SOME ASPECTS OF MELANIN PIGMENTATION

IN AMPHIBIA

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CONTENTS

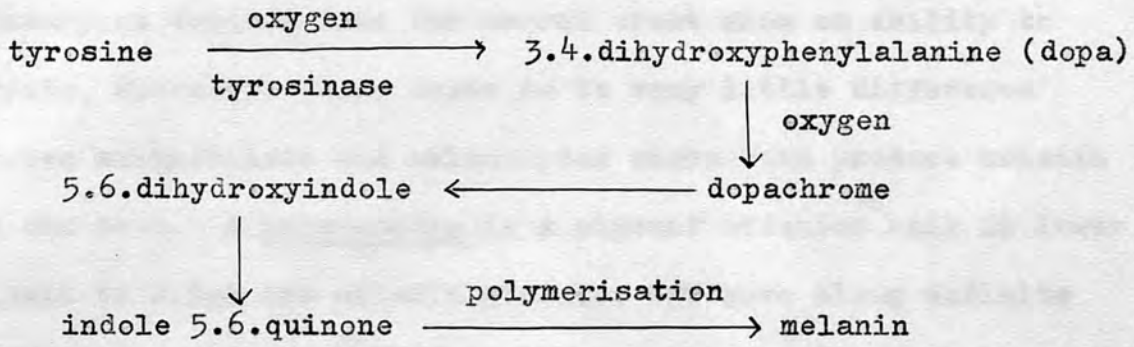
INTRODUCTION

Black and brown pigments are widely distributed in animals, and most of them are it is melanin. In spite of the vast amount of biological, biochemical and chemical research on melanin, it remains ill defined chemically and difficult to identify. The chemical structure of naturally occurring melanin is unknown and there is still some uncertainty about its elementary composition, because of the difficulty of extracting it.

Synthetic and natural melanins have been defined by Thomson (1962) as dark coloured biochromes. They are nitrogenous polymers of high molecular weight formed by the enzymatic oxidation of phenols. This definition, however, would include a whole range of products derived by oxidation of many phenolic substrates. Thus Thomson derives melanin from tyramine and 3-hydroxy-tyramine. However, the generally accepted definition is restricted to the pigments formed by oxidation of tyrosine or dihydroxyphenylalanine (dopa) by tyrosinase. The melanin formed in vitro from tyramine and 3-hydroxytyramine is chemically different from tyrosine melanin. The melanin monomer is thought to be an indole unit, but this has not yet been proved. Natural tyrosine melanin is frequently bound to protein and the linkage is believed to occur by sulphydryl groups, between the C-4 position in the melanin and an S-atom in the protein.

The biochemical and chemical investigations into melanogenesis have been summarised by Mason (1959) and Fox and Vevers

(1960). The accepted scheme for the formation of melanin from tyrosine was advanced as a model scheme by Raper (1927). As a result of his experiments on the action of tyrosinase, isolated from potato, mushroom and the mealworm, on tyrosine, he proposed the following:



This scheme was confirmed by Mason (1948). By investigating human skin, he found that when melanogenesis begins, the small amount of dopa first formed by relatively inactive tyrosinase functions as a catalyst activating progressively more of the enzyme.

Certain properties of melanin enable one to identify it at least provisionally. Lison (1953) states that a pigment is melanin if it is insoluble in all organic solvents and mineral acids, bleached by oxidising agents such as hydrogen peroxide, and if it reduces an ammoniacal silver nitrate solution to silver. A more complete list of the chemical tests for the identification of melanin appears below.

It is now necessary to clarify the terminology of melanin-bearing cells. Gordon (1959) attempted to produce a suitable

terminology, proposing the names melanoblast, melanocyte and melanophore. These are all definite cells and not syncytial structures. Gordon's definitions are as follows: a melanoblast is an embryonic cell capable of producing melanin. A melanocyte is a mature cell, producing and containing melanin. Cultures of melanocytes derived from the neural crest show an ability to migrate, therefore there seems to be very little difference between melanoblasts and melanocytes since both produce melanin and can move. A melanophore is a pigment effector cell in lower animals in which the melanin granules can move along definite cytoplasmic paths. Melanophores have been the subject of a lengthy controversy. They were once thought to be amoeboid cells and when their cell boundary contracted, it resulted in the concentration of the pigment into a small sphere. Conversely, as the cell boundary expanded and pseudopodia were produced, the pigment was dispersed. Now it is generally believed that the melanophore has a fixed outline and a permanent arborised form and that the melanin granules disperse and aggregate. However, cephalopod melanophores can expand and contract because associated with the highly elastic cell membrane are from six to twenty smooth-muscle fibres. These all contract simultaneously, stretching out the small spherical central melanophore into a disc whose diameter is twenty times that of the original sphere.

Hadley and Quevedo (1966) state that melanocytes frequently appear to secrete melanin granules into surrounding cells, and

therefore the term melanocyte has been endowed with this special significance when applied to the mammalian epidermis (Fitzpatrick, 1965). Now such a particular use of the term melanocyte has been extended by Hadley and Quevedo to describe the pigment-bearing cells in the amphibian epidermis, because they showed that these cells can not only produce melanin but also secrete it into surrounding epidermal cells. The cells can also disperse and aggregate their contained granules and so possess qualities of both melanocytes and melanophores. It would appear that in the case of amphibians the distinct terms defined above cannot be used so specifically.

I therefore propose to use the term melanophore in the more general sense of a pigment-bearing cell in which melanin is formed.

Additional terms that have been proposed merit some comment. Thus melanosome is applied by Seiji et al. (1963) to discrete organelles of varying size with tyrosinase activity and a distinctive internal structure. These are transformed into melanin granules, at the same time losing their tyrosinase activity. Seiji also distinguished a premelanosome. This is defined as a stage prior to the melanosome in which the enzyme molecules are aligned in an ordered pattern but not yet active. The term premelanin granule proposed by Wellings and Siegel (1960) in their studies of the origin of melanin granules in mammalian melanomas, is apparently synonymous with the term melanosome.

The function of melanin has often been a matter of argument. It aids survival in some animals where it provides adaptive colouration; it may assist in temperature control and perhaps act as a protective screen against the harmful effects of ultra violet radiation; or it may have some excretory significance. It may simply be a metabolic accident. Melanin present in certain malignant tumours is of obvious interest.

Melanin is not only widespread in the animal kingdom but is distributed widely in particular animals. Among the early accounts showing the latter is that of Weidenreich in 1912. He described vertebrate melanin in the following places: in the skin (both dermis and epidermis), surrounding the central nervous system, in a layer around the coelom and in the walls of the blood vessels. One of the early accounts of peritoneal melanophores is that of Ballowitz (1919) who described them in teleosts. However, much of this earlier work merely recorded the existence or distribution of black pigments, the chemical character of which was not shown. Moreover, they did not distinguish between intracellular and extracellular pigment.

The distribution of melanin in the body of various amphibian larvae has been reported by Sims (1961, 1962) and in more detail by Millott and Lynn (1954, 1965 and 1966). All these workers demonstrated the inhibition of melanogenesis by phenylthiourea. As a result Millott and Lynn inferred a turnover of melanin in amphibian larvae, although Sims found no evidence of such activity.

It has been shown too that physical and chemical factors such as temperature, humidity, hydrogen-ion concentration, oxidation-reduction potentials, and electrolyte concentration all affect the rate of melanogenesis.

This work was undertaken in order to investigate the distribution of melanin in certain amphibians, and to find any possible relationship between distribution and formation of melanin, the occurrence of the enzyme tyrosinase and its substrate tyrosine, and the inhibition of melanogenesis in developing tadpoles.

1. Distribution and identification of melanin in amphibians

There are very few accounts of the general distribution of black or brown pigment in adult amphibians. Yamamoto (1937) described melanophores in the epidermis, dermis and peritoneum of Rana rugosa, but such pigment is more widespread in other species of Amphibia. Accordingly a selection of adult amphibians was examined, namely: the anurans Rana temporaria (Linné), Rana pipiens (Schreber), Hyla arborea (Linné), Hyla versicolor (Le Conte) and Xenopus laevis (Daudin) and the urodeles Triturus cristatus (Laurenti) and Triturus vulgaris (Linné). Significant quantities of the pigment were found internally only in X. laevis, T. cristatus and T. vulgaris, although it was found in the skin of R. temporaria and Ambystoma mexicanum.

The black and brown pigment was identified as melanin in all the animals examined by the series of chemical tests described below.

In X. laevis melanin was found in the skin, eyes, around the myotomes, and in the parietal and visceral peritoneum. It was found in the last around the liver, lungs, pancreas, gonads, intestine, kidney, heart and major blood vessels. In all these places melanin occurs in melanophores. The latter also overlie the spinal cord and are found around some of the spinal nerves. Extracellular melanin is found in the liver, pancreas, kidney and in the heart.

The distribution of melanin in T. cristatus and T. vulgaris is very similar except that there is less melanin associated with the myotomes, and the parietal peritoneum has melanophores evenly dispersed dorsally and laterally but they are not present in the mid ventral line. Despite the relatively small amount of internal pigment in R. temporaria it is still found over the dorsal surface of the spinal cord, and around the roots of the spinal nerves.

Melanin is much more widespread in larval than in adult amphibians. The widespread larval pigmentation was reported by Sims (1961 and 1962) for the larvae of X. laevis, and by Millott and Lynn (1965 and 1966) for the larvae of Ambystoma mexicanum, Rana pipiens, Rana temporaria and X. laevis. The present investigation confirms these findings in Xenopus.

An examination of Xenopus larvae reared in water shows that pigment, identified as melanin by the means described below, becomes more widely distributed in the body as the larva develops. It appears in several forms in the situations listed below and can be seen as granules evenly dispersed or aggregated in the body cells, in between the cells, or in the lumen of an organ. The pigment occurs too in highly branched, nucleated melanophores whose processes may penetrate into the tissue of an organ. Thus intracellular pigment is seen in the cells of the dermis and epidermis (at very early stages of larval development), in the eye, in the cells of the liver, pancreas, olfactory epithelium

and sucker. Extracellular melanin is found in the epidermis (very early in development), between the yolk cells, in the epithelial lining and lumen of the gut, among the muscle fibres, in the cerebral ventricles, walls of the forming brain, in the canal of the neural tube and in the walls of the membranous labyrinth. Pigment occurs in specialised branched melanophores in the dermis, peritoneum (both parietal and visceral) meninges, connective tissue around the myotomes, walls of the major blood vessels, pericardium, pleural membranes and around the pro- and mesonephros though not in the wall or lumen of the nephric tubule.

The identification of melanin, especially when quantities are small, is not easy. It must be distinguished from other brown pigments such as ommochromes and lipofuscins. Pigment in a variety of situations was examined in serial sections of R. temporaria and X. laevis larvae. Peritoneal pigment was examined in excised strips. The pigment was identified by its solubility, reaction to bleaching agents and its reducing properties.

To test solubility, strips of tissue or sections containing the pigment were left for twenty four hours, in each of the following solvents: benzene, chloroform, di-ethyl ether, acetone, pyridine, dilute oxalic acid, dilute nitric acid, 10 M formic acid, five per cent hydrochloric acid, acid methanol and a normal solution of sodium hydroxide. To test solubility in concentrated sulphuric acid a drop was put on the tissue and allowed to remain for a few minutes. The pigment proved to be soluble only in

sodium hydroxide. This eliminates ommochromes which are soluble in 10 M formic acid and acid methanol. Further the characteristic purple colour which they produce with concentrated sulphuric acid was not observed. Again the pigment was not reduced by a solution of potassium borohydride, thus confirming that it is not an ommochrome which gives a yellow colour when treated with this substance (Fox and Vevers, 1959).

The pigment in the sections and strips was readily oxidised and bleached by a thirty volume solution of hydrogen peroxide in twenty four to forty eight hours. It was also bleached by other methods, for example, by twenty four hours treatment with a 0.1 per cent solution of potassium permanganate followed by brief treatment with a 1 per cent solution of oxalic acid; by treatment for twenty four hours with a 1 per cent solution of bromine, or with a hypochloric acid solution for forty eight hours. The oxidation of a lipofuscin takes much longer than this (Fox and Vevers, 1959).

In order to confirm that the pigment is melanin three more tests were performed. First the reaction to ammoniacal silver nitrate was determined. This depends on the ability of melanin to reduce such solutions of this salt with the deposition of silver (Pearse, 1953). The pigment under examination behaved in this way. Lipofuscins, however, lack this argentaffin property. Second, the ferrous iron test (Lillie, 1957) was applied. This depends on the formation of a complex ferrous salt, which,

reacting with ferricyanide ions forms Turnbull's blue. This on top of the dark brown melanin produces a dark green colour. However, these results were difficult to assess because the definitive green colour was only seen in areas where the original pigment was brown. This may be due to the fact that with increasing density, the original blackness of the pigment is difficult to distinguish from any dark green colour formed as a result of the test. Third, the Nile Blue test (Lillie, 1956) was used. This depends on the appearance of a dark green colour after the pigment is stained with a solution of Nile Blue. Although the results of this test were again difficult to assess, the blue green colour resulting when a lipofuscin is stained with Nile Blue was not seen.

Ehrlich's haematoxylin-basic fuchsin test (Gurr, 1956) was also applied. The pigment remained brownish-black and was thus distinguished from haemofuscin which stains bright red after this test.

From all these tests the pigment was judged to be melanin.

2. Observations on living melanophores

Because of their large size and accessibility the melanophores in the peritoneum from adults of X. laevis and T. cristatus were selected for study. Peritoneum was stretched out on a slide in amphibian Ringer's solution, and examined microscopically. There are many melanophores in this tissue, the majority being stellate in shape with highly branched processes radiating out from a central body, when the melanophores are fully expanded (fig. 1). The tips of these processes appear to meet and make contact with those of neighbouring melanophores to form an apparently continuous network. Some melanophores are more punctate in shape with few processes. Such cells are often seen in the region of a blood vessel or nerve.

There was no evidence of a consistent group response of the type described by Yamamoto (1931) in the peritoneal melanophores of a minnow, where some of the melanophores were said to behave as a group. In such cases, smaller cells surround a larger central cell, and when stimulated, they disperse and aggregate their granules simultaneously with this cell.

Yamamoto (1937) and Kuleman (1960), describing the peritoneal melanophores of Rana rugosa and the embryonal melanophores of the "ganglia" (unspecified) of Xenopus laevis respectively, both state that the pigment in the melanophores reacts to light by dispersion and to darkness by aggregation. An experiment performed to verify this in the peritoneal melanophores of X. laevis is reported in

Fig. 1. Triturus cristatus

Whole mount of peritoneum showing stellate melanophores, in the central area of which nuclei can be discerned. The scattered nuclei are those of the surrounding peritoneal cells.

Fixed: Bouin's fluid.

Stained: Mayer's alum carmine.

Scale: 240 μ

3. Observations on fixed melanophores

Strips of peritoneum containing melanophores from T. cristatus were fixed in a number of fixatives, the action of which has been discussed by Baker (1945). These were: formol-acetic, 10 per cent formaldehyde, Bouin, Helly, Zenker and Altmann. Of these 10 per cent formaldehyde was the best, for although some shrinkage occurred in the melanophores, there was no distortion. Bouin's fluid was less satisfactory because it caused the melanin granules to aggregate around the nucleus. For studying the relationship between mitochondria and melanin granules tissue was fixed in Altmann's fixative, followed by his method for post-chroming (Baker, 1945).

Peritoneal melanophores of both X. laevis and T. cristatus have large nuclei (150 to 250 μ in diameter). These were particularly evident in sections cut parallel to the surface of the peritoneum, at a thickness of 6 μ. To reveal the nuclei it was necessary to bleach the melanophores with 30 volume hydrogen peroxide until the melanin was a faint brown in colour. The cells were subsequently stained in methyl green-pyronin G or Feulgen's stain using the methods given by Pearse (1953), or in Heidenhain's iron alum haematoxylin, which although not specifically a nuclear stain, proved very successful with this particular tissue (fig. 2).

An attempt was made to determine the effect of light on these cells. Similar pieces of peritoneum from Xenopus were kept

Section 3, page 17, but the result is relevant here, namely that there was no appreciable difference in the distribution of melanin granules, in the light and in the dark.

Melanin granules in and around the processes of peritoneal melanophores were observed to move, but not in a definite pattern. They exhibited a bobbing movement, jostling each other. This was also described by Marsland (1944) in the scale melanophores of Fundulus heteroclitus.

The observation of granule movement agrees with Burgers (1957) who examined the melanophores of X. laevis and reported not only movement but also the effects of adrenaline and other phenyl alkyl amines on the dispersion and aggregation of melanin.

Van Oordt and Burgers (1959) also working on Xenopus melanophores stated that those found internally are fundamentally different from those in the dermis. This is because intermedin causes a maximal pigment dispersion in dermal melanophores, but has hardly any effect on those in the wall of the intestine. In view of the observed movement of granules, the lack of evidence of dispersion and aggregation of pigment comparable to that reported by Yamamoto and Kuleman was unexpected. In this connection it is possible that the properties of melanophores change with age. However, insufficient experiments using photic stimulation were performed on these melanophores to permit drawing any valid conclusions.

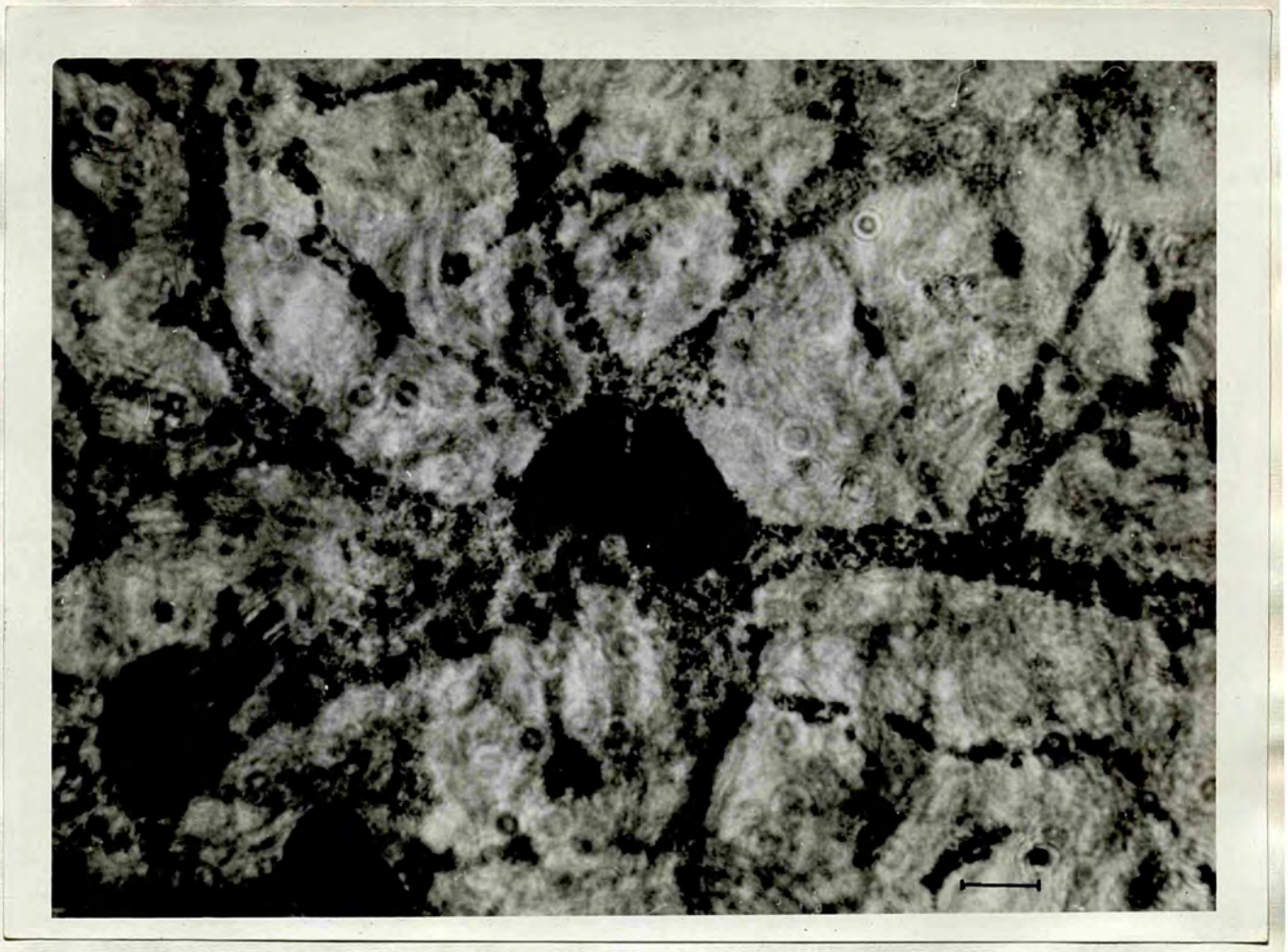


Fig. 2. Triturus cristatus

A melanophore from a section of peritoneum cut parallel to the surface. The melanophore shows evenly dispersed melanin granules which had been bleached until light brown in colour, prior to staining. Note the large central bi-lobed nucleus.

Fixed: Bouin's fluid.

Section: 6 µ in thickness.

Bleached: 30 volume hydrogen peroxide for 4 hours.

Stained: Heidenhain's iron alum haematoxylin.

Scale: 100 µ

separately in the light or in the dark for twenty-four hours at the same temperature and humidity. They were then fixed immediately in Bouin's fluid. There was no appreciable difference in the distribution of granules in melanophores from the two sets of experiments. But because Bouin's fluid has a slight effect on the distribution of the granules, it was difficult to be sure that light had no effect.

4. The development of melanin pigmentation in Xenopus laevis larvae

The origin and development of amphibian melanophores has long been the subject of research. Bytinski-Salz (1938) showed that anural pigment cells originate in the neural crest. Niu (1950) showed that amphibian melanophores also originate in the cranial and caudal portions of the medullary plate. These contribute respectively to the pigment developed in the head and tail. He also demonstrated that cells other than melanoblasts can be induced, under proper conditions, to become pigment cells.

