenyl-thiourea on this process. The drug might act on the enzyme tyrosinase, the substrate, the intermediates, the formation of the promelanin granule, the production of tyrosinase, or another metabolic process of the cell essential for its normal activity. If the action of the drug is on the last 3 of these possibilities it would not be revealed by an experiment performed *in vitro*.

In the course of work already reported (Sims, 1961) it was shown that in tadpoles of *Xenopus laevis* melanogenesis is inhibited by phenyl-thiourea and that a preparation of melanophores with no pigment in them is easily obtained by amputating the tails of the tadpoles and allowing regeneration to occur while the tadpoles are treated with the drug.

Material and method

Ovulation and amplexus were induced in adult *X. laevis* with chorionic gonadotrophin. The eggs were allowed to develop in glass vessels kept on a matt black surface in a cupboard, the temperature of which was maintained at 20° C. When necessary the water was aerated and the tadpoles were fed with a suspension of nettle-powder in water. The methods used to rear and stage the animals were those recommended by Nieuwkoop and Faber (1956). The experimental animals were kept under the same conditions.

Tadpoles were taken for operation when their hind-limb buds were conical in shape and possessed pigmented melanophores: that is stage of development number 51 in the scheme published by Nieuwkoop and Faber (1956). The tadpoles were anaesthetized with a 1:5,000 solution of M.S. 222 (Sandoz) and the caudal two-thirds of their tails amputated with a pair of fine scissors. The stump of the tail which remained on the animal had no melanin in the dorsal and ventral fins. The operated animals were divided into 2 groups. One group was kept in tap-water and the other was kept in a solution of 0·01% phenyl-thiourea in tap-water while regeneration of the tails took place. The water and treatment solution were changed daily. One month after the operation the tail was cut off at the level of the anus, so that the tail-stump was

preserved with the regenerate, for histological examination. At this time normal tails were available from unoperated tadpoles at the same stage of development as the experimental groups and reared from the same batch of eggs. In this way 3 groups of tails were obtained: first a group of normal untreated tails; second a group of untreated regenerates with their stumps; and third a group of treated regenerates with their stumps. For each histochemical test 3 tails from each group were used and they were stained and mounted whole. The experiment was repeated on tadpoles obtained from 2 different adults.

A set of unstained whole mounts was prepared to show the distribution of pigmented cells under the conditions of the experiment. The tails 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

They were dehydrated in ascending grades of alcohol and passed through xylene into Canada balsam.

The presence of the enzyme tyrosinase was demonstrated by the DOPA-reaction. The method used was that given by Pearse (1960), modified to suit the tissue. The substrate was dissolved in 0·05 M phosphate buffer at pH 7·4 and the tissue incubated for 15 h at 20° C. The fixation performed after incubation was made with the formalin-acetic acid mixture used for the unstained specimens. Billingham and Medawar (1953) suggested that the ability of living melanocytes in human and guinea-pig skin to keep methylene blue in the coloured oxidized state is a consequence of their oxidase content. Therefore intravital staining with methylene blue was employed in this investigation. A solution of 0·01% methylene blue in Holtfreter's solution containing 0·05% sodium bicarbonate was injected through a fine glass micropipette into the third aortic arch of living anaesthetized tadpoles. The tadpoles were observed for a period of 3 h.

The Bensley and Gersh modification of the Millon reaction (Pearse, 1960) was used to demonstrate the presence of tyrosine.

Melanin granules and promelanin granules were stained by Gomori's hexamine-silver method and Schmorl's reaction according to the methods given by Pearse.

Observations

Unstained material

Normal tail (fig. 1, A). The melanin of pigmented regions appeared evenly distributed to the naked eye. With a dissecting microscope melanophores were seen on the superficial surface of the muscle with occasional cells among the muscle-fibres, in the meninges, around the caudal artery, around the dorsal tributary of the caudal vein, and in the fins of the terminal two-thirds of the tail. There was no melanin in the region of the ventral fin immediately behind

the anus. The melanophores contained melanin which was black or dark brown. When their pigment was dispersed their apparent size varied from cells whose branches occupied a circle of 0·05 mm diameter to cells whose branches occupied a circle of 0·25 mm diameter (fig. 1, D, E). When the pigment granules were aggregated they occupied a circle of not more than 0·04 mm diameter (fig. 1, F). The branches of neighbouring cells did not overlap when their pigment was dispersed.