Presumptive melanophores are dispersed from their site of origin by active migration and the causes of their dispersal have been analysed *in vivo* and *in vitro*. Amongst the accounts of the origin and migration of amphibian melanophores are those of De Lanney (1941), Twitty (1944), Flickinger (1945), Stevens (1954), Niu (1954), Lehman (1957) and Bagnara (1960). Twitty reported the presence of an unidentified diffusible substance or substances produced by pro-pigment cells which excites adjacent cells to migratory activity. Later Niu (1959) suggested that pro-pigment cells are moving partly in response to the concentration gradients of diffusible metabolic products. Actively migrating pigment cells may be prevented from inter-mingling and over-growing one another by "contact inhibition" (Niu, 1959). Twitty (1944) said that the onset of melanisation in pro-pigment cells does not preclude their further migratory activity, but Lehman (1957) found

that these cells become less active as they acquire pigment.

The period of pigment formation is characterised by a rising oxygen consumption (Flickinger, 1945). This could be due to the oxidative transformation of pigment precursors into melanin. The rate of pigment formation is dependent on the amount of available substrate, and the presence of active enzyme and molecular oxygen.

In view of these claims I decided to investigate the sequence of melanisation in X. laevis larvae. Experiments were designed to study the formation and distribution of melanin during the period of development from the egg until metamorphosis.

Technique

The dorsal lymph sac of males and females was injected with a proprietary solution of gonadotrophin ("Pregnyl" made by Organon Laboratories) just anterior to the thigh. Two injections were given. On the first evening 0.4 mls. of the substance were administered, and on the following evening 0.4 mls. was given to males and 0.8 mls. to females. The animals were placed in a large deep trough of water, the temperature of which was thermostatically maintained at approximately 25°C. Eggs were usually obtained early in the morning following the last injection. They were removed from the trough, divided into batches of twenty to thirty, and kept in shallow dishes in tap water at room temperature. All groups of animals were kept under the same conditions. The water in the dishes was changed daily.

The developing embryos were fed on a suspension of dry nettle powder, or as a control, for the reasons given later, with dry milk powder. All animals were staged according to the scheme of Nieuwkoop and Faber (1956). Animals in the control batches were fixed at intervals in 10 per cent formol so that a series of animals from stages 23 to 53 was obtained. These were subjected to a standard procedure of embedding, sectioning and staining. They were stained by Mallory's triple method.

As regards the precise appearance of melanin in the sites listed on pages 10,11, Stevens (1954) describes the melanophores of X. laevis as first appearing simultaneously along the dorsal border of the intestinal tract and on the surface of the brain and eye. He reports melanin appearing later along all of the dorsal ridges of the myotomes, and over the neural tube. It has been found, however, by Millott and Lynn (1965, 1966) that melanin is present in these larvae long before melanophores appear.

In agreement with these workers, an examination of larvae at stage 26 showed small amounts of melanin to be already present in the cells of the dermis, epidermis and sucker. Extracellular pigment was present in scattered amounts among the yolk cells associated with the organising gut, and in between the cells of the neural tube. This extracellular pigment is unaffected by phenyl-thiourea (see later) and differs in its disposition from that formed later. Thus the pigment seen initially among the yolk cells and in the neural tube consisted of isolated granules lying on

the outside surface of the yolk and neural cells, whereas that seen later in these sites consisted of many more extracellular granules aggregated together (Figs. 5 and 6). It therefore appears that the extracellular pigment seen at these early stages is pigment carried over from the egg. Judging by the amounts which accumulate in the epidermis, foregut and neural tube, it is possible that the pigment has been swept into these places by the morphogenetic movements of the developing embryo and eventually eliminated (see below). Since much more melanin appears later in the neural tube and gut this again suggests an independent origin of the early pigment.

Between stages 26 and 36 the presumed egg pigment is still seen in the epidermis, dermis and among the yolk cells (fig. 3) but melanin has begun to develop in other areas too. From stage 32 onwards it appears in the eye, where amounts rapidly increase, in the peritoneum, dermis, connective tissue around the myotomes and around the neural tube (fig. 4). In the last four places the pigment is in melanophores. Some extracellular pigment is seen at this stage among the muscle fibres. About this time too increasing amounts of melanin are seen in the walls of the brain and in the epithelial lining of the gut. Histological appearances suggest that here granules of pigment are being cast off from the free borders of the brain walls into the cerebral ventricles (fig. 5) and from the epithelial lining of the gut into the lumen. The pigment in the epithelial lining and lumen of the gut is melanin and is not derived from the food of the larvae, because

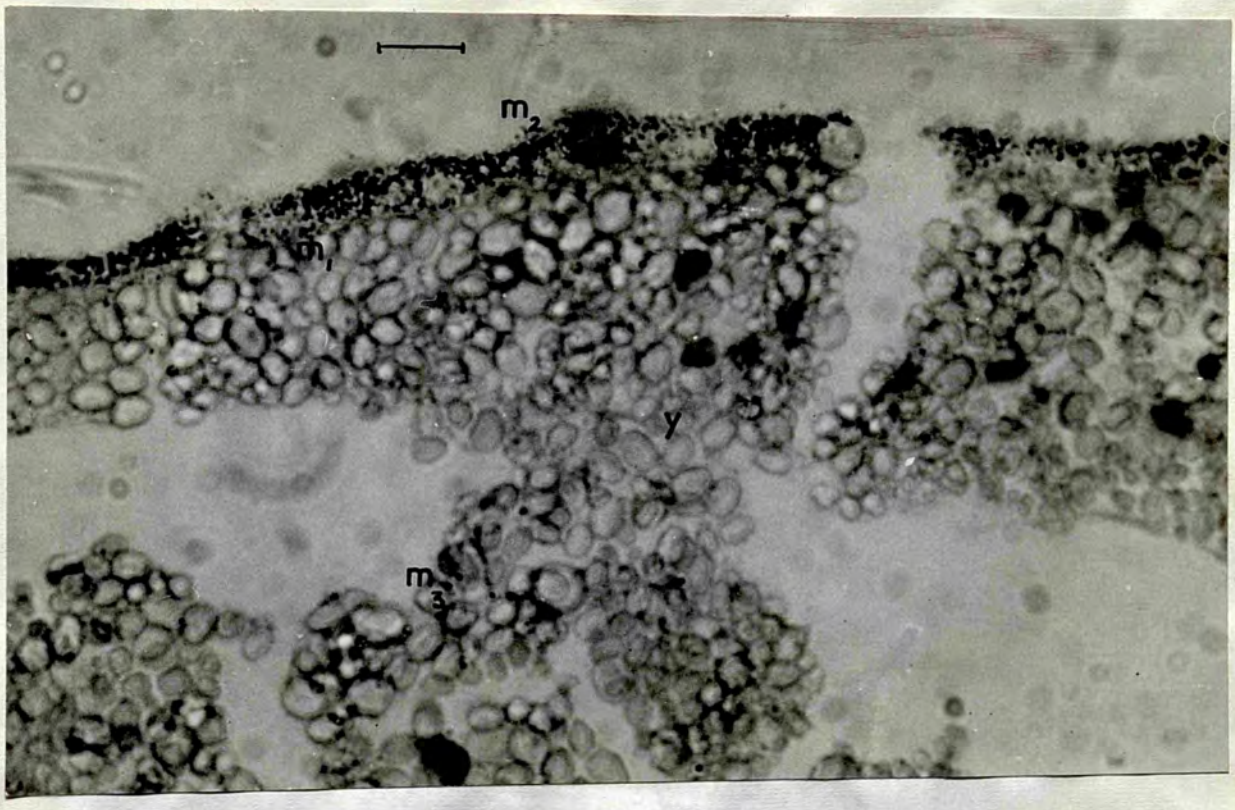


Fig. 3. Xenopus laevis

Distribution of melanin granules as seen in a section through the abdominal region of a larva at stage 32 which had been reared in water. Pigment, probably carried over from the egg, is seen in the dermis (m_1), epidermis (m_2) and among the yolk cells (m_3).

y = yolk cells

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 180 µ

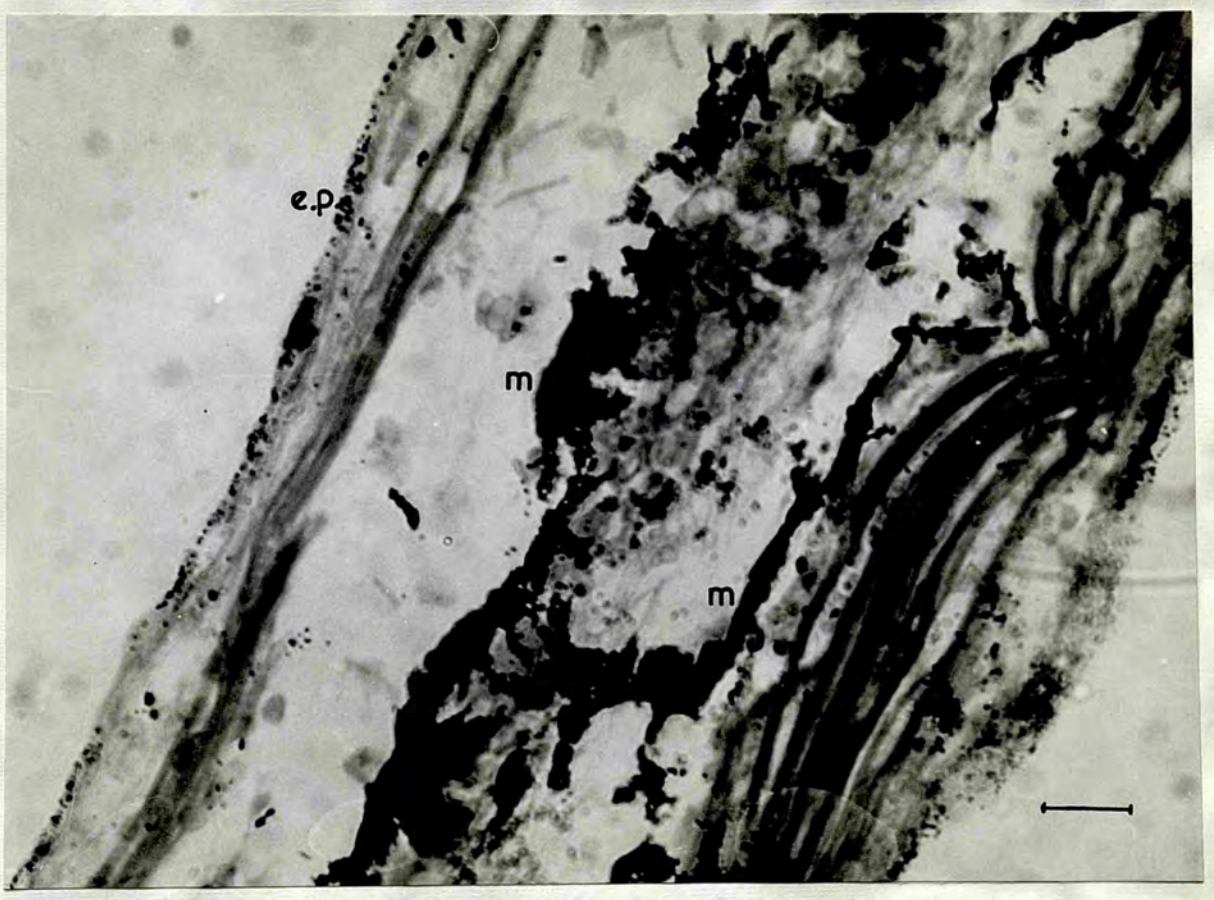


Fig. 4. Xenopus laevis

Melanophores (m) around the neural tube (nt) and myotomes (my) as seen in an oblique frontal section of the tail region of a larva at stage 35. The larva had been reared in water. Note that egg pigment is present in the epidermis (ep) and that some extracellular melanin appears in between the muscle fibres.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 180 µ

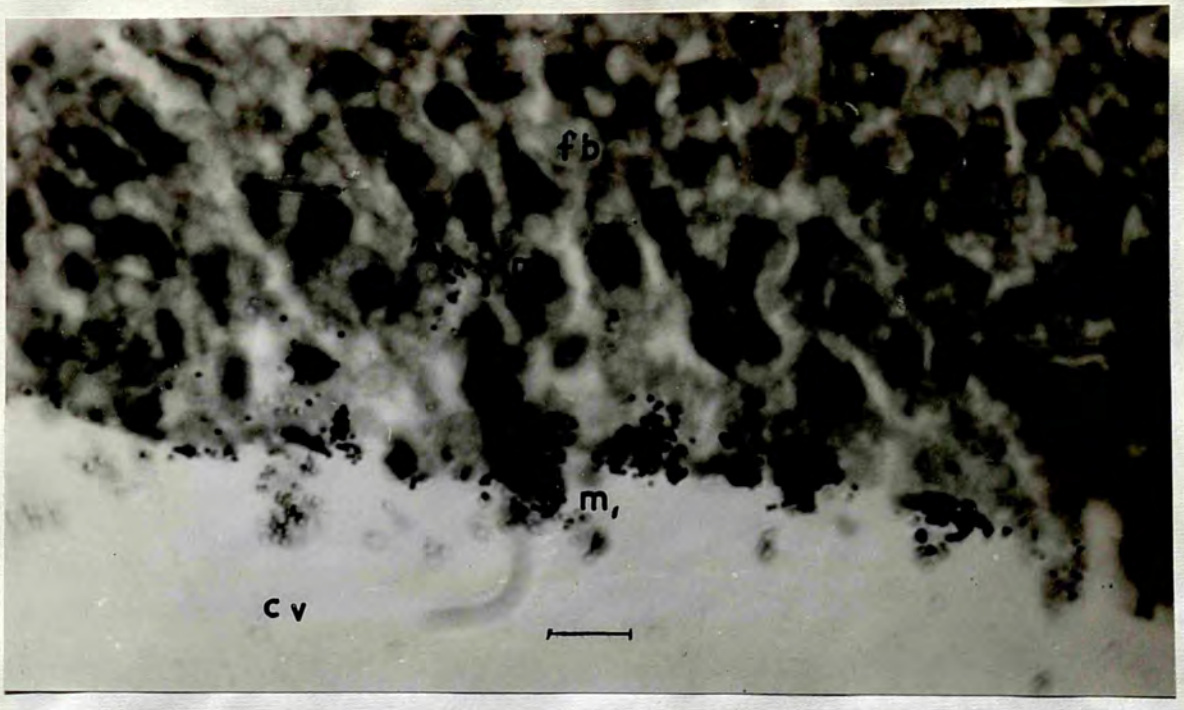


Fig. 5. Xenopus laevis

Melanin (m_1) being cast off from the wall of the forebrain (fb) into the cerebral ventricle (cv) as seen in an oblique section of a larva at stage 33. The larva had been reared in water. Melanin (m_2) is also present in between the cells of the forebrain.

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 100 μ

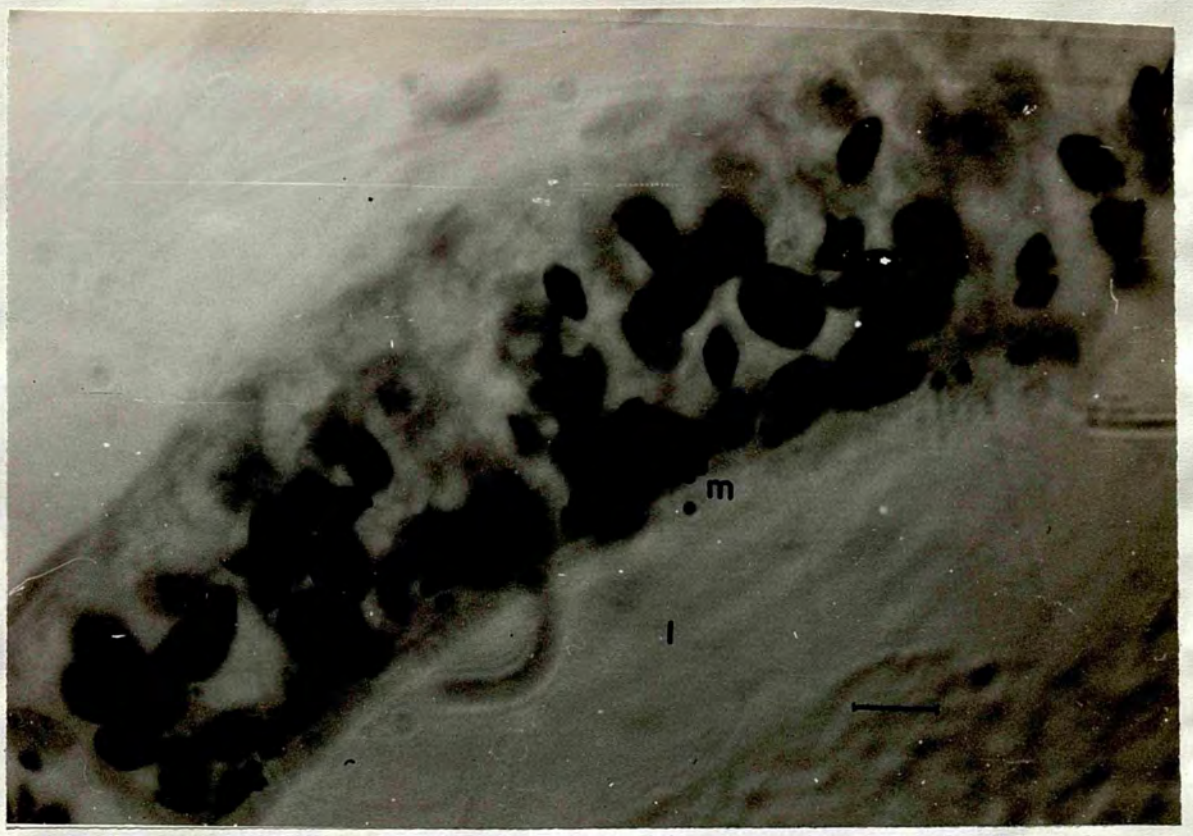


Fig. 6. Xenopus laevis

Melanin (m) accumulating at the free border of the intestinal epithelium (ie) prior to being cast off into the lumen (l) of the gut. As seen in a larva at stage 41. The larva had been reared in water, and cut longitudinally.

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 180 μ

when animals are starved or fed on milk powder the same pigment is seen in these sites.

It is noteworthy that after stage 36, which is the time of hatching of the embryo, no more melanin appears in the epidermis until stage 58 when the adult skin pattern develops. This again suggests that the epidermal melanin present before stage 36 is egg pigment. Its accumulation on the outside surface further suggests that it is being excreted.

More melanin appears between stages 36 and 44. Melanophores appear around the nasal pit at stage 39, and about the same time they are seen in the meninges and around the pronephric tubules. Traces of melanin appear in the liver, and increased amounts are seen in the gut (fig. 6), cerebral ventricles and in the eye. Extracellular pigment continues to appear in the connective tissue between the muscle fibres, in addition to the pigment in the melanophores surrounding the myotomes. At stages 39 and 40 traces of melanin are seen in the walls of the membranous labyrinth.

Between stages 44 and 48 pigment continues to accumulate in the peritoneum, meninges and retina until at stage 47 the histological picture presented is of a uniform black sheet in these areas. In contrast, from stage 45 the amount of melanin in the gut and cerebral ventricles decreases, but melanin is still seen in the central canal of the neural tube (fig. 7). Here the melanin granules appear to have been compacted together to produce large black aggregates. At stage 47 more melanin appears

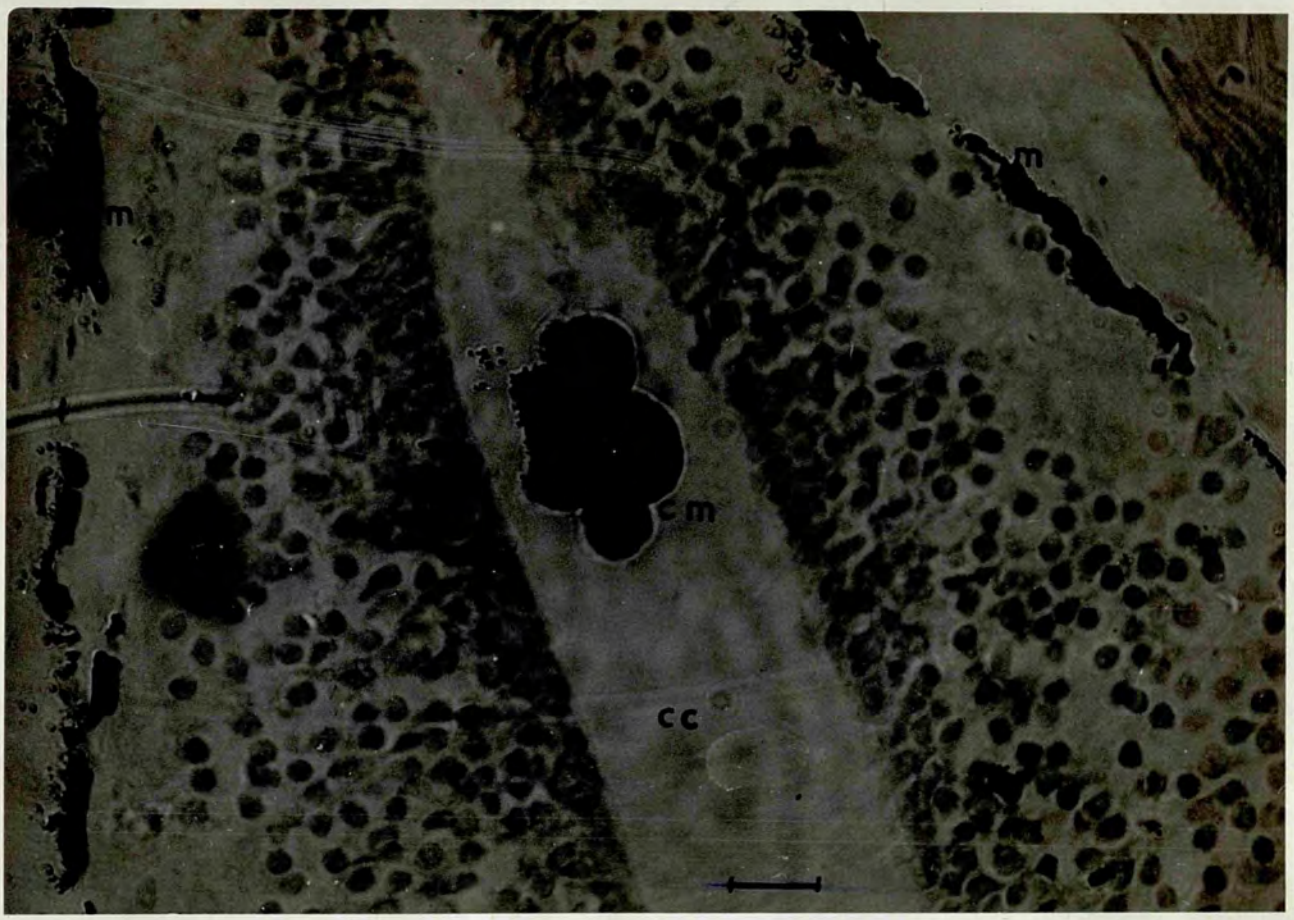


Fig. 7. Xenopus laevis

Compacted melanin (cm) in the central canal (cc) of the medulla oblongata of a larva at stage 46. The larva had been reared in water, and was cut frontally. Note also the melanophores (m) in the meninges around the medulla.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 240 µ

in the liver and pancreas but the amounts are still small.

After stage 49 no more melanin is seen in the gut, cerebral ventricles or neural tube, but at this stage some is present around the nerves. From stages 49 to 53 (the last stage examined in detail) amounts of melanin increase in the following places: the melanophores around the myotomes and mesonephros, in the pericardium, walls of the major blood vessels, pleural membranes, liver and pancreas. The melanin tends to form a sheet surrounding many of the organs, but penetrates only a few, e.g. the liver and pancreas, where the amounts increase up to metamorphosis.

From this examination of the normal development of pigmentation in X. laevis larvae, several points emerge. It appears as though the young embryo first excretes the pigment carried over from the egg (stages 26 to 36). Following this, and partially overlapping it, there is a period of rapid and extensive melanisation. This apparently occurs in two phases. Up to stage 46 increasing amounts of melanin appear in the parietal peritoneum, meninges, eye, olfactory epithelium, gut and neural tube. In the latter two places the amount of melanin then decreases until finally there is none, but in the remainder no change is apparent (possibly because a maximal amount has been formed here). The second phase occurs between stages 49 to 53 when melanin appears in the liver and pancreas, and increases in amount in these organs as well as in the pericardium, walls of the major blood vessels, pleural membranes and around the

myotomes and mesonephros. The dermis is exceptional in that the amount of melanin within it accumulates steadily from stage 32 to stage 50. In this case the increase in pigmentation can be seen to be due to an increase in the number of melanophores as well as to the accumulation of pigment within these cells. The increase in pigmentation of the peritoneum and meninges up to stage 46 is brought about in the same two ways.

5. The effect of phenylthiourea

The enzyme tyrosinase, concerned in melanogenesis, requires copper ions for its successful activity. Substances which combine with copper inactivate the enzyme, although this inactivation is sometimes reversed by the addition of excess cupric ions. Phenylthiocarbamide combines with copper and this was found to be a very effective inhibitor of tyrosinase by Bernheim and Bernheim (1942). Later Dubois and Erway (1946) reported that phenylthiourea, α- naphthylthiourea, allylthiourea, thiourea and thiouracil all inhibit tyrosinase. Lerner et al (1950) investigating the mechanism of this inhibition found that most "thio-" compounds that inhibit tyrosinase do so by combining with copper. However, they stated that phenylthiourea may inhibit tyrosinase in a way other than by binding copper, because inhibition cannot always be completely reversed by addition of cupric ions. Other substances, for example cysteine, have also been found to inhibit tyrosinase activity (Lerner and Fitzpatrick, 1953).

Many people have investigated the effect of these inhibiting substances on the pigmentation of both mammals and amphibians. Lynn and Marie (1946) found that thiouracil made the melanophores of tadpoles lighter in colour. It also caused the pigment to aggregate. Dieke (1947) found the same and observed the return of pigment in the skin of rats after cessation of phenylthiourea treatment. Millott and Lynn (1954) studied the effect of phenylthiourea on the melanin pigmentation of the developing frog

Eleutherodactylus martinicensis. They found that progressively longer treatment with phenylthiourea caused the sub-epidermal and retinal melanophores to show decreased melanin content. This was due partly to a diminution in the number of melanin granules and partly to a decrease in density of the granules themselves, so that they appeared brown.

Lehman (1957) observed that in the axolotl, the diminution of melanin pigmentation produced by phenylthiourea, does not affect the migratory ability of potential melanophores even though their ability to synthesize melanin is impeded. Since then Sims (1961, 1962) and Millott and Lynn (1965, 1966) have analysed the effect of phenylthiourea on melanogenesis in X. laevis, and X. laevis, R. temporaria, R. pipiens and Ambystoma mexicanum respectively.

In the light of this work I decided to study the effect of phenylthiourea on melanin formation, particularly its effects on isolated melanophores and their contained granules in the larvae of X. laevis. Certain precautions are necessary in designing the experiments; thus, in agreement with Millott and Lynn it was found that phenylthiourea not only affects pigmentation but also retards development. For example, two groups of eggs taken from the same batch and therefore of the same age and parentage were subjected to different treatment. One group was reared in water and the other in a 0.01 per cent solution of phenylthiourea from stage 23. After ten days animals in water were at stage 46 and treated animals

were at stage 43 of development. Therefore when comparing treated animals with controls, it is necessary that all should be at the same developmental stage, in order to assess the effect of the drug on pigmentation.

For experimental purposes larvae were reared in a 0.01 or 0.001 per cent solution of phenylthiourea, but in other respects they were subjected to the same conditions as those already described (page 21). The phenylthiourea solutions were changed daily. Larvae were subjected to the phenylthiourea treatment which began at various stages from 23 to 40 and lasted for graded lengths of time. Throughout the experiments treated larvae were compared with controls at the same developmental stage. In order to reveal the effect of phenylthiourea on individual melanophores, larvae at stage 36 (by which time dermal melanophores have developed) were treated with phenylthiourea for seven days until very little melanin remained. They were then returned to water in order to observe the return of pigment in the melanophores.

In all these experiments, animals were fixed at regular intervals in a standard fashion using 10 per cent formol. Serial sections, 6 μ in thickness, were prepared and stained by Mallory's triple method.

The first group of experiments consisted of rearing larvae from stage 23 in phenylthiourea. After one day's treatment, when larvae in both drug solutions were at stage 26, they were compared with a control animal. No effect was apparent except in the

meninges where the pigment was slightly decreased in amount.

After one and a half days' treatment, by which time larvae in both solutions were at stage 36, more extensive effects of phenylthiourea appeared, but most significantly they were not the same in all areas. Thus, the treated larvae showed reduced amounts of melanin in the dermis, peritoneum, meninges, eye and sucker, and apparently increased amounts in the cerebral ventricles. In the epidermis and gut pigmentation remained unaffected. This lack of effect of the drug on the pigment in the epidermis and gut sets these areas apart and suggests that their pigment at this stage is not in the process of formation, but may have been carried over from the egg. Moreover, the appearances already described (page 23) suggest that the pigment is being eliminated in these two areas.

The inhibitory effect of the drug depends on concentration. It was seen that in these experiments larvae at stage 36 taken from the 0.01 per cent solution possessed no melanin in the peritoneum and meninges, but those of the same stage from the 0.001 per cent solution showed some melanin in these areas. Thus the weaker solution is less effective than the stronger.

After three days' treatment, larvae from both solutions examined at stage 39 showed the same effects on the amount of melanin present, but the differences between the experimentals and the controls were more striking. When examined at stage 42 larvae treated with either concentration of phenylthiourea showed

decreased amounts of melanin in the same areas as those noted above, but in addition there was less in the olfactory epithelium. On the other hand, there were similar amounts of melanin in the cerebral ventricles in comparison with the controls.

By stage 46 when larvae had been in either drug solution for fifteen days, there was no dermal melanin and only traces in the peritoneum, meninges, eye, connective tissue around the myotomes and olfactory epithelium. At this stage the control animals showed much melanin in all the above areas. The effect of the drug is therefore clearly inhibitory but in areas such as the gut its effect is to increase slightly the amounts of melanin present. The small amounts of pigment present in the liver and pancreas at this stage were just perceptibly less in treated animals as compared with that present in controls.

A second series of experiments was performed in which phenylthiourea treatment was begun at stage 33. The same two concentrations of the drug were used, but in some cases, particularly with the stronger solutions, the larvae failed to survive.

After three days' treatment in the 0.01 per cent solution, by which time larvae were at stage 42, the amount of melanin was found to have diminished in the following areas: dermis, peritoneum, meninges, eye, connective tissue around the myotomes and in the olfactory epithelium.

After nine days' treatment in the 0.001 per cent solution, larvae which were then at stage 45, showed only a very slight

decrease in melanin content of the following areas: peritoneum, meninges and olfactory epithelium. In all the other areas the amount of melanin appeared to be unaffected by the drug. After seventeen days' treatment, larvae which were then at stage 47, in addition showed a reduction in retinal and dermal melanin. However, there was a slight increase in the amount of melanin in the gut. Larvae treated for twenty-three days in the same solution showed roughly the same amounts of melanin distributed in the same organs as those seen at stage 47.

It is evident from these experiments that the inhibitory effect of phenylthiourea declines not only as concentration decreases but also as development proceeds. Thus five days' treatment with a 0.01 per cent solution, begun at stage 39, only slightly reduces the melanin content of the dermis, peritoneum, meninges and olfactory epithelium. It has no effect on the retinal melanin, or on the melanophores associated with the myotomes (fig. 8). Six days' treatment begun at stage 46 produces no effect on the melanin content of the majority of the organs, but it slightly reduces the melanin in the liver and pancreas.

In general these findings agree with Sims (1961) who found that phenylthiourea under normal circumstances ceased to affect melanogenesis after stage 46. Nevertheless, pigment must still be produced because as the animal grows it does not become paler. That pigment is still being formed in the dermis for a limited time after stage 46 and in the liver and pancreas up until

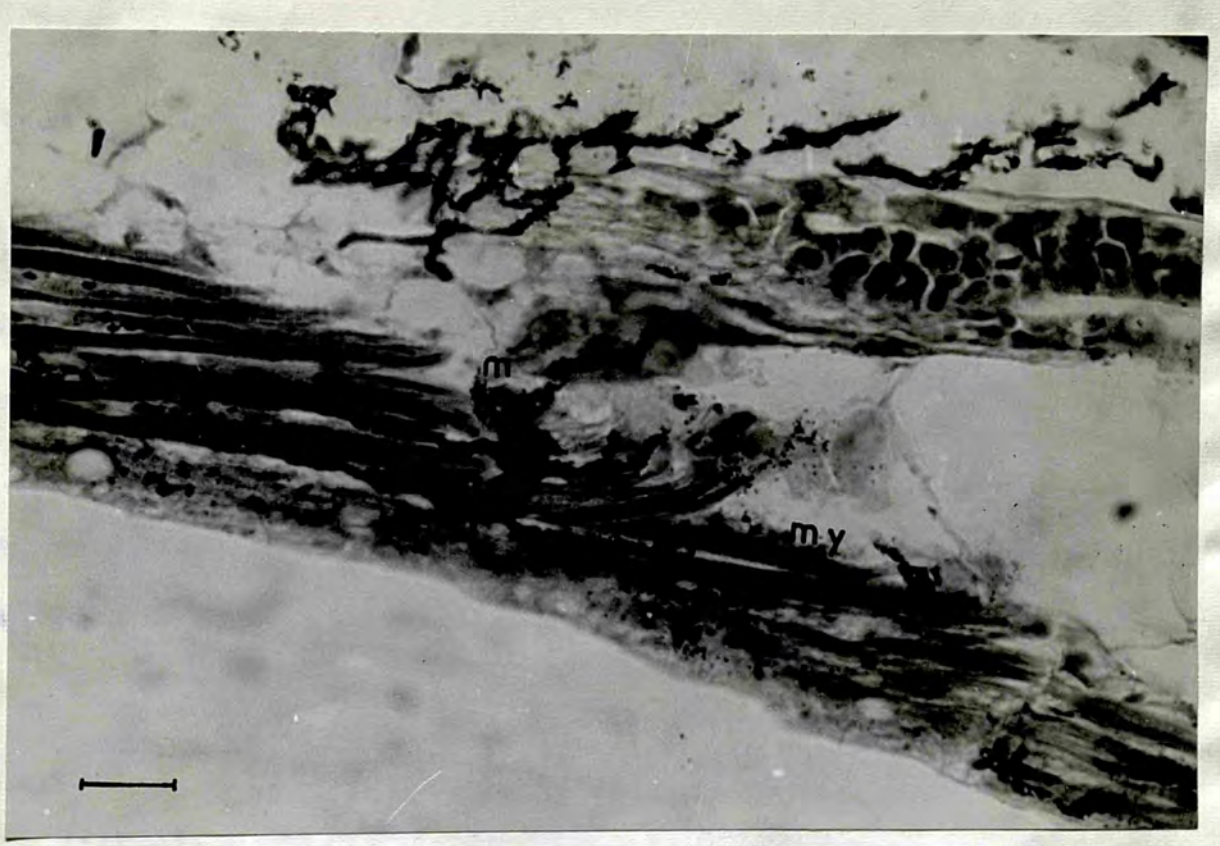


Fig. 8. Xenopus laevis

Melanophore (m) from the tail region of a larva at stage 42 as seen in a longitudinal section, following treatment for five days with a 0.01 per cent solution of phenylthiourea, with effect from stage 39. The prominent melanophore (m), which retains its dense pigment, is lying on top of the myotome (my), with its processes in between the groups of muscle fibres.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 180 µ

metamorphosis, can be shown by keeping larvae for seven days in phenylthiourea with effect from stage 36. By this time they have reached stage 45 or 46 and are very pale. On returning them to water, pigment is re-developed in the dermis, liver and pancreas and traces re-appear in the peritoneum and meninges.

Unfortunately it has not yet been possible to devise a quantitative means of estimating the amount of melanin at various sites, but the foregoing experiments show that phenylthiourea is not always effective and, further, when it is,it may increase as well as decrease pigmentation. The gut and cerebral ventricles are notable areas where the drug increases pigmentation. The effect on the amount of melanin was manifest in two ways: on the number of melanin granules and on the density of melanin of these granules.

It is obvious that phenylthiourea not only produces the effects already described but also has some effect on the shape of the melanophores.

In order to make a detailed examination of these effects, the peritoneal melanophores and the dermal melanophores dorsal to the neural tube of X. laevis were selected for observation. Larvae at stage 36 were reared in a 0.01 per cent solution of phenylthiourea. At this stage the majority of the egg pigment had disappeared from the epidermis, and well formed dermal melanophores were present (fig. 11a). Sections of these larvae were prepared and the melanophores were examined at regular

intervals during the treatment. After seven days' treatment only a few granules remained in the melanophores under observation, and the overall amount of melanin in the body of the tadpole was reduced. At this point the larvae were returned to water and the pigment was observed as it re-developed. In order to give an accurate account of the effect of the drug on the melanophores and their granules, the cells were drawn, for when they were photographed, it was difficult to see all the details in one focal plane.

The effects of phenylthiourea on peritoneal melanophores were as follows. When subject to ten or twelve days' treatment, these cells showed a diminution in the number of melanin granules mainly in the central areas. There was no apparent effect on granule size, but a few granules in the central areas were less dense than the others and this was taken to mean a decreased melanin content (fig. 9). Some peritoneal melanophores showed an aggregation of the granules into the centre of the cell, leaving few granules in the cell processes; but this could also be explained as due to a disappearance of melanin which is more marked at the periphery (fig. 10).

After one day in phenylthiourea the dermal melanophores examined already showed a reduction in the amount of melanin present (fig. 11b). Not only were there less granules, but noticeably many granules were lighter in colour than normal ones, thus showing a reduced melanin content. Thus, some granules were certainly lighter but there was some evidence that some of them

Fig. 9. Xenopus laevis

A peritoneal melanophore from a larva which had been treated with a 0.001 per cent solution of phenylthiourea for twelve days from stage 33. Note the decreased number of melanin granules in the central area of the cell, and also some lighter granules with a decreased melanin content.

Fig. 10. Xenopus laevis

A peritoneal melanophore from a larva which had been treated with a 0.001 per cent solution of phenylthiourea for ten days from stage 33. The effect shown could be due to a disappearance of melanin which is more marked at the periphery, or an aggregation of the granules, so that only one or two remain in the processes.

Fig. 11a. Xenopus laevis

Control: A dermal melanophore from the region dorsal to the neural tube from a larva at stage 36 which had not been treated with phenylthiourea. Note the central nucleus and the apparently dense rind of granules around the periphery of the cell. Near the end of some of the processes some of the pigment has lost its granular form so that melanin appears to have been liberated into the cytoplasm.

All fixed: 10 per cent formol

Sections: 6 µ

Stained: Mallory's triple

Scale: 6.3 µ

N = nucleus

LMG = melanin granules lighter in colour than normal ones

MC = melanin in cytoplasm

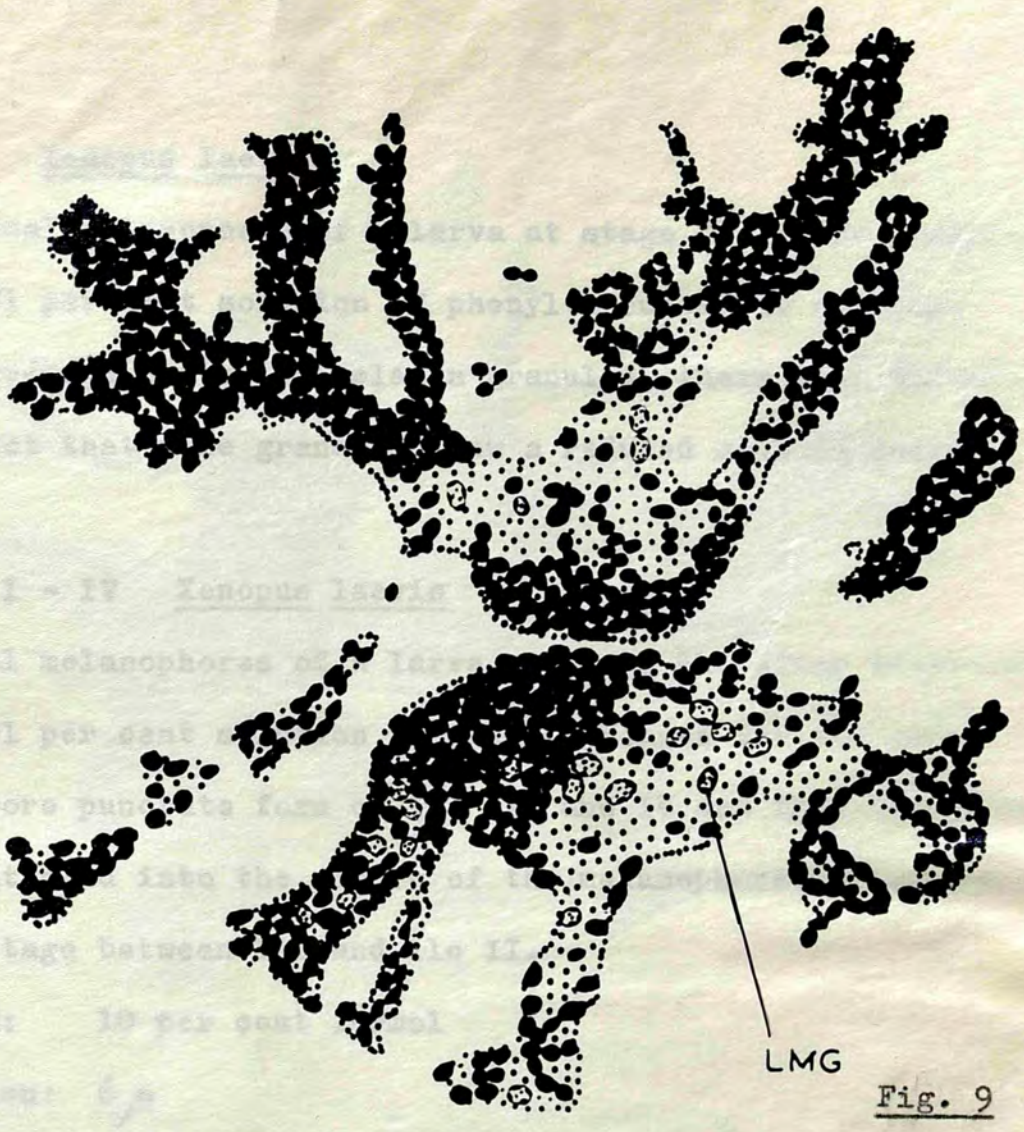


Fig. 9

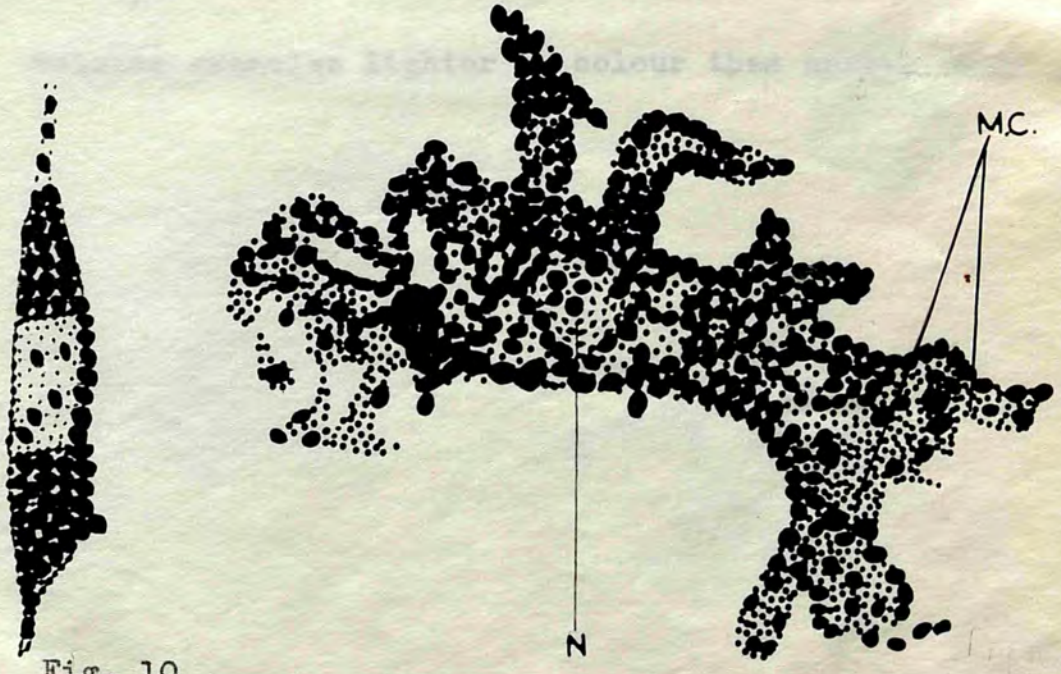


Fig. 10

Fig. 11a

Fig. 11b. Xenopus laevis

A dermal melanophore of a larva at stage 39, after treatment with a 0.01 per cent solution of phenylthiourea for one day. Note the reduced number of melanin granules, their size difference and the fact that some granules show a reduced melanin content.