Untreated regenerate (fig. 1, B). The state of the tail-stump resembled that of the same region in the normal tail. The regenerated part of the tail was recognized by the absence of the dorsal fin. The pigmentation over the musculature of the regenerate appeared more intense to the naked eye than that elsewhere in the specimen. The distribution of melanophores, their size, and the colour of their melanin, was similar to that of the normal tail. However pigment was present only in a narrow strip of the ventral fin at its junction with the tail musculature. The branches of neighbouring cells overlapped when their pigment was dispersed. This gave the impression that there were more melanophores per unit area in the regenerate than in the tail-stump.

Treated regenerate (fig. 1, C). The state of the tail-stump resembled that of the same region in the normal tail. The regenerated part of the tail was recognized by the absence of the dorsal fin. The number of melanophores and the amount of pigment in them decreased rapidly at the amputation site. The terminal three-quarters of the regenerate contained no melanin.

DOPA reaction

Normal tail. DOPA-positive cells were found only in regions containing no melanophores. They were most evident in the region of the ventral fin immediately behind the anus. The stained cells were situated beneath the epidermis, were branched, and were accommodated by a circle of 0·05 mm diameter. Many such cells were surrounded by a halo of black stain, which is a diffusion artifact associated with a positive result. At the boundary of the pigmented

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FIG. 1 (plate). A, distribution of melanin in an unstained whole-mount of a tail from normal *Xenopus*.

B, distribution of melanin in an unstained whole-mount of a tail with regenerate from untreated *Xenopus*.

C, distribution of melanin in an unstained whole-mount of tail with regenerate from *Xenopus* treated with phenyl-thiourea.

D, small melanophores in unstained whole-mount of a tail from normal *Xenopus*.

E, large melanophores with pigment granules dispersed in unstained whole-mount of a tail from normal *Xenopus*.

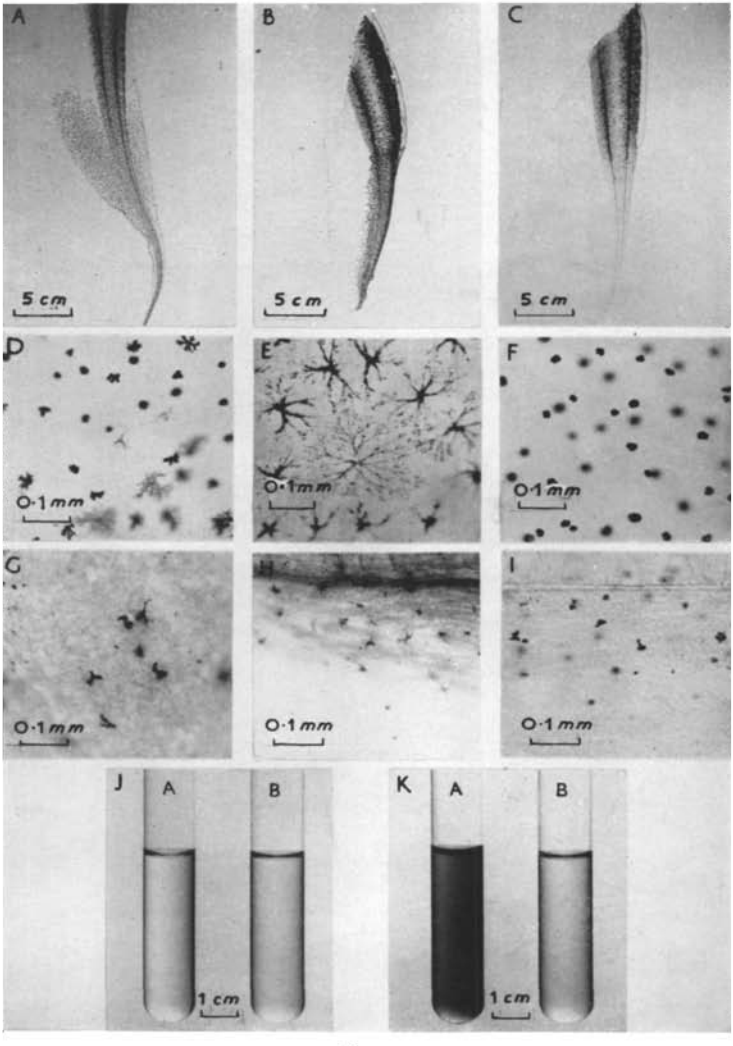
F, melanophores with pigment granules aggregated in unstained whole-mount of a tail from normal *Xenopus*.