Fig. 11c. I - IV Xenopus laevis

Dermal melanophores of a larva at stage 41, after treatment with a 0.01 per cent solution of phenylthiourea for two days. Note the more punctate form of II, III and IV and that the granules are concentrated into the centre of the melanophore. Fig. 11c I may be a stage between 11b and 11c II.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 6.3 µ

LMG = melanin granules lighter in colour than normal ones

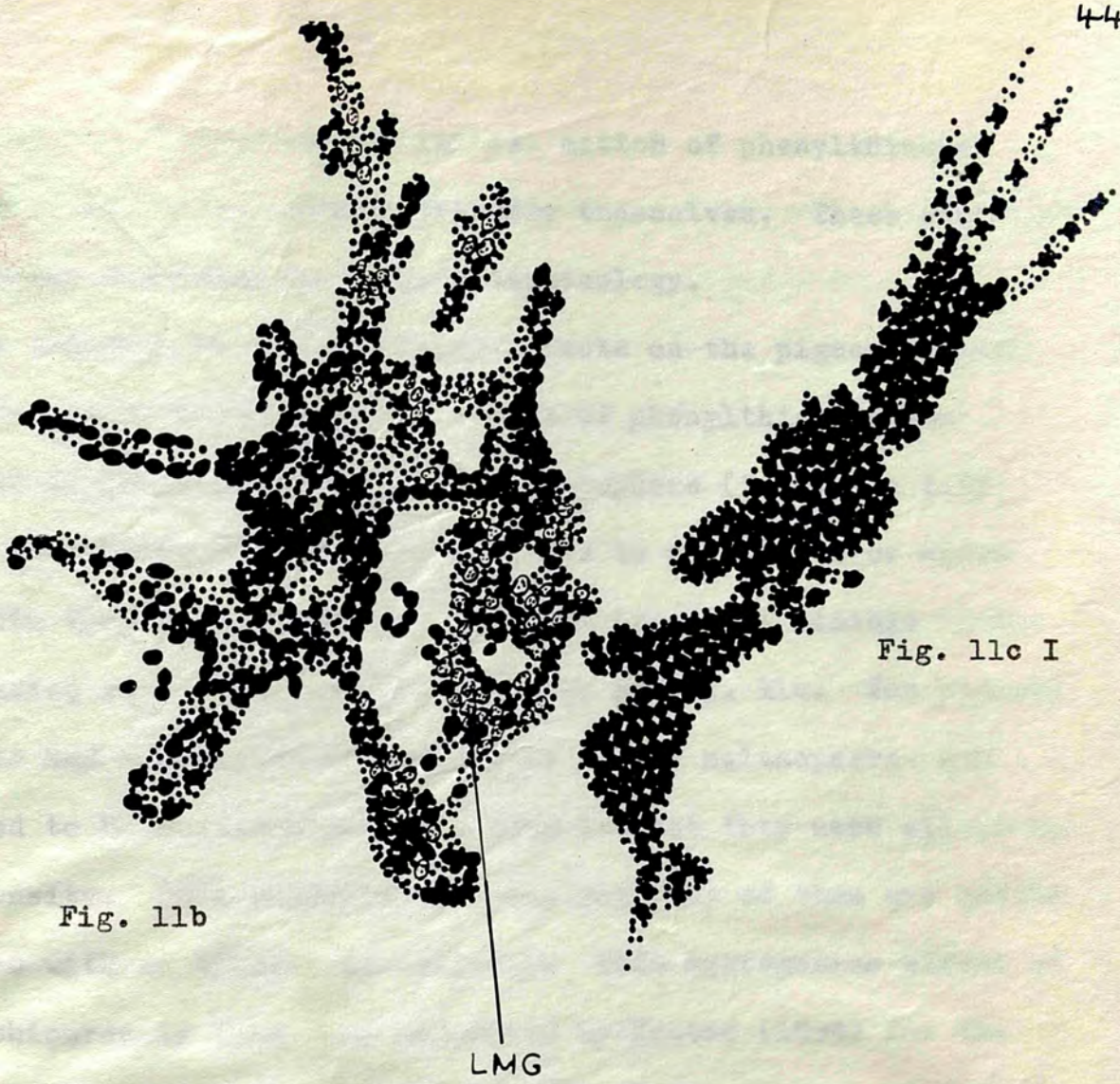
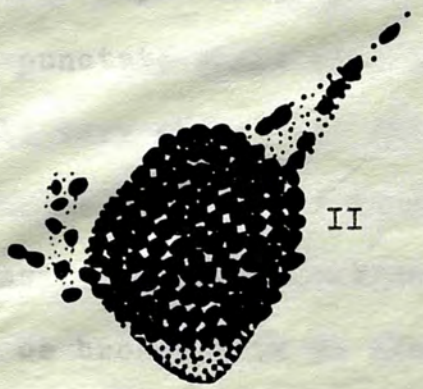


Fig. 11b

Fig. 11c I

III



II

IV

Fig. 11c

were smaller. Therefore the initial action of phenylthiourea appears to be on the forming granules themselves. These would be melanosomes according to Seiji's terminology.

In addition to the foregoing effects on the pigment itself, after two days' treatment, the effect of phenylthiourea was extended to other features of the melanophore (figs. 11c I-IV). It had changed in shape from a stellate to a punctate or round cell with very few processes. The only processes visible in the preparation were those which were drawn in fig. 11c. The pigment granules had aggregated in the centre of the melanophore, and appeared to be unaffected by the drug because they were all of an even density. This suggests that the majority of them are mature granules with no tyrosinase activity. This aggregation effect of phenylthiourea is like that described by Foster (1959) for the effect on the skin melanophores of Rana clamitans of beef pineal extract which he found to cause aggregation of melanin granules and a reduction of their melanin content.

After four days' treatment, the melanophores retain their roundish or punctate shape with very few discernible processes which are all very short. At this stage four effects appear: melanin granules are reduced in number, they become more uneven in size and density, and in addition the granular nature of the pigment may be broken down so that it is liberated into the cytoplasm (fig. 11d I-III). Nevertheless, since phenylthiourea still affects the granules at this stage, it appears as if some melanin is still being formed.

After seven days' treatment there is a further reduction in number of the melanin granules until only a few remain in the melanophores (fig. 11e I,II). Several melanophores were observed at this stage which contained only one granule. Nearly all the melanophores were round or punctate in shape and I could see no de-melanised processes even after heavy staining. The only evidence of processes at this stage was in a melanophore such as that seen in fig. 11e I. The majority of the melanophores had clear cytoplasm, but in some the cytoplasm contained small amounts of melanin. One explanation of this is that phenylthiourea in some way breaks down the mature melanin granule, so liberating melanin into the cytoplasm. It is also possible that even in some presumed mature melanin granules there is still some tyrosinase activity and therefore turnover of the pigment. This would explain the colour difference of the granules, for by suppressing pigment formation, the drug would lighten those granules still forming melanin.

At this stage some of the larvae were returned to tap water so that pigment was allowed to re-develop in the melanophores. Figs. 11f - 11k show the return of melanin after one to fifteen days' treatment in water. Initially more granules appear, but the melanophore itself remains round in shape (figs. 11f I,II). Accompanying this increase in number of granules, there appears to be a size increase in some granules. Some granules are light brown in colour and this may indicate that they are in the initial

Fig. 11d. I - III Xenopus laevis

Dermal melanophores from a larva at stage 43, after being treated with a 0.01 per cent solution of phenylthiourea for four days. Note that there are fewer granules than in previous figures, and some are lighter in colour. Figure 11d III shows that the drug is affecting pigment in the centre of the cell more than at the periphery.

Fig. 11e. I, II Xenopus laevis

Dermal melanophores from a larva, at stage 45, after being treated with a 0.01 per cent solution of phenylthiourea for seven days. Only a few granules remain in the melanophore. Note that in this figure and figure 11f the melanophores are apparently punctate.

Fig. 11f. I, II Xenopus laevis

Dermal melanophores from a larva, at stage 45, after being treated with a 0.01 per cent solution of phenylthiourea for seven days, followed by transfer to water for one day. Note that as compared with figure 11e there is already an increase in number of melanin granules.

All fixed: 10 per cent formol

Sections: 6 μ

Stained: Mallory's triple

Scale: 6.3 μ

N = nucleus

LMG = melanin granules lighter in colour than normal ones

Fig. 11d

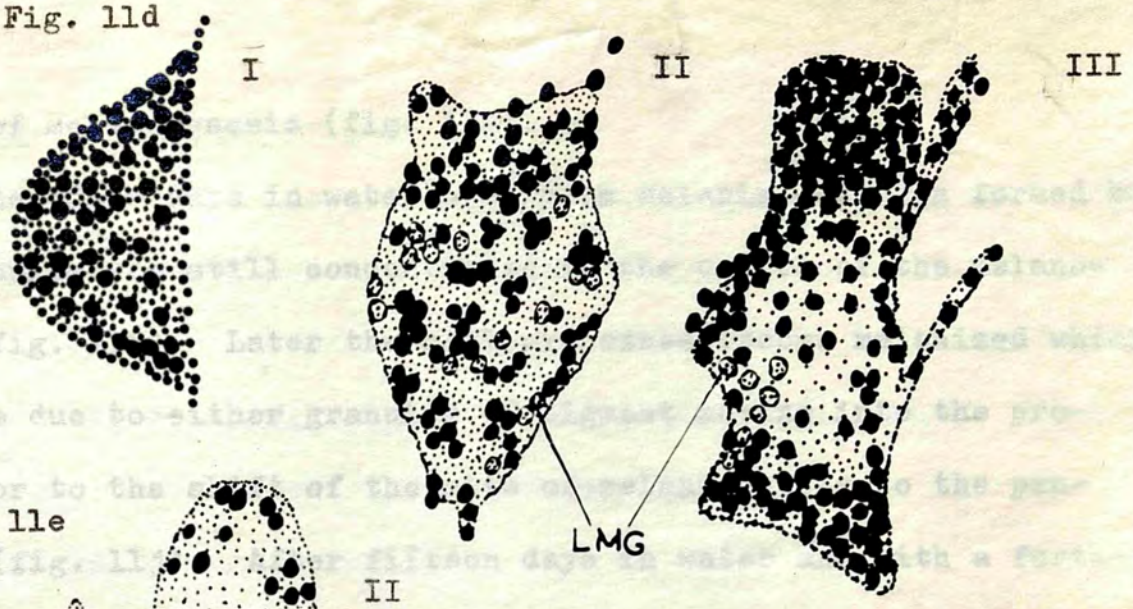


Fig. 11e

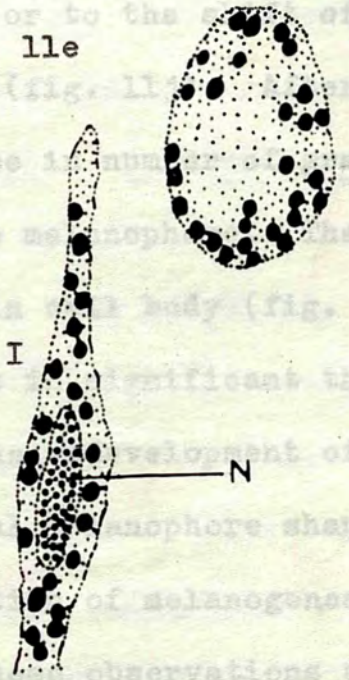
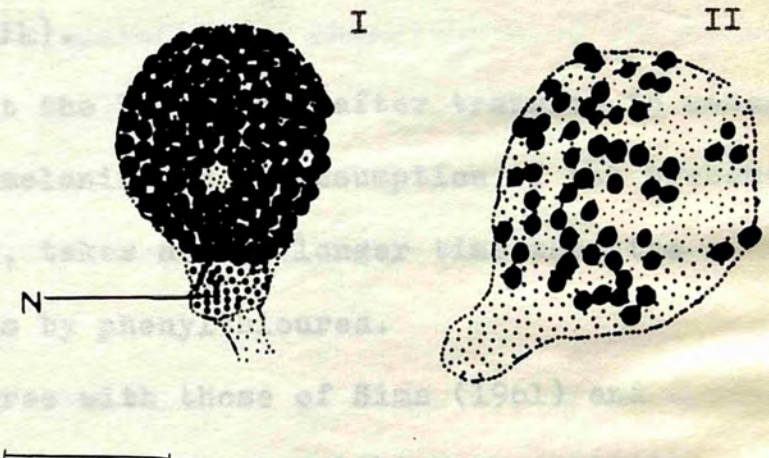


Fig. 11f



stages of melanogenesis (fig. 11g).

After four days in water much more melanin has been formed but the granules are still concentrated in the centre of the melanophore (fig. 11h). Later the cell processes become melanized which could be due to either granules of pigment moving into the processes or to the shift of the site of melanogenesis to the processes (fig. 11j). After fifteen days in water and with a further increase in number of granules, the latter are dispersed throughout the melanophore. The cell shows many processes extending from the main cell body (fig. 11k).

It is significant that the "recovery" after transfer to water viz. the redevelopment of melanin and re-assumption of the presumed original melanophore shape, takes a much longer time than the inhibition of melanogenesis by phenylthiourea.

These observations agree with those of Sims (1961) and Millott and Lynn (1954) who said that phenylthiourea inhibits melanogenesis by a direct effect on the melanophore. However, contrary to Sims' conclusions the drug does seem to have a definite effect e.g. suppression of formation, on the pigment itself.

All fixed: 10 per cent formol

Sections: 6 μ

Stained: Mallory's triple

Scale: 6.3 μ

N = nucleus

Fig. 11g. Xenopus laevis

A dermal melanophore from a larva, at stage 45, after being treated with a 0.01 per cent solution of phenylthiourea for seven days, followed by transfer to water for two days. Note that as compared with figure 11f. more granules have been formed, but some are lighter than others, showing that they are in the initial stages of re-forming melanin.

Fig. 11h. Xenopus laevis

A dermal melanophore from a larva, at stage 46, after being treated with a 0.01 per cent solution of phenylthiourea for seven days, followed by transfer to water for four days. Note, as compared with figure 11g, the further increase in number of the granules, some being slightly larger than in previous figures.

Fig. 11i. Xenopus laevis

A dermal melanophore from a larva, at stage 47, after being treated with a 0.01 per cent solution of phenylthiourea for seven days, followed by transfer to water for seven days. Note that as compared with figure 11h there is only a slight increase in amount of melanin but the granules are still concentrated in the centre of the cell.

Fig. 11j. Xenopus laevis

A dermal melanophore from a larva, at stage 48, after being treated with a 0.01 per cent solution of phenylthiourea for seven days, followed by transfer to water for nine days. Note that as compared with figures 11f - i, one or two granules of pigment are now in the cell processes.

Fig. 11g

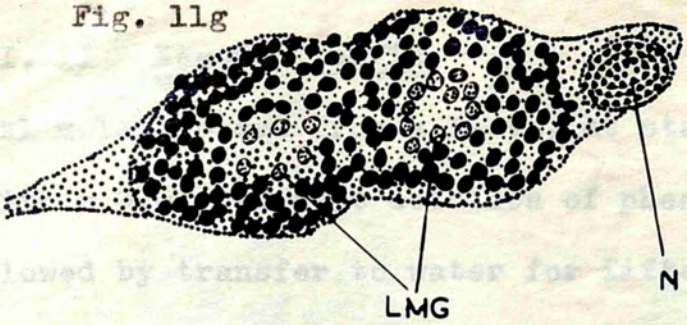


Fig. 11h

Fig. 11i

Fig. 11j

Fig. 11k I, II *Xenopus laevis*

Dermal melanophores from a larva, at stage 49, after being treated with a 0.01 per cent solution of phenylthiourea for seven days, followed by transfer to water for fifteen days. Note that with a still further increase in number of granules, they are becoming more dispersed throughout the melanophore, which has enlarged and is developing processes.

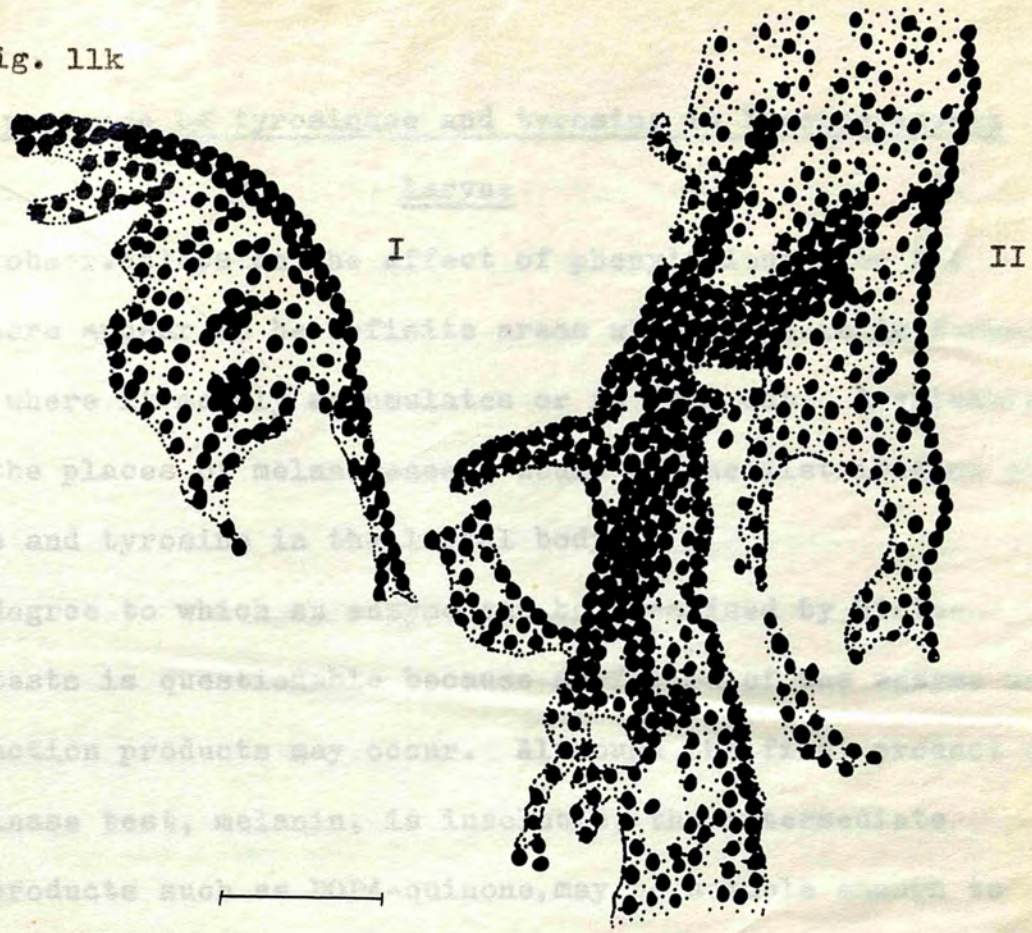
Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 6.3 μ

Fig. 11k



6. The presence of tyrosinase and tyrosine in Xenopus laevis larvae

From observations on the effect of phenylthiourea on the larvae, there appear to be definite areas where melanin is formed and areas where it merely accumulates or is cast out. A valuable index to the places of melanogenesis would be the distribution of tyrosinase and tyrosine in the larval body.

The degree to which an enzyme can be localised by histochemical tests is questionable because diffusion of the enzyme and/or the reaction products may occur. Although the final product of the tyrosinase test, melanin, is insoluble, the intermediate reaction products such as DOPA-quinone, may be soluble enough to diffuse from the site of their production. However, in mammals at least most of the tyrosinase is supposed to be contained in the intercellular melanosomes and it has been found that it does not diffuse easily from them. If this were also true for the melanin granules forming melanin in amphibians, then a good histochemical test showing the presence of tyrosinase might be a useful guide.

Bearing in mind these difficulties attempts were made to demonstrate the presence of tyrosinase by the DOPA-oxidase reaction using the method given by Becker *et al* (1935) and modified by Pearse (1953). *X. laevis* larvae at stages ranging from 26 to 52 were reared in water. They were fixed for one hour in 10 per cent formalin, and then incubated with a solution of DOPA for sixteen hours at 37°C. Subsequently fixation was continued in Bouin's fluid, and prepared sections were stained by Mallory's triple method.

These were compared with sections of larvae of the same stages that had not been incubated with a DOPA solution but in other respects had been treated in the same way. In this test the presence of tyrosinase is indicated by dense aggregates of black pigment, formed by the enzymatic oxidation of the substrate artificially supplied.

A factor mentioned in the histochemical DOPA test is that the size of the tissue incubated with DOPA should be 3-5 mms. thick. Accordingly some of the larger larvae from about stages 42 to 52 were cut into pieces of the required size before incubation. The results of the DOPA test on these pieces were the same as those from tested whole larvae at the same stages.

The results of the DOPA tests which show the presence of tyrosinase in various areas of the larval body are shown in Table 1. Often the results were not easy to assess, particularly in the peritoneum, meninges and eye of a larva at stage 47. In these areas of a control animal there is already so much melanin present that they appear a uniform black colour, and this presents a difficulty in trying to assess the result of the DOPA test, as any additional pigment produced as a result of the test does not show up. With this severe limitation, the only conclusion that can be drawn from such experiments in themselves is that active tyrosinase may be present in the peritoneum, meninges and eye from stage 47 onwards, but the result of the DOPA test does not give an accurate picture. Since tyrosinase is present in these three areas before stage 47,

and since phenylthiourea decreases the amount of melanin at stage 47 and later, it is probable that tyrosinase is active in these areas, unless the effect of the drug is to cause discharge of melanin from these areas. However, as confirmatory evidence it has been noted elsewhere (page 39) that in larvae which have been treated with phenylthiourea until very pale, and then returned to water at stage 46, pigment redevelops in the peritoneum and meninges, and sometimes traces re-appear in the eye.

However, the test was good enough to reveal significant differences between experimental and control larvae in the case of the dermal pigmentation (figs. 12 and 13).

In the gut there was no evidence of tyrosinase activity at any stage of development, and this confirms the idea that here melanin is being accumulated and excreted. In the cerebral ventricles and central canal of the neural tube there was a slight indication of tyrosinase activity up to stage 47 but none after this. Here the results from the phenylthiourea tests and the DOPA tests do not appear to correspond, for phenylthiourea actually increases the amount of pigment present or is without effect. The pigment occurs in between the cells of the brain wall, in the cerebral ventricles and lumen of the neural tube. From its disposition it is likely that it is excreted from the inter-cellular spaces of the brain. It is possible that this pigment is passed into the brain and neural tube from the processes of the melanophores which surround these organs. Judging also from the effect of phenylthiourea on

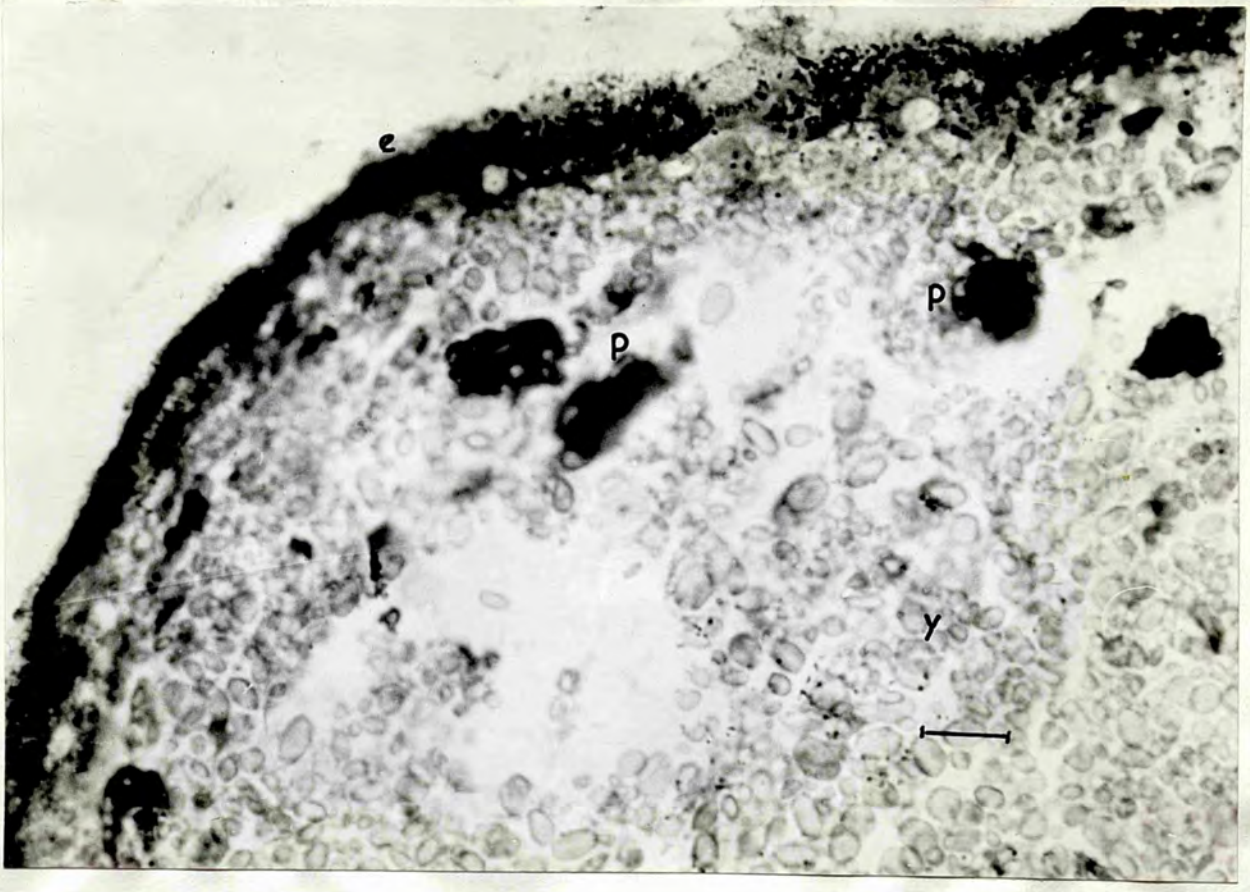


Fig. 12. Xenopus laevis

Portion of a sagittal section of a larva at stage 32, which had been incubated with a DOPA solution for sixteen hours. Note here the large black masses of pigment (p) which indicate the presence of tyrosinase among the yolk cells (y). Compare with fig. 3 which is a similar section that has not been incubated with DOPA. The definitive epidermis (e) shows more pigment here as compared with fig. 3.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 180 µ

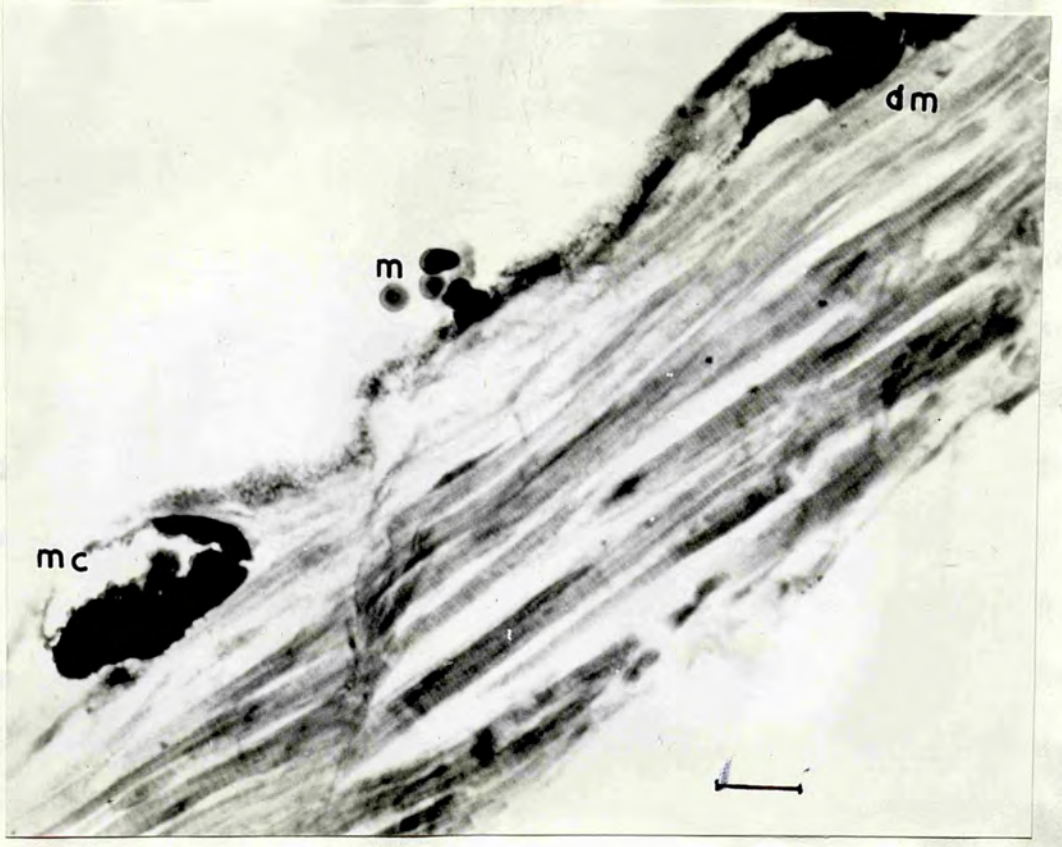


Fig. 13. Xenopus laevis

Portion of a sagittal section through the tail region of a larva, at stage 46, which had been incubated with a solution of DOPA for sixteen hours. Note that the mucous cells (mc) in the epidermis give a positive response to DOPA. What appears to be large aggregations of melanin (m) being liberated from the surface of the epidermis is probably mucus showing a positive response to DOPA. Note also the dermal melanophore (dm) which shows a positive response to DOPA.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 100 µ

the pigmentation of these sites it appears as if melanin is excreted from them. The DOPA test may be unreliable in this situation because of the apparent greater density of the pigment in the cerebral ventricles and neural tube of the experimental animals when compared with controls. This makes any change in blackness difficult to judge. It is therefore doubtful whether tyrosinase is active in melanogenesis in these areas.

Since the results of the DOPA tests are often difficult to assess, the pigmentation of some larvae was partially bleached with hydrogen peroxide, after the initial fixing in formalin. Then after washing for two to three minutes in tap water, the animals were incubated with a DOPA solution as described above. The results showed that the DOPA reaction had been inhibited in some way, for the DOPA solution remained colourless whereas it is normally brown after incubation. Also no new melanin was formed in any of the larval sites following incubation. Although the larvae were washed after bleaching, if any hydrogen peroxide still remained it would probably bleach any new melanin formed after incubation with DOPA. It is possible that the hydrogen peroxide affected the pH of the reaction, since in the histochemical test a pH of 6.4 is recommended, or that it denatured the tyrosinase.

Although the DOPA test has been used extensively to demonstrate histochemically the presence of tyrosinase, it is not fully reliable. Pearse (1953) states that erythrocytes and leucocytes have sometimes been found to oxidise a DOPA solution, and here a

peroxidase is thought to be responsible. Therefore it is necessary to interpret DOPA test results with great caution.

In general the results of the DOPA test showed a positive reaction in the same areas as those in which phenylthiourea inhibited melanogenesis. The inference made on page ^ that melanogenesis was occurring in these areas is therefore confirmed, but there are some anomalies. Thus, certain large epidermal cells in larvae from stages 39 to 46 gave a positive result when incubated with DOPA. The picture presented (fig. 13) was of large aggregations of DOPA positive substance in large epidermal glands, some of the substance apparently being discharged as secretion. Such dark areas were not found in the epidermis of the control larvae, where after stage 36 no melanin was present at all. In appearance and distribution the DOPA positive cells resemble mucous glands. The above results for the epidermis agree with those of Sims (1962) who said that in Amphibia a positive response to the DOPA reaction is not always confined to cells capable of forming melanin. Again Dushane (1936) found that the epidermal and mucus secreting cells of Axolotl and Triturus skin were all DOPA positive.

It therefore appears that these large DOPA positive epidermal cells could be secreting a mucus that reacts positively to DOPA. To test these ideas, sections of larvae from stages 39 to 45, with or without incubation with DOPA, were examined for mucus. The methods used were staining in Alcian Blue (Steedman, 1950,

modified by Pearse, 1953) and the colloidal iron test (Mowry, 1958). After incubation with DOPA solution the majority of tissues in the sections were stained light brown. In the case of the sections which had not been incubated with DOPA the epidermal mucus glands responded to stain in the manner expected, but after such incubation, because the sections turned brown, subsequent staining produced colours much darker than normal. The mucus cells of Xenopus skin therefore react positively to DOPA, although melanin is not formed in these cells.

Another interesting feature, revealed when sections of larvae at stages 33 and 36 were stained with these mucoid stains, was that egg pigment was mixed with the mucus discharged from the surface of the epidermis. From these sections it was again apparent that the DOPA positive substance described above, being discharged from the epidermis, was a secretion containing mucus.

It is now necessary to show the presence of a substrate. Tyrosine was tested for and revealed in sections of larvae from stages 26 to 52, by the method of Glenner and Lillie (1959) which is based on diazotization of tyrosine, followed by coupling of the product with "S-acid" (8 amino-1 naphthol-5 sulphonic acid) to yield a stable deep violet chromogen. The results of these tests are shown in Table 1.

It was found that tyrosine is widely distributed and not confined to the areas of melanogenesis. Thus it is present in strikingly large amounts in yolk cells. This is not surprising since tyrosine is an essential amino acid as well as being the

substrate for melanogenesis. It was found in the majority of the areas where melanin is formed, except in the peritoneum and meninges where the tyrosine test did not give a clear positive result. From stage 45 onwards the result of the test was clearly negative in these areas. This is surprising since melanin is formed there at least up to stage 47. It is possible that melanin in these two areas is formed from a substrate other than tyrosine, for example DOPA or from a colourless intermediate compound. Tyrosine is not found in the epidermis except noticeably in the mucus cells.

The significance of the results in Table 1 will be discussed later, but two points may be noted here. First, that in the liver and pancreas, tyrosine is present from stage 40 onwards, but no melanin is seen until stage 47. On the basis of what was revealed by the DOPA tests this appears understandable because tyrosinase appears at stage 47. Second, that although tyrosine is present in the mucus glands, they do not normally produce melanin and yet they can oxidise DOPA. Here it appears as though DOPA may be oxidised independently of tyrosinase, possibly by a peroxidase system as in erythrocytes and leucocytes. Another possibility is that the mucus cells can trap coloured autoxidation products from the DOPA incubating medium.

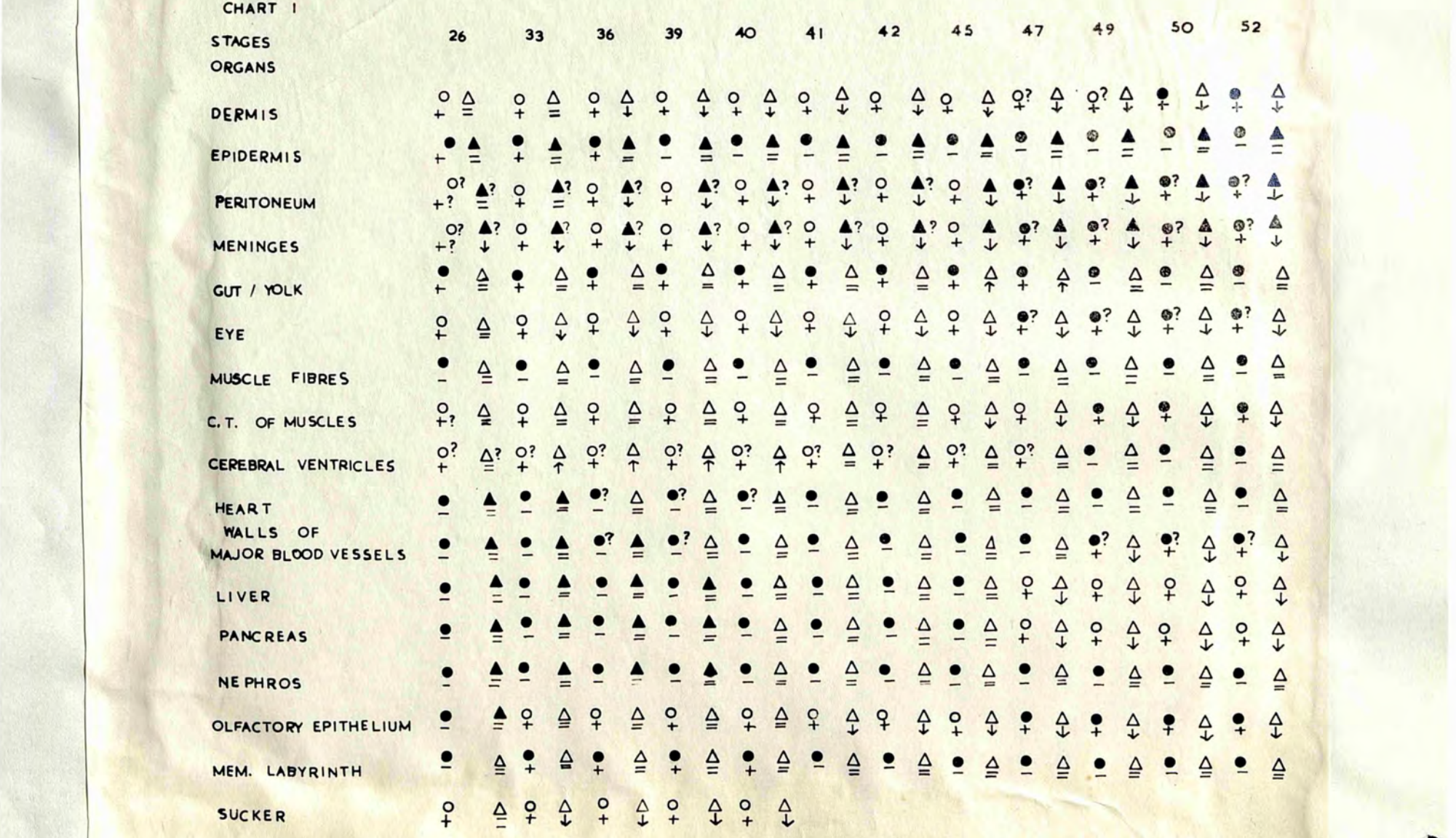
Table 1

This correlates the experimental demonstration of tyrosine and tyrosinase with the presence of melanin in, and the gross effect of phenylthiourea during the development of Xenopus laevis.

Stages are according to Nieukwoop and Faber, and indicate the times when the larvae were removed from the drug and fixed. The data for phenylthiourea were derived from the experiments using 0.01 per cent solutions in which the larvae were reared with effect from stage 23. Stage 36 corresponds to the time of hatching. Note that in the peritoneum, meninges and eye, after stage 47, the results of the DOPA test are difficult to assess (see page 56) so that although the results are negative, an active enzyme may still be present.

+	presence of melanin
-	absence of melanin
O	presence of tyrosinase
●	absence of tyrosinase
◬	presence of tyrosine
▲	absence of tyrosine
=	phenylthiourea has no effect
↓	phenylthiourea decreases the amount of melanin
↑	phenylthiourea increases the amount of melanin
?	a doubtful result

CHART I


STAGES
ORGANS
26
33
36
39
40
41
42
45
47
49
50
52
DERMIS
EPIDERMIS
PERITONEUM
MENINGES
GUT / YOLK
EYE
MUSCLE FIBRES
C.T. OF MUSCLES
CEREBRAL VENTRICLES
HEART
WALLS OF MAJOR BLOOD VESSELS
LIVER
PANCREAS
NEPHROS
OLFACTORY EPITHELIUM
MEM. LABYRINTH
SUCKER

7. The development of melanin pigmentation in Rana temporaria larvae and the effect of phenylthiourea

The foregoing experiments with Xenopus show some results that need verification and clarification, so by way of comparison, the development of pigmentation and the effect of phenylthiourea were studied in R. temporaria larvae. The experiments on Rana were less extensive than those on Xenopus, but the results resolved certain of the difficulties that arose in the study of the latter.

The experimental methods used for Rana larvae were the same as those used for Xenopus. Small amounts of frog spawn were reared in tap water in shallow dishes at 15°C. The developing larvae were fixed daily from egg stage until sixteen days old (equivalent to stage 51 of Xenopus development). Subsequent preparation of sections was the same as that described for Xenopus.

Two groups of experiments were performed to investigate the effect of phenylthiourea. In one, frog spawn was reared in 0.01 per cent solutions for fourteen days from the egg stage. In the other, tadpoles were reared in the same concentrations but treatment was begun nine days after the eggs were laid. The effect was examined from one to seventeen days after treatment began. Again in these experiments larvae were fixed daily and sections compared with controls at the same stage of development.

In general much more melanin is present in Rana larvae than in those of Xenopus, and the effect of the drug is much clearer. When the distribution of melanin is compared in the two species, in addition to the sites where melanin is found in Xenopus, in Rana

it is found in the muscle fibres themselves, in the heart, nephric tubules and around the gonads. When the amounts of pigment in the two species is compared in such situations as the muscles and brain, in Xenopus melanin was seen in between the muscle fibres and in the walls of the forming brain, but in Rana much greater amounts are found in these two areas.

During the first two to four days in development (from gastrula to neural tube stage) large quantities of pigment are present in the cells of the definitive epidermis, in those of the sucker and olfactory epithelium. Extracellular pigment is present surrounding the dermal cells and around the yolk cells of the forming gut. All the epidermal melanin is in a finely divided state.

The epidermal pigment like that in the yolk and dermis is unaffected by three days' treatment with phenylthiourea, and so appears to be egg pigment. Isolated melanophores are often seen in the epidermis at this stage. The familiar signs of accumulation and excretion of extracellular pigment were evident in the walls of the forming brain and lumen of the neural tube. Such pigment at this stage is unaffected by phenylthiourea, although later the pigment is increased by treatment with the drug.

The amount of pigment taken over from the egg steadily diminishes in the period of development from four to ten days.

At the neural tube stage of development only traces of pigment are present in the peritoneum, meninges, eye, connective tissue around the myotomes and in the membranous labyrinth. Of this

pigment only that in the peritoneum, meninges and membranous labyrinth is affected at this stage by three days' treatment with phenylthiourea.

Melanophores, at first round in form, begin to appear in the dermis at six days and increase in number, especially in the tail region (fig. 15). Five days' treatment with phenylthiourea, beginning at gastrulation, decreases the melanin content of these dermal melanophores. Increasing amounts of melanin appear with age in the peritoneum, meninges and eye, and this remains susceptible to phenylthiourea. Thus pigment in these areas and in the olfactory epithelium, membranous labyrinth and sucker is decreased in amount by the drug, but in the last three areas it is clearly less susceptible to it than in the peritoneum, meninges and eye.

Small amounts of melanin are seen in the muscle fibres, in between them and around the myotomes, but this pigment is unaffected by five to seven days' treatment with phenylthiourea. However, after eight days' treatment, beginning at gastrulation, the amount of melanin in the muscle fibres is increased, and this increase becomes more apparent with age and longer treatment. The melanin content of the gut, cerebral ventricles and forming brain is also very obviously increased by the same treatment with phenylthiourea.