G, DOPA-positive cells in tail regenerate of *Xenopus* treated with phenyl-thiourea.

H, cells stained by Schmorl's reaction in tail regenerate of *Xenopus* treated with phenyl-thiourea.

I, cells stained by the hexamine-silver method in tail regenerate of *Xenopus* treated with phenyl-thiourea.

J and K, both test tubes contain a solution of DOPA at pH 7·4. The solution in tube *B* is saturated with phenyl-thiourea. J, Before incubation. K, After 24 h incubation.


A
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K
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FIG. 1

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zone no intermingling of the DOPA-positive cells and melanophores occurred. The melanophores were DOPA-negative. The melanin granules present may have obscured the stain but none of the melanophores showed the diffusion artifact and cells with aggregated pigment had no stain in their peripheral cytoplasm.

Untreated regenerate. DOPA-positive cells with the same characteristics as those in the normal tail were confined to regions containing no melanophores. The stained cells were intermingled with the melanophores at the boundary of the pigmented zone. It was at this boundary region that the similarities in position, size, and shape of the DOPA-positive cell and the melanophores were most evident. As in the normal tail the melanophores were DOPA-negative.

Treated regenerates. The unpigmented ventral fin and regenerate contained many DOPA-positive cells with the same characteristics as those in the untreated material (fig. 1, G). The stump of the original tail had DOPA-positive cells among its melanophores. The melanophores were in the upper region of the size-range and contrasted with the size of the DOPA-positive cells.

Intravital methylene blue

Normal tail, untreated and treated regenerate. Erythrocytes, endothelial cells of blood-vessels, axons of peripheral nerves, and Schwann cell nuclei were stained bright blue. The melanophores and DOPA-positive cells were not stained.

Millon reaction

Normal tail, untreated and treated regenerate. There was no reaction in melanophores and DOPA-positive cells.

Hexamine-silver reaction

Normal tail and untreated regenerate. There were no cells stained.

Treated regenerate. No cells were stained in the stump of the original tail. In the regenerate, cells similar in size and shape to DOPA-positive cells contained a brown granular deposit. They were distributed over the muscle with an occasional cell in the dorsal part of the ventral fin (fig. 1, I).

Schmorl's reaction

Normal tail and untreated regenerate. There were no cells stained.

Treated regenerate. No cells were stained in the stump of the original tail. In the regenerate the cytoplasm of cells similar in size and shape to DOPA-positive cells was stained dark green and had a granular appearance. They were distributed over the muscle with an occasional cell in the dorsal part of the ventral fin (fig. 1, H).

Test-tube experiment

50 c.c. of a solution containing 0·1% DOPA in 0·05 M phosphate buffer at pH 7·4 was divided into two equal parts which were designated A and B. Solution B was saturated with phenyl-thiourea and filtered. The pH of both

solutions was measured with a glass-electrode pH meter and they were found to be identical at pH 7·4. They were incubated for 24 h at 20° C. Before incubation both solutions were colourless: after incubation solution A was black and solution B was colourless.

Discussion

In Amphibia a positive response to the DOPA-reaction may not be confined to cells capable of forming melanin. Dushane (1936) found that epidermal cells, mucus secreting cells, and red cells of *Axolotl* and *Triturus* skin were all DOPA-positive in addition to the melanophores. It may be that some of these cell types stained because the tissues were incubated at 36° C for 6 to 11 h. In the experiments reported here the tissues were incubated at the much lower temperature of 20° C for 15 h and only one type of DOPA-positive cell was found. The distribution and morphology of the DOPA-positive cells in the tail of *Xenopus* do not resemble the distribution and morphology of any of the cell types found to be DOPA-positive by Dushane (1936), so it may be stated that the DOPA-positive cells demonstrated in the *Xenopus* tail are not epidermal cells, mucus secreting cells, red cells, or melanophores. In normal *Xenopus* tails and untreated tail regenerates the DOPA-positive cells were situated only in regions which contained no pigmented melanophores. It is unlikely that any type of cell would have such a distribution unless it had some association with melanophores. The cells most likely to possess this distribution and be DOPA-positive are melanoblasts. The existence of melanoblasts has not been demonstrated in Anura but their presence has been revealed in Urodela by ectodermal transplants (De Lanney, 1941). The same experiments also showed that ectoderm may have a role in the control of melanin synthesis by melanophores. Twitty and Bodenstein (1944) grafted neural-crest tissue to different positions on *Triturus* embryos and found that the conditions favouring melanin formation varied in different regions. It is possible that the DOPA-positive cells and the melanophores in *Xenopus* tails represent a single type of cell, the former situated in an environment unfavourable for pigment synthesis, the latter situated in an environment favourable for pigment synthesis. If this is so then tyrosinase can be present in melanoblasts without promelanin granules demonstrable by the hexamine-silver technique or Schmorl's reaction. These methods revealed no promelanin granules in normal *Xenopus* tails and untreated regenerates. The DOPA-positive cells among the melanophores of the tail-stump of the tadpoles treated with phenyl-thiourea can be regarded as cells which normally would have developed pigment as growth occurred. The treatment prevented melanogenesis and arrested the differentiation of the cells at the melanoblast stage.