At six and seven days, traces of melanin appear in the muscular coats of the heart, major blood vessels and around the nerves. This pigment is unaffected by nine days' treatment with

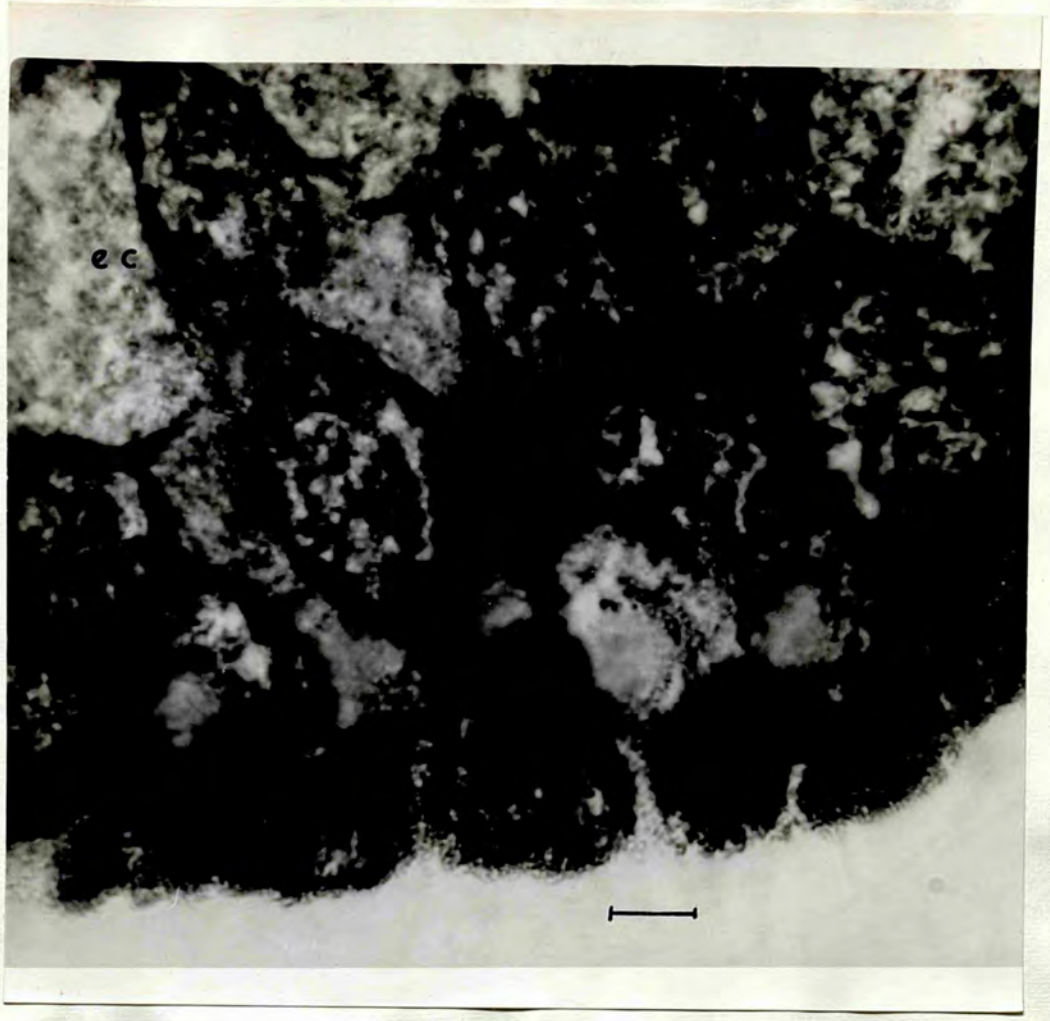


Fig. 14. Rana temporaria

A region of the epidermis showing epidermal cells (ec) full of egg pigment, and what appears to be an isolated melanophore in the centre of the photograph, although this is probably an epidermal cell, but packed with dense pigment. From a para-sagittal section of a gastrula.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 100 µ

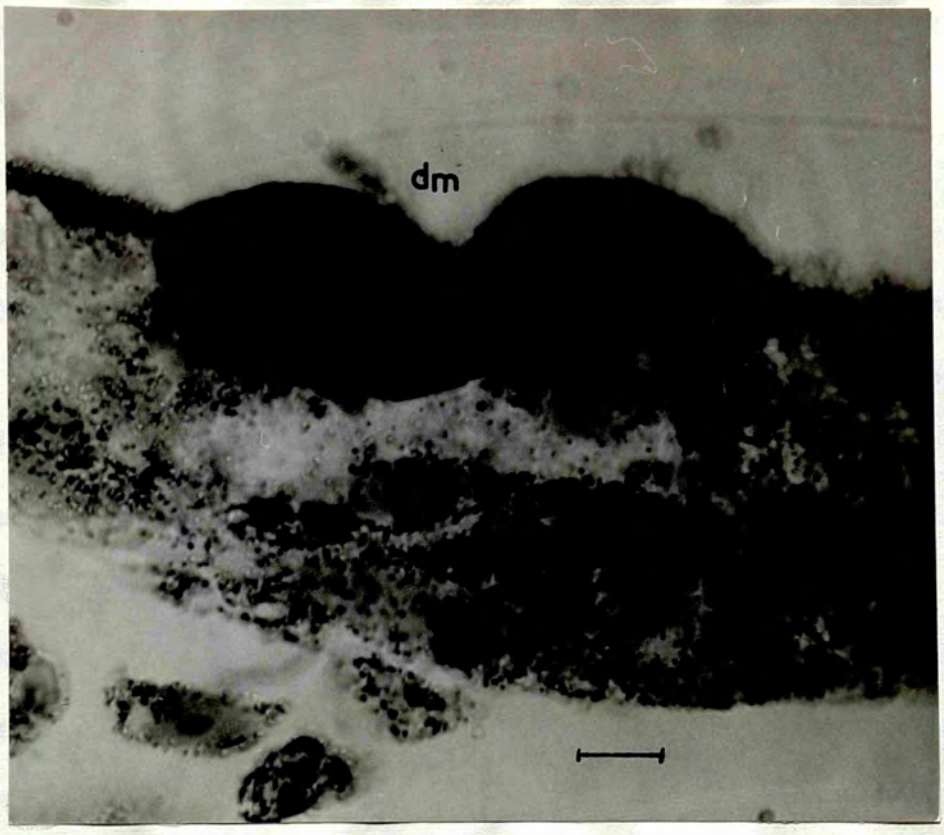


Fig. 15. Rana temporaria

Two dermal melanophores (dm) as seen in a longitudinal section of the tail region of a seven days old larva reared in water. Note also the melanin in the muscle fibres (mf) below the dermis.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 100 µ

phenylthiourea but after twelve days' treatment, there are slight indications of increased melanin in the heart.

Although very little melanin is normally present in the liver at nine days, larvae at this stage, which have been treated with phenylthiourea for eight days, may show considerable amounts. Thus the effect of the drug on the liver of Rana is the opposite of that seen in Xenopus. A similar situation is seen in the pronephric tubules where melanin appears at six days and similarly small amounts appear later in the mesonephric tubules. The effect of the drug in both of these cases is to increase the amount of melanin. This again is different from the situation in Xenopus where melanin is not present in the nephric tubules, and does not appear even after twenty-three days' treatment with phenylthiourea.

Considerable amounts of melanin are seen in the membranous labyrinth at nine days and it appears to be discharged into the otic vesicle (fig. 16).

At thirteen and fourteen days there are many more dermal and epidermal melanophores. The processes of these cells can often be seen to be alongside unpigmented epidermal cells, but a fuller discussion of their appearance is reserved for a later section. Intracellular pigment which is not in melanophores, still remains in the epidermis, but whereas up to this stage the amount of this pigment was unaffected or increased by phenylthiourea, after thirteen days' treatment beginning at gastrulation, there are signs of a decrease in epidermal pigmentation (see Table 2).

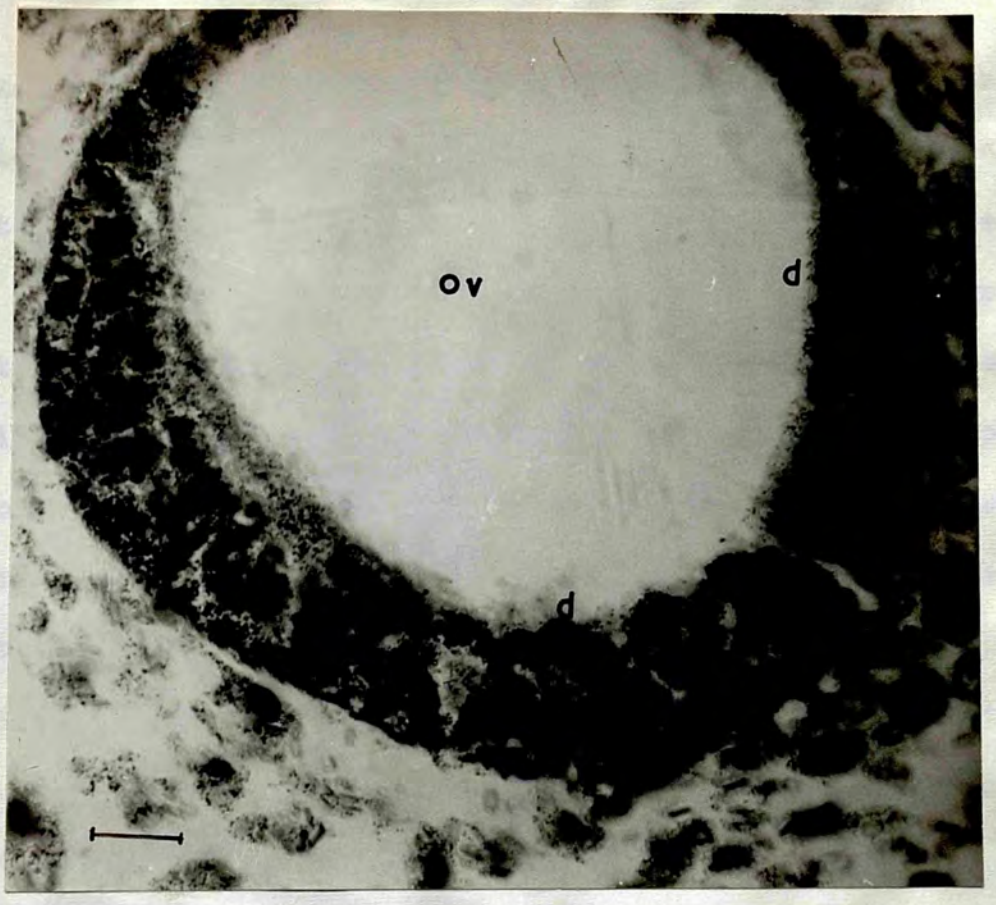


Fig. 16. Rana temporaria

Melanin in the developing membranous labyrinth as seen in a parasagittal section of a larva, nine days old, taken from water.

ov, otic vesicle

d, melanin apparently being discharged into the otic vesicle

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 100 μ

After seventeen days' treatment beginning in larvae that are nine days old there is quite a considerable decrease in epidermal pigment, but here the effect of the drug would appear to be a simple inhibition of melanogenesis in the epidermal melanophores.

At fourteen days there is still more melanin in the muscles (fig. 17), heart and walls of the major blood vessels. Experiments on treated larvae, in which the treatment began at gastrulation or when nine days old, showed the following effects: a decrease of the pigment in the dermis, peritoneum, meninges, eye, olfactory epithelium and membranous labyrinth; an increase of pigment in the gut, muscle fibres, cerebral ventricles and brain, liver and nephric tubules. It is noteworthy that when treatment was begun at nine days the general effects of the drug were less pronounced than those produced by treatment which started earlier.

Figs. 17 to 26 illustrate the points made above. These agree with the conclusions of Millott and Lynn (1965) except that they found that the amount of pigment in the cerebral ventricles remained unaltered after treatment, whereas my results show a slight increase in the pigment.

Fig. 17. Rana temporaria

Melanin (m) inside muscle fibres (mf), in between them (i) and in the connective tissue around the myotomes (ct), as seen in a frontal section of the tail region of a larva, thirteen days old, taken from water.

Fixed: 10 per cent formol

Sections: 6 µ

Stained: Mallory's triple

Scale: 180 µ

Fig. 18. Rana temporaria

Melanin granules (m) in the liver lobules and large melanin aggregations (a) outside the lobules seen in the liver of a larva, fourteen days old, taken from water.

Compare with Fig. 19.

Fig. 19. Rana temporaria

Increased amounts of both intra- (m) and extra-lobular melanin (a) seen in the liver of a larva, fourteen days old, which had been treated with a 0.01 per cent solution of phenyl-thiourea for five days, starting when the larva was nine days old.

Both fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 100 µ

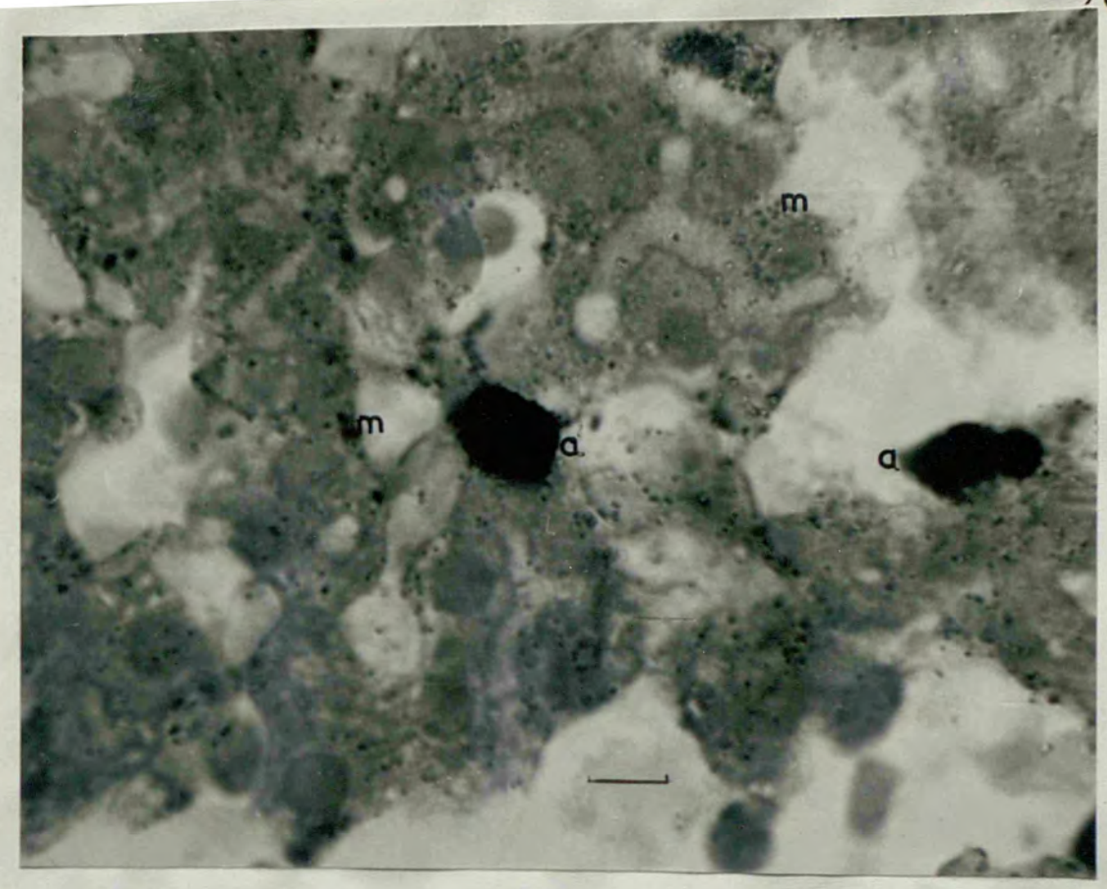


Fig. 18.

Fig. 19.

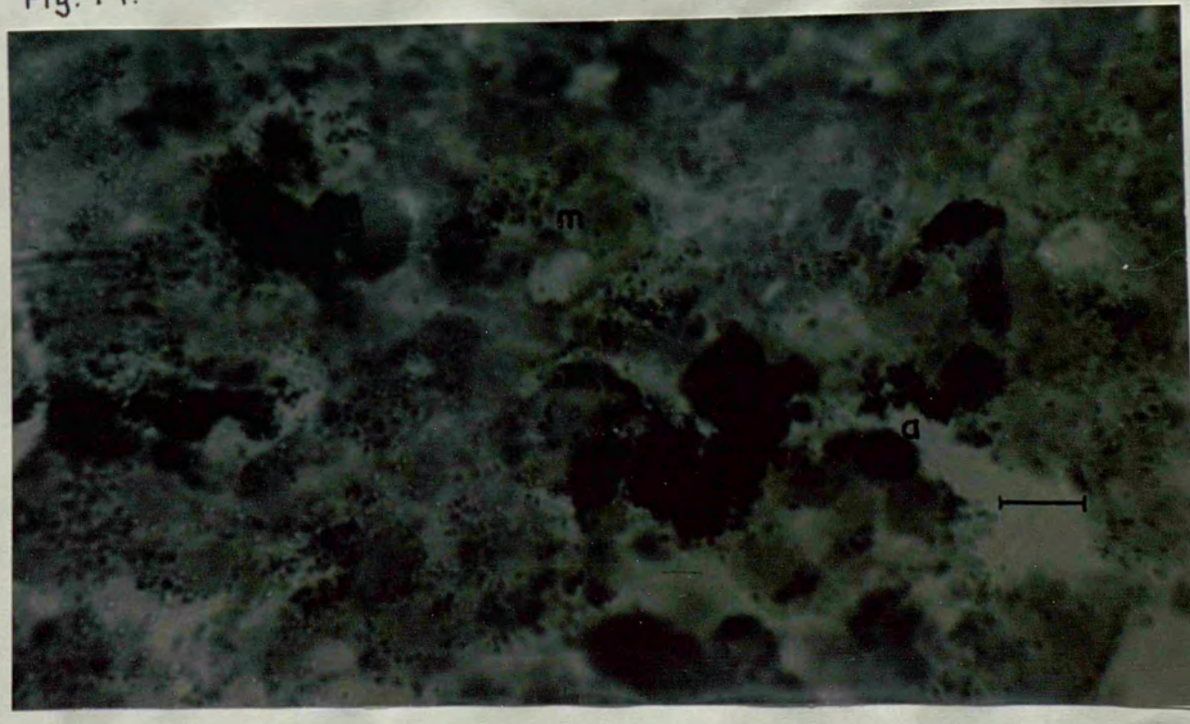


Fig. 20. Rana temporaria

Melanin granules in the epidermis (me), dermal melanophores (dm) and dense melanin in the peritoneum (mp) as seen in a control larva when fourteen days old. Note that the process (p) of the dermal melanophore in the centre appears to be giving off granules into an epidermal cell at the point marked by an arrow.

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 100 μ

Compare with Fig. 21.

Fig. 21. Rana temporaria

Similar section to that in Fig. 20 taken from a larva which had been treated with a 0.01 per cent solution of phenylthiourea for five days, starting when nine days old. Note that the epidermal pigment (e) is unaffected, but there is now no pigment in the dermis. Melanin is still present in the peritoneum (mp) although it has been reduced considerably. The peritoneum seen in the section lines the body cavity and has been pressed against the skin. Note too that melanin is still present in the muscle fibres (mf).

Fixed, sectioned and stained as in Fig. 20.

Scale as in Fig. 20.

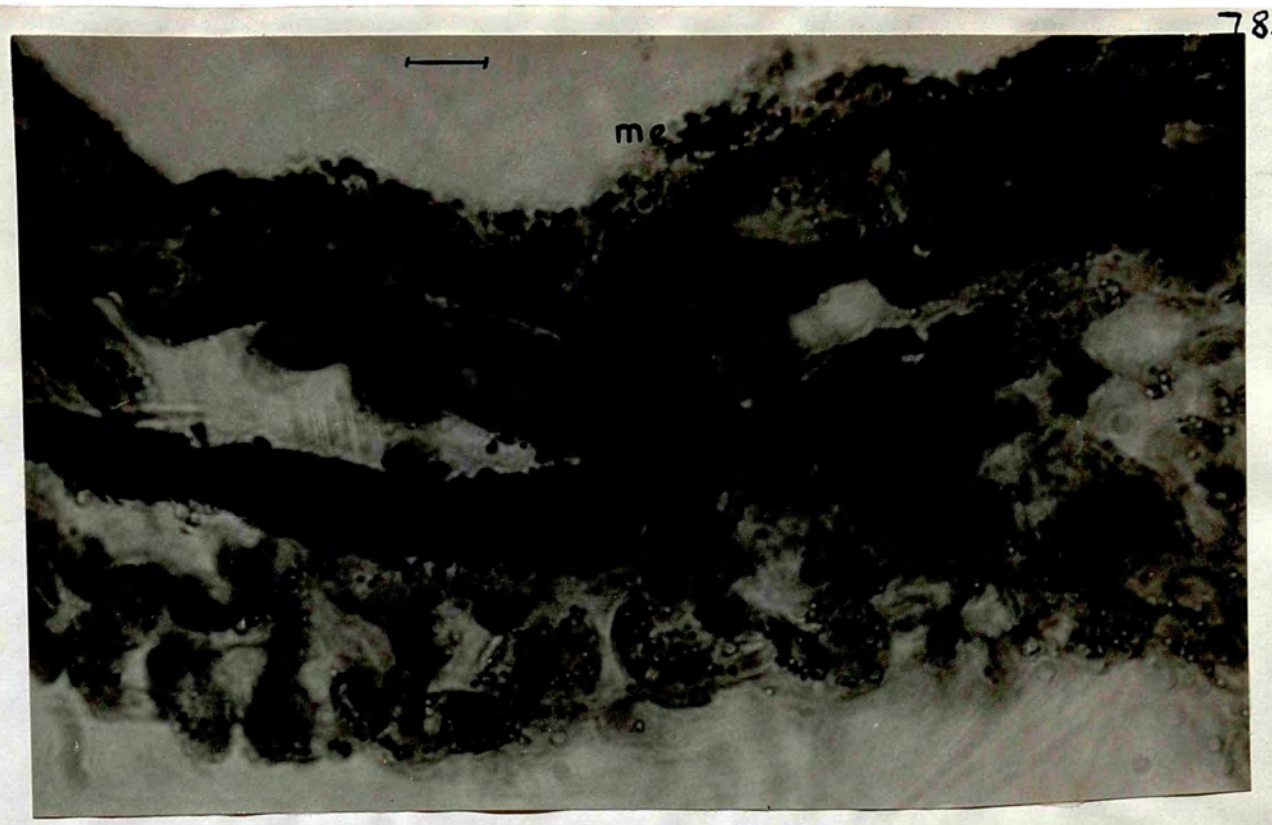


Fig. 20

Fig. 21.

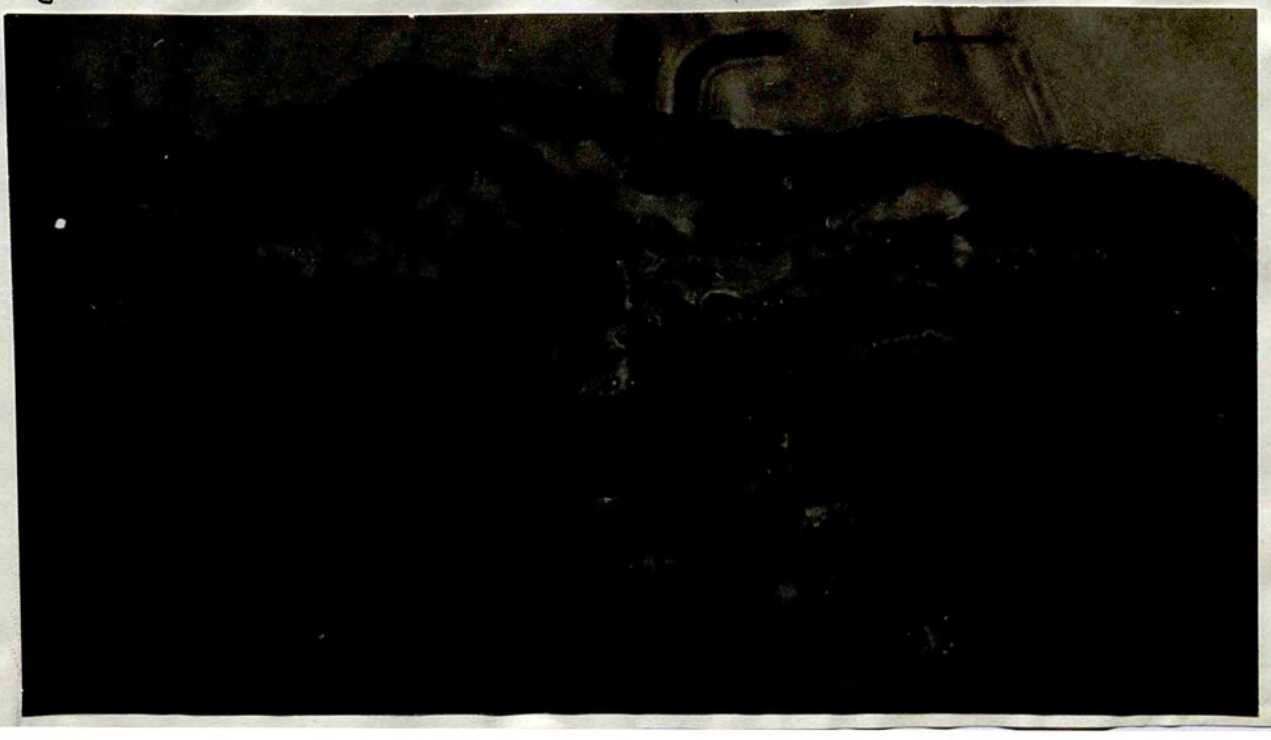


Fig. 22. Rana temporaria

Melanin (m) in the walls of the diencephalon, some of which appears to be discharged into the third ventricle (v) as seen in a frontal section of a control larva, thirteen days old.

Compare with Fig. 23.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 240 µ

Fig. 23. Rana temporaria

Compare with Fig. 22 and note the slightly increased amounts of melanin in the walls (w) of the hind brain, larger amounts of which appear to be discharged into the fourth ventricle at the position marked by the arrow. A similar section from a larva of the same age which had been treated with a 0.01 per cent solution of phenylthiourea for twelve days.

Fixed, sectioned and stained as in Fig. 22.

Scale as in Fig. 22.

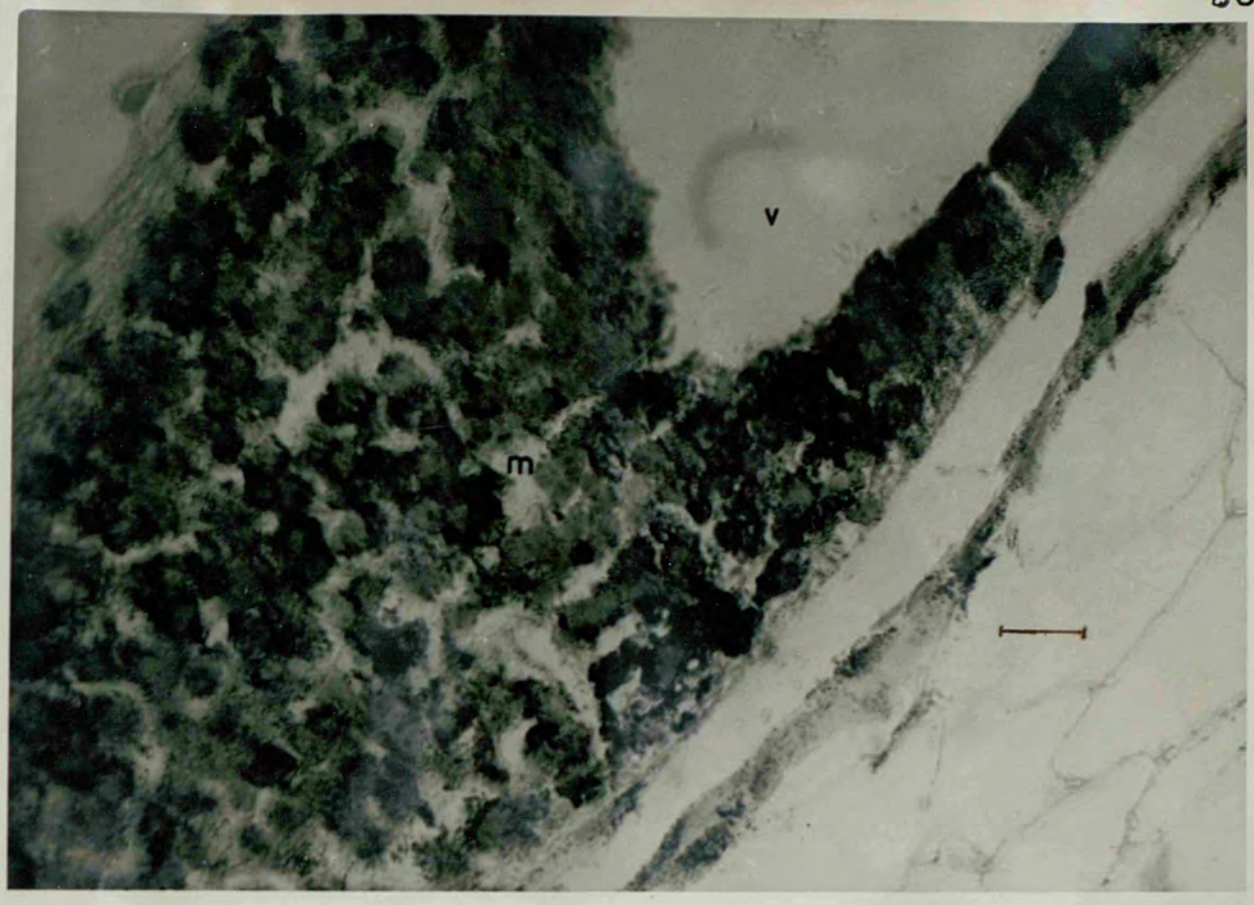


Fig. 22.

Fig. 23.

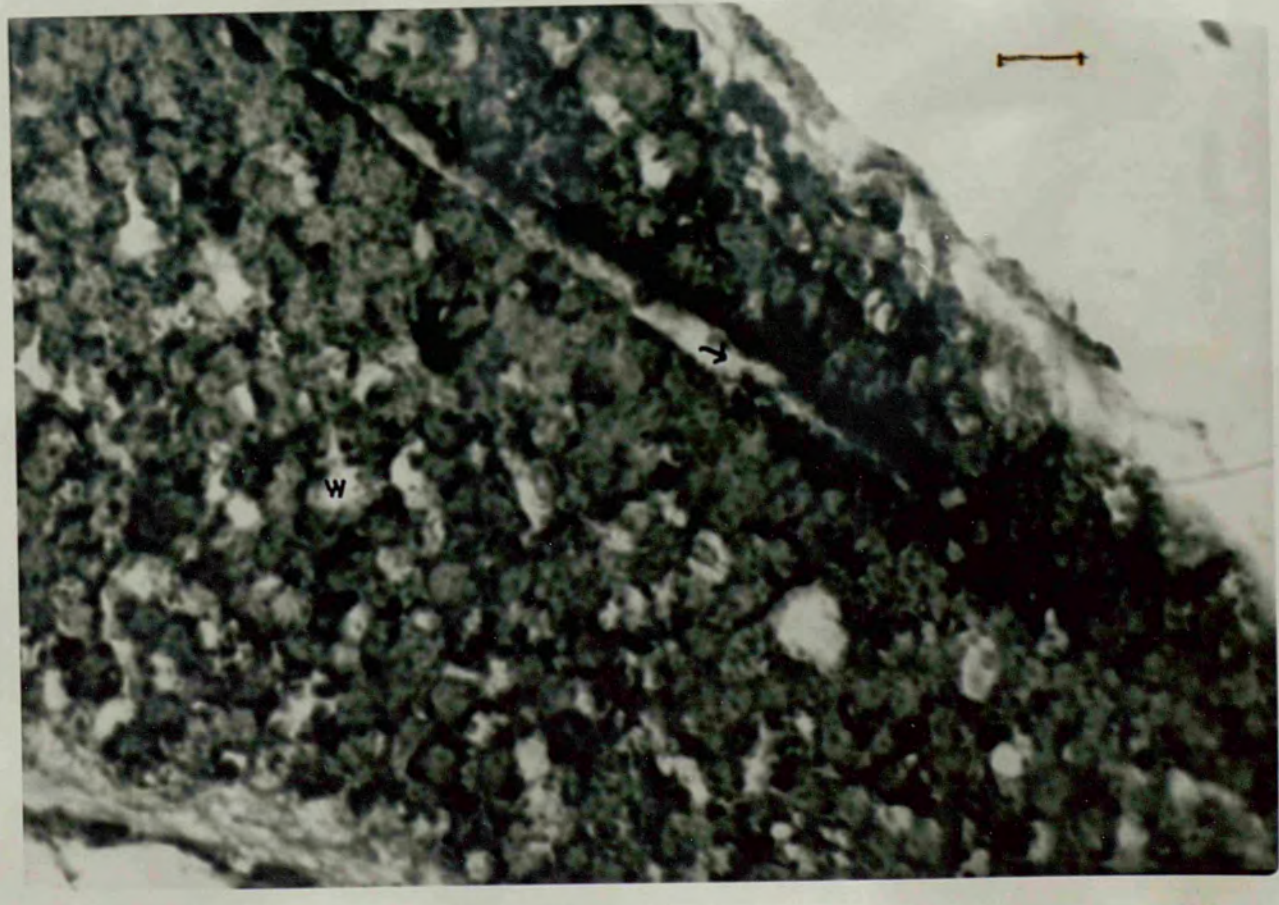


Fig. 24. Rana temporaria

Melanin in the epithelial cells lining the gut as seen in a control larva, fourteen days old. At X some granules are apparently being discharged into the gut lumen (gl). The granules in the lumen are not due to food, because these animals were fed on "Complan" milk powder. Note also that the peritoneum (p) is black with melanin.

Compare with Fig. 25.

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 100 μ

Fig. 25. Rana temporaria

Compare with Fig. 24 and note the much greater amount of melanin in the cells of the gut wall (w). A similar section from a larva of the same age which had been treated with a 0.01 per cent solution of phenylthiourea for five days, starting when nine days old.

Fixed, sectioned and stained as in Fig. 24.

Scale as in Fig. 24.

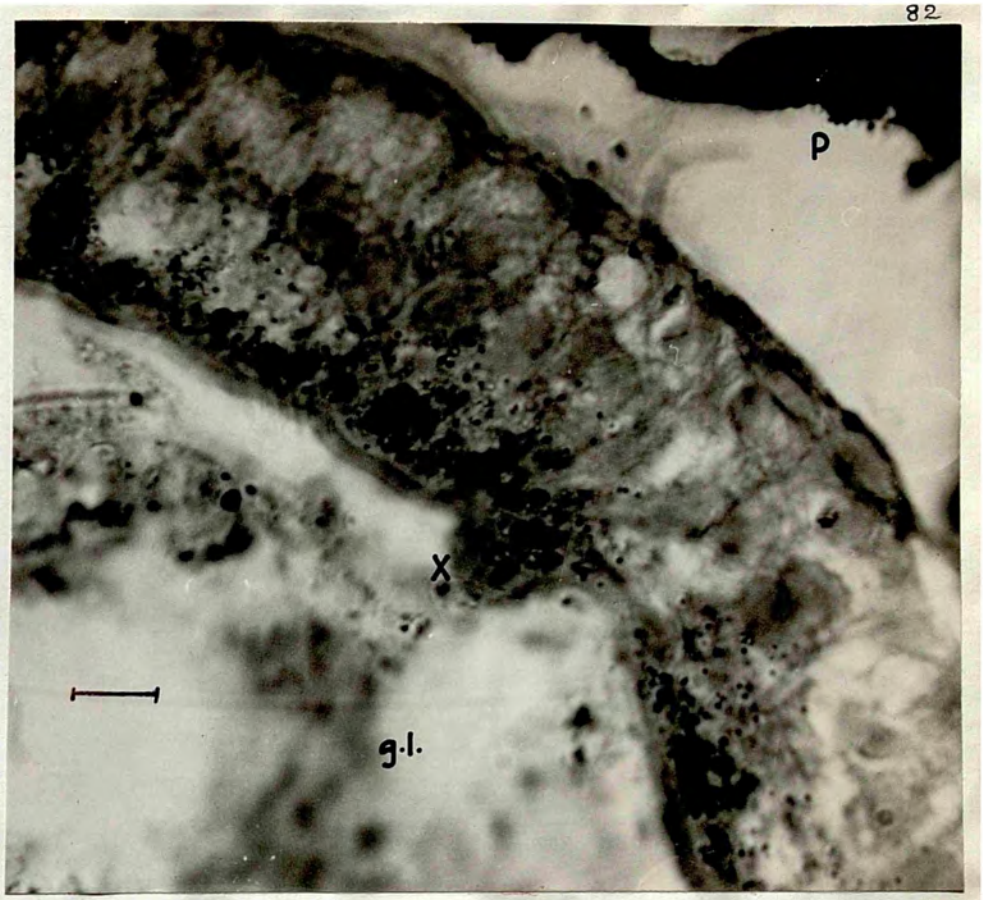


Fig. 24.

Fig. 25.

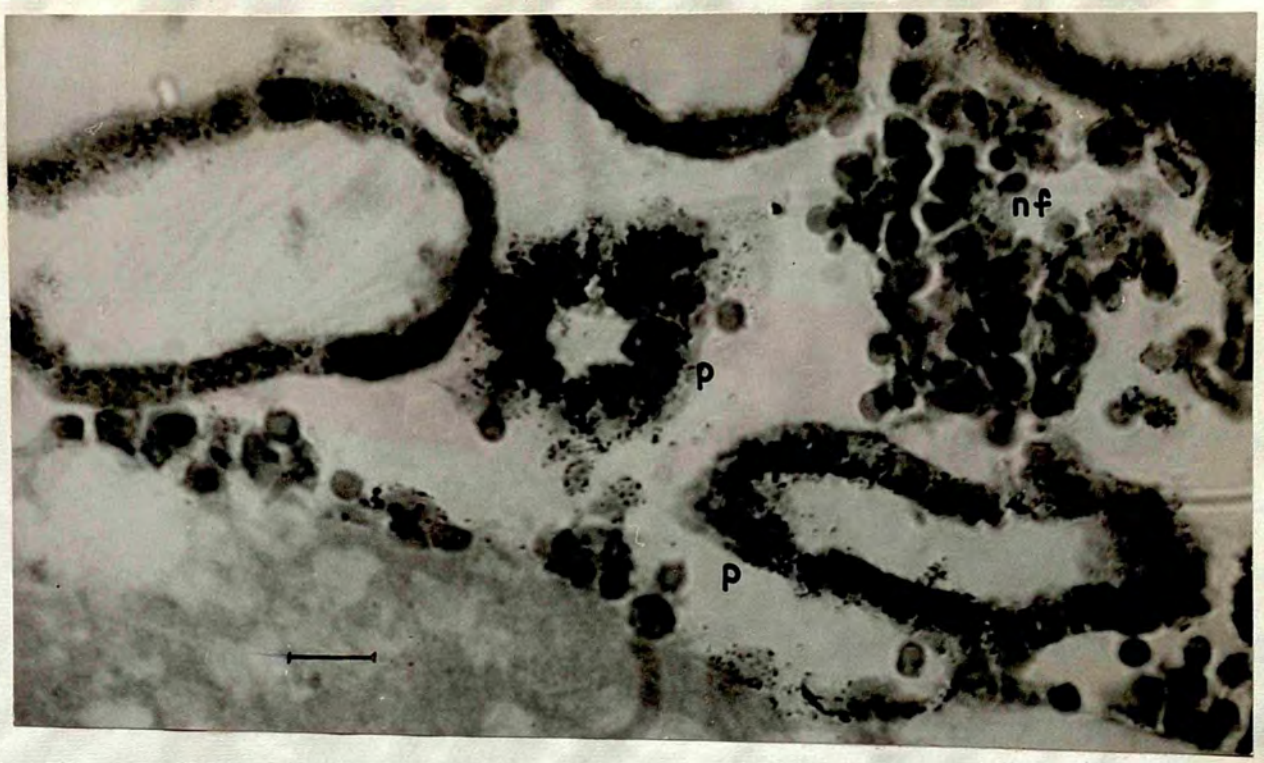


Fig. 26. Rana temporaria

Melanin in the walls of the pronephric tubules (p) as seen in a frontal section of a control larva, thirteen days old. A bundle of nerve fibres (nf) is also shown with some melanin in between the nerves.

Fixed: 10 per cent formol

Section: 6 µ

Stained: Mallory's triple

Scale: 180 µ

8. The presence of tyrosinase and tyrosine in Rana temporaria larvae

The DOPA oxidase test and the tyrosine test (Glenner and Lillie, 1959) were performed on Rana temporaria larvae at stages ranging from gastrulae to fourteen days (about ten days after hatching). The experimental methods were the same as those described for Xenopus laevae. The results of both sets of tests are shown in Table 2.

Tyrosinase, indicated by the DOPA reaction, was found at all stages, with effect from the time the structures could be recognised, in the following instances: dermis, peritoneum, meninges, eye, olfactory epithelium, membranous labyrinth and sucker. As already reported (page 73) phenylthiourea had an inhibitory effect on melanogenesis in all these areas. In these areas too the Glenner and Lillie test showed that tyrosine was present. There is thus consistent evidence that melanin is being formed in these areas, at least in embryos up to fourteen days old.

Neither tyrosine nor tyrosinase are present in any of the epidermal cells up to the age of thirteen days. Nevertheless, from the earliest stages examined melanin was present in all epidermal cells. After thirteen days, although no tyrosine could be demonstrated in the epidermis, a positive indication of tyrosinase appears which coincides with the appearance of an inhibitory effect of phenylthiourea on melanogenesis in the epidermal melanophores. These indications of melanin formation appear to be confined to the epidermal melanophores and are not seen in the

other epidermal cells.

The enzyme was not found in the gut, muscle fibres or connective tissue around the muscles, in the brain wall or cerebral ventricles, in the heart, walls of the major blood vessels, liver or nephric tubules. There is thus no evidence that pigment is being formed in these areas. However, as stated previously the DOPA test is not fully reliable, for it would not show the existence of an inactivated enzyme system. Nevertheless, it may be noted that in the sites listed above, where tyrosinase appears to be absent, phenylthiourea increases pigmentation or is without effect. The pigment that is found in these sites may therefore be accumulated following synthesis elsewhere between four to thirteen days. In addition, there is evidence that it is being eliminated in the gut, for by the time the larva is thirteen days old the pigment begins to diminish in amount in this area. There are clear signs too of this elimination in the case of the cerebral ventricles (figs. 22, 23). In this connexion it is significant to note that the amount of pigment eliminated after treatment with phenylthiourea increased.

In Rana larvae tyrosine is not confined to the sites of melanogenesis. In agreement with what was found in Xenopus larvae, the greatest amounts of tyrosine are found in the yolk cells and the forming gut. Considerable amounts are also present in the muscle fibres, brain and neural tube. Tyrosine may be present in very small amounts or bound in a protein, in which

cases it may not be stainable, but when amounts increase or are freed from the protein then they can be stained. This could explain the situation seen in the heart, walls of the major blood vessels, liver and nephric tubules where tyrosine was only demonstrated after seven days. However, no enzyme was found in these areas, which presumably explains why melanogenesis does not occur in them, and why phenylthiourea has no inhibitory effect on the pigment there.