The negative response of the normal melanophores to the DOPA-reaction was unexpected. It could mean that they do not produce melanin themselves but obtain it from another cell. The absence of DOPA-positive cells, which might supply the melanin, in regions populated by melanophores makes this hypothesis unlikely. It seems more reasonable to suggest that tyrosinase

activity is lost when a melanophore has formed its complement of melanin. As there is evidence which indicates that no turnover of pigment takes place in *Xenopus* melanophores (Sims, 1961), tyrosinase activity need not be present throughout the life of the cell.

In the tail regenerates of the tadpoles treated with phenyl-thiourea cells were found which were stained by the hexamine-silver technique and Schmorl's reaction. Similar cells were not found in the tail regenerates of the untreated tadpoles so their presence must be related to treatment with the drug. As they were absent from the tail-stump of the treated animals they cannot represent a general reaction to the drug and must be an expression of a difference in the local cells. In the untreated regenerates the corresponding region was occupied by pigmented melanophores so in the treated regenerates the stained cells could represent melanophores which contained propigment granules, the formation of melanin on the propigment granules having been prevented by the treatment with phenyl-thiourea. The staining properties of the cells in the tail regenerates of tadpoles treated with phenyl-thiourea as found in these experiments would be compatible with the presence of melanophores in three different states. In the ventral fin were cells which were DOPA-positive and possessed no granules, over the tail musculature were cells which were DOPA-positive and possessed unpigmented granules, and in the tail-stump were cells which were DOPA-negative and possessed pigmented granules. This possibility is compatible with the method of production of melanin granules proposed by Seiji, Fitzpatrick, and Birbeck (1961).

The presence of DOPA-positive cells in the tail regenerates of animals treated with phenyl-thiourea suggests that the drug does not affect the synthesis and action of tyrosinase. This view conflicts with the conclusions from all the work on this problem which has been performed *in vitro*. The presence of DOPA-positive cells in the regenerate is further evidence which supports Lehman's (1957) observation that inhibition of pigmentation by phenyl-thiourea has no influence on the distribution of potential melanophores. As the distribution of these cells was not affected, phenyl-thiourea does not act on a metabolic process which is essential for their normal activity. An action of the drug on the formation of promelanin granules is excluded by the demonstration of promelanin granules in the treated regenerates with Schmorl's reaction and the hexamine-silver technique. The possibility of the drug inhibiting melanogenesis by combining with intermediates formed during the process could not be tested by an experiment *in vivo*. The test-tube experiment described showed that phenyl-thiourea prevented the formation of DOPA-melanin from DOPA even when tyrosinase was absent from the system. On this evidence it is concluded that phenyl-thiourea inhibits melanogenesis *in vivo* by combining with DOPA or some other intermediate of the reaction. As this conclusion is in conflict with all previous views on the mode of action of the drug the reports of the *in vitro* experiments were carefully re-examined. Only Bernheim and Bernheim (1942) mention the performance of an experiment on the autoxidation of DOPA and they found that phenyl-

thiourea did not inhibit this process at pH 7·8. They measured the oxidation by estimating oxygen consumption in the Warburg apparatus, but did not record the concentrations of DOPA and phenyl-thiourea in the test mixtures so a comparison with the experiment reported here is not possible. If the suggested mode of action of phenyl-thiourea on melanogenesis is confirmed it may prove possible to control the pigmentation which accompanies chronic conjunctivitis in dogs by topical administration of the drug.

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