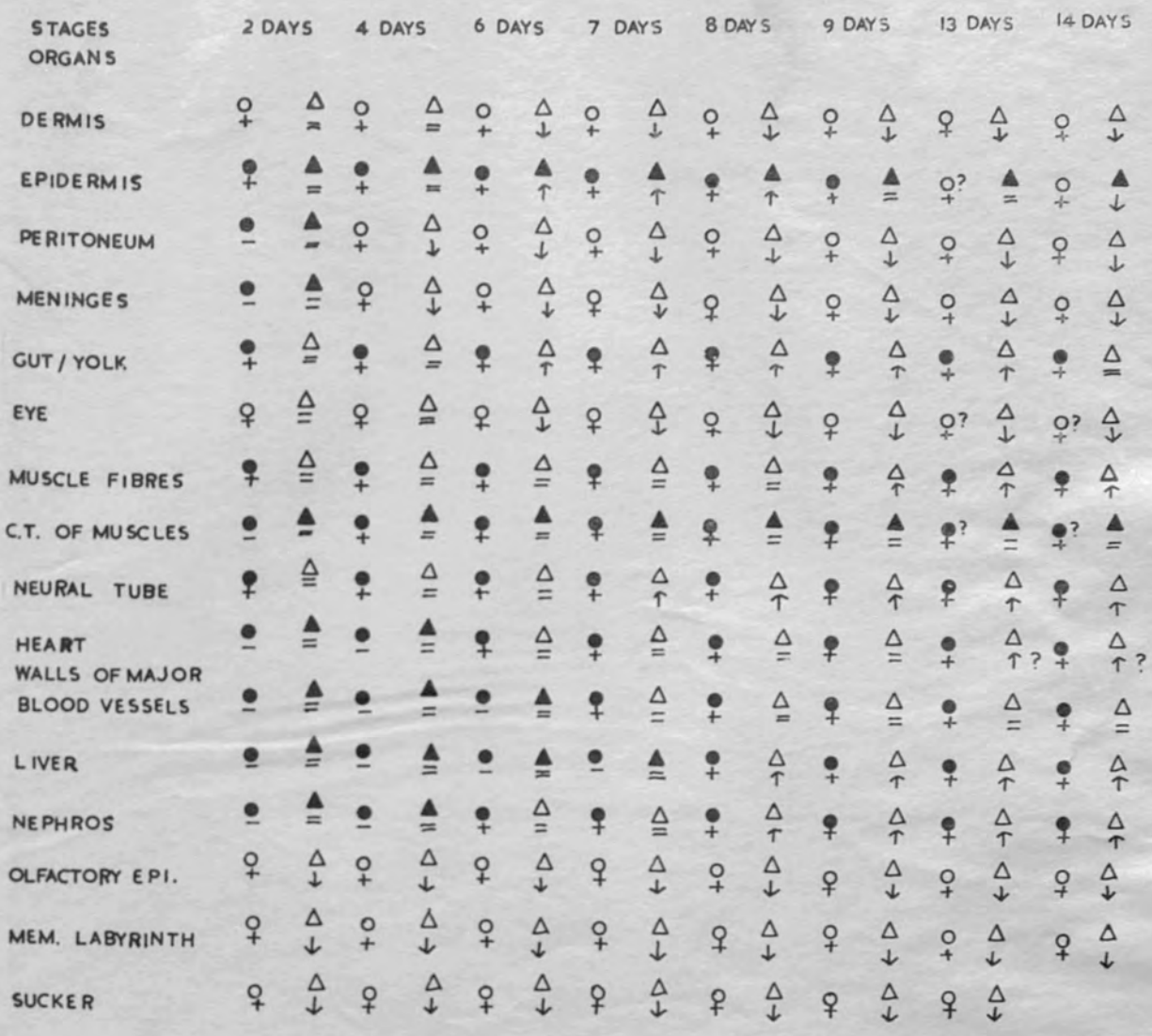
The distribution of tyrosine and <u>active</u> tyrosinase agrees well with the distribution of the areas where phenylthiourea was found to have an inhibitory effect on pigment formation. Again the pigmented areas lacking active enzyme correspond with those from which pigment appears to be eliminated, and in which phenylthiourea appears to increase the amount discharged.

Table 2

This shows the presence of tyrosine, tyrosinase and melanin in Rana temporaria larvae. It also indicates the effect of phenylthiourea (see section 7). The stages indicated at the top of the Table are those at which the larvae were removed from the drug and fixed. Phenylthiourea was used in a 0.01 per cent solution and eggs were put in it when one day old (at gastrula stage).

+	presence of melanin
-	absence of melanin
O	presence of tyrosinase
●	absence of tyrosinase
Δ	presence of tyrosine
▲	absence of tyrosine
=	phenylthiourea has no effect on the melanin
↓	phenylthiourea decreases the amount of melanin
↑	phenylthiourea increases the amount of melanin
?	a doubtful result

CHART 2


STAGES
ORGANS
2 DAYS
4 DAYS
6 DAYS
7 DAYS
8 DAYS
9 DAYS
13 DAYS
14 DAYS
DERMIS
EPIDERMIS
PERITONEUM
MENINGES
GUT / YOLK
EYE
MUSCLE FIBRES
C.T. OF MUSCLES
NEURAL TUBE
HEART
WALLS OF MAJOR
BLOOD VESSELS
LIVER
NEPHROS
OLFACTORY EPI.
MEM. LABYRINTH
SUCKER

9. The relationship of mitochondria to melanin granules

There are three major theories about the precise mode of origin of melanin granules. They are all based on considerations of mammalian material. Meirowsky (1908) proposed that melanin granules are composed of extruded nuclear material. In the light of later cytological investigations on cell components, this theory was disproved. Secondly, Du Buy et al (1949) derived the granules from mitochondria. This hypothesis was based on data provided by vital staining and biochemical enzymatic assays of mouse melanoma. However, subsequent electron microscope studies by Fitzpatrick et al (1960) and Seiji et al (1963) have suggested that melanin granules and mitochondria are distinct subcellular particles. The third theory advanced by Birbeck and Barnicot (1958), Wellings and Siegel (1959) and Seiji et al (1963) suggests that the melanin granules are formed in the Golgi apparatus. This rests on their observation of small vesicles in the Golgi zone that increase in size and then concentrate to form larger granules, in which the melanin polymer is deposited.

None of the above theories is directly relevant to amphibian material, but Niu (1954), working on Triturus torosus embryos, found that melanin first appeared around the nucleus, where mitochondria were most abundant. He therefore suggested that the metabolic activity of mitochondria may help in the initial stages of pigment synthesis. Birbeck and Barnicot, however, suggested that the reason for the large numbers of mitochondria at the site of melanin synthesis in human hair, is that they help to synthesize

the protein matrix of the melanin granule.

It is therefore pertinent to investigate possible relationships between mitochondria and melanin granules in amphibian tissue. Accordingly pieces of parietal peritoneum and liver were removed from Xenopus tadpoles, which had been reared in water, from which they were taken at stage 56. Peritoneum was chosen because of its high content of melanin at this stage, and liver because of its large number of mitochondria and considerable amounts of melanin. 1 to 2 mm. cubes of tissue were fixed in Altmann's fluid for twenty-four hours, followed by his method for post-chroming as given by Baker (1945). Tissue was sectioned at 3 μ, and stained by Metzner's aniline acid fuchsin method (Metzner and Krause, 1928), after which the mitochondria are stained bright red.

Most of the peritoneal melanophores contained much melanin, so that it was necessary to confine observations to the ends of the melanophore processes where melanin granules and mitochondria could be distinguished. The pigment granules were 0.8 to 0.9 μ, and the mitochondria were about 0.8 μ in diameter. The two were sometimes closely associated. Thus, one or two mitochondria were observed with a cap of melanin overlying them (fig. 27) and others with a ring of melanin around them (fig. 28). It must be emphasised that such appearances were rare and the vast majority of mitochondria showed no relationship at all with the melanin granules. The significance of these associations is not yet clear.

Fig. 27. Xenopus laevis

Two types of association between melanin and mitochondria (see page 27) occasionally seen in tangential sections of peritoneum.

A From a larva at stage 56. Note the melanin granules (M.G.) and mitochondria (Mi) with caps of melanin (M) to one side of them.

B Xenopus laevis

Another type of association between melanin (M) and mitochondria (Mi) (see page 27) seen in a larva at stage 56.

Both sections fixed: Altmann's fluid

Sections: 3/u

Stained: Metzner's aniline acid fuchsin

Scale: 2.7/u

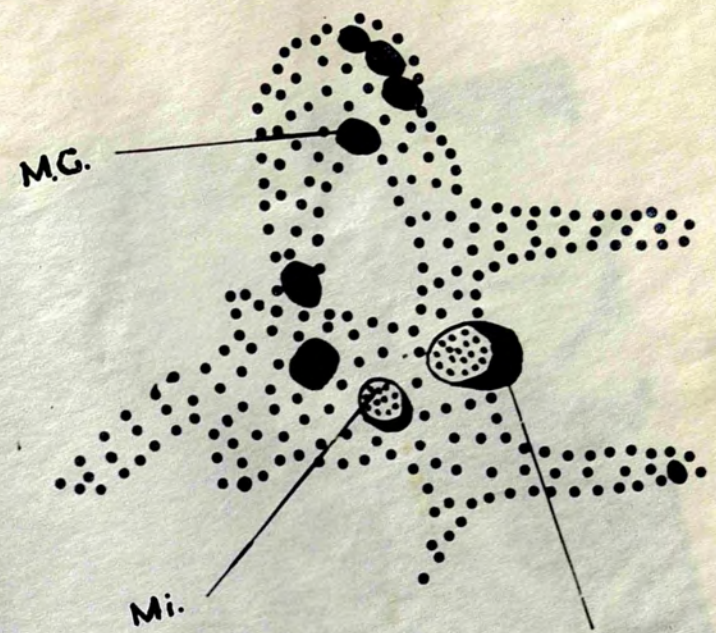


Fig. 27 A

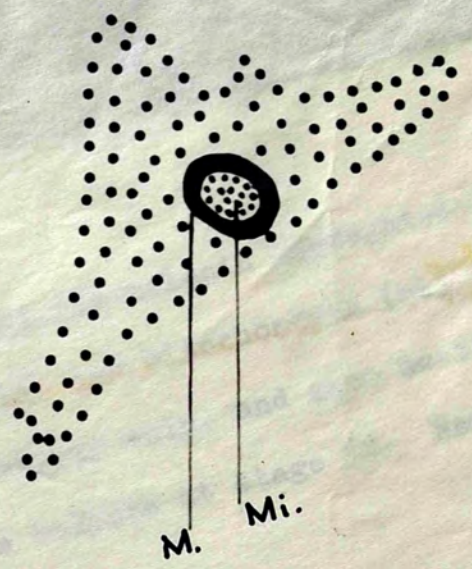


Fig. 27 B

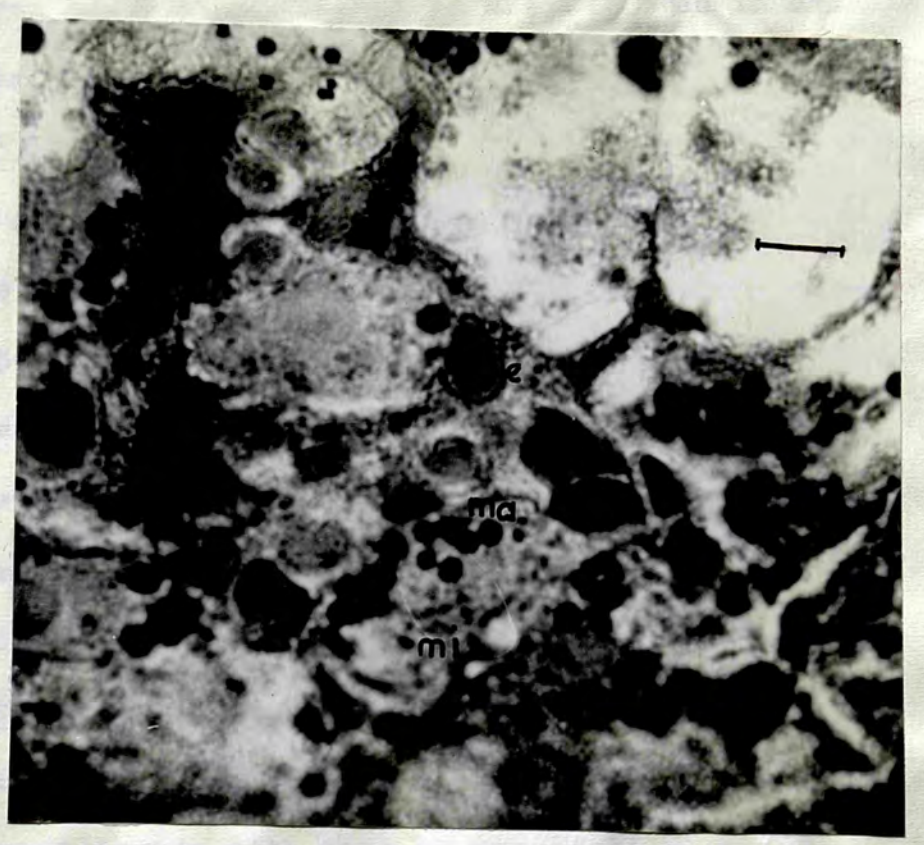


Fig. 28. Xenopus laevis

Section of liver showing the melanin aggregates (ma) in the cortex of a liver lobule, and mitochondria (mi) concentrated around the central intralobular vein, and also scattered throughout the cytoplasm, from a tadpole at stage 56. Note the large oval erythrocytes (e).

Fixed: Altmann's fluid

Section: 3 µ

Stained: Metzner's aniline acid fuchsin

Scale: 20 µ

It could mean that the mitochondria are involved in melanin synthesis as already suggested for amphibians by Niu (1954) and Algard (1953). But it is unlikely that the mitochondria are actually transformed directly into melanin granules, because in cases where they have been studied under the electron microscope, the two types of body show a very different arrangement of their internal membranes (Birbeck and Barnicot, 1959).

The hepatic cells contain very large amounts of melanin. This occurred in two forms; as isolated granules of the size seen in other tissues, and as large round aggregations varying from 1.5 to 10 μ in diameter. These aggregates were massed together in the liver lobules (fig. 28) usually around the periphery. This statement agrees with that of Prasad et al (1965) based on their electron microscope studies on the liver of Amphiuma. They also found that the melanocytes in the liver contained several round organelles 2.2 μ in diameter, which were composed of several melanin granules as well as many concentrically laminated "sub-units" (not defined). The round aggregates of melanin seen under the light microscope in Xenopus liver resemble the round organelles seen under the electron microscope in Amphiuma liver (described by Prasad et al).

The numerous mitochondria in Xenopus liver were scattered uniformly throughout the cytoplasm, but very often were concentrated around the central vein, a branch of the hepatic vein. They showed no discernible spatial relationship with the melanin aggregates.

In an attempt to overcome the obscuring effects of the dense pigment, sections of Xenopus liver were bleached for four hours in a 6 per cent solution of hydrogen peroxide. After this the melanin could no longer be seen, but it was possible to see spaces in the cytoplasm where the melanin granules and aggregates had been (figs. 29 and 30). In such bleached sections the mitochondria were again distributed throughout the cytoplasm, but they tended to aggregate around the central vein and not in the areas where melanin had been dense. However, in some parts they appeared to be situated around the periphery of the space left by the bleached melanin (fig. 30). Whether any definite relationship between the mitochondria and melanin can be deduced from the observations is uncertain. Van Woert et al (1965) showed that mitochondrial functions such as certain enzyme activities were localised in the melanin organelles described by Prasad et al in Amphiuma liver. It is therefore possible that in Xenopus liver the appearances noted above might imply that some biochemical relationship exists between mitochondria and melanin aggregates. However, such a relationship has not yet been confirmed by any of the histochemical staining techniques used here, because the mitochondria in situations where they are found alongside melanin granules do not respond to histochemical tests in the same way as the melanin.

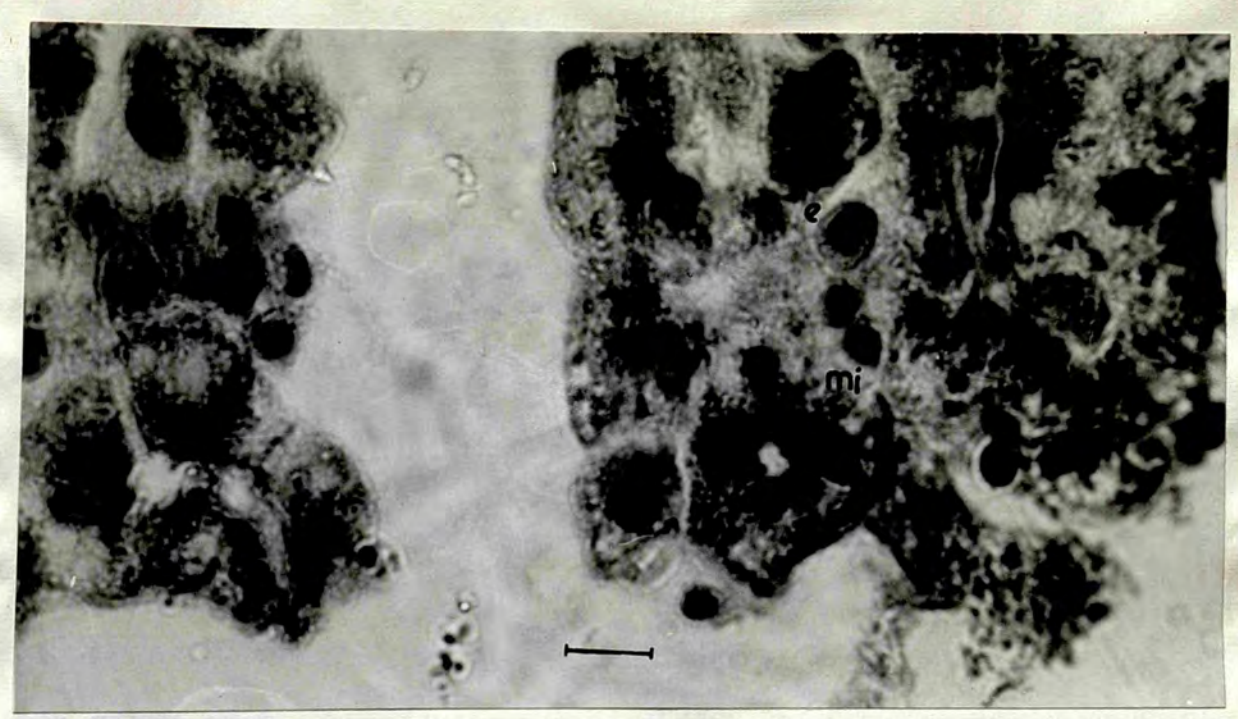


Fig. 29. Xenopus laevis

Section of liver showing mitochondria (mi) scattered throughout the cytoplasm, but tending to be concentrated around the central vein. From a tadpole, at stage 56, sections of which had been bleached for four hours in a 6 per cent solution of hydrogen peroxide prior to staining. Note the large oval erythrocytes (e).

Fixed: Altmann's fluid

Section: 3 µ

Stained: Metzner's aniline acid fuchsin

Scale: 20 µ

Fig. 30. Xenopus laevis

Mitochondria (Mi) in a liver cell of a tadpole at stage 56 as they appear in an area similar to that shown in fig. 29. Note the mitochondria present in the cytoplasm, and that they tend to be concentrated around the central vein (C.V.). The large amounts of melanin present had been bleached leaving clear areas such as that labelled A.M.G. It is then possible to see that around the periphery of these, mitochondria are often seen.

Fixed: Altmann's fluid

Section: 3/u

Stained: Metzner's aniline acid fuchsin

Scale: 8/u

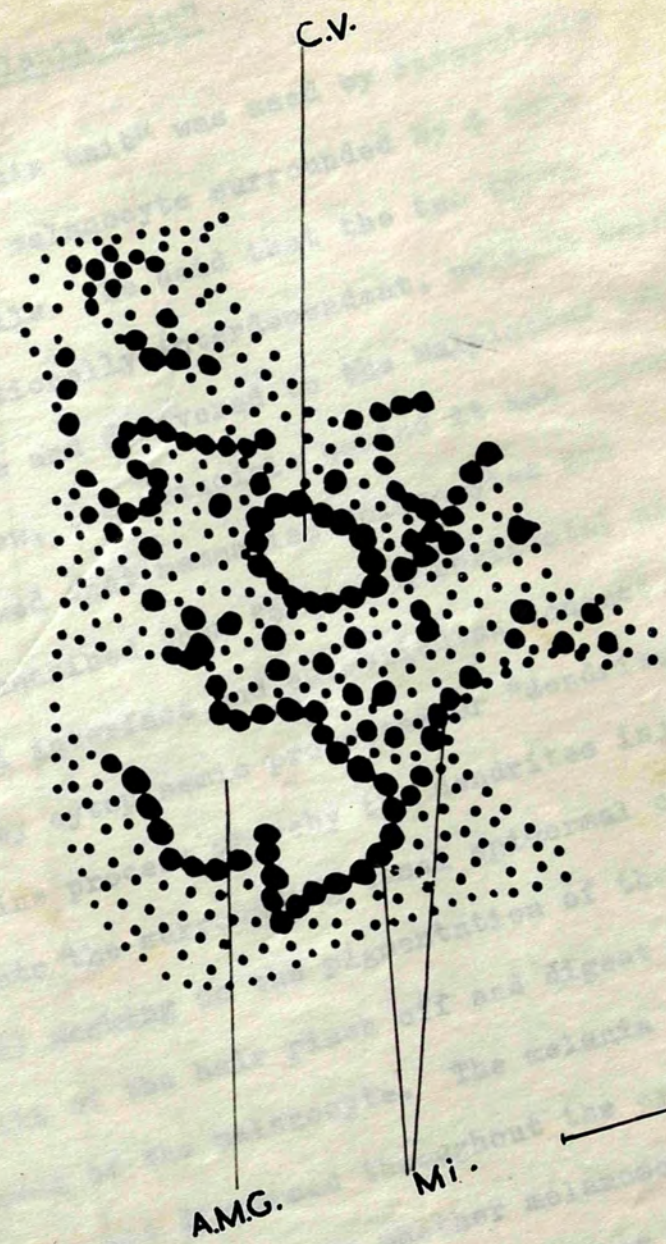


Fig. 30

10. The "epidermal-dermal melanin unit"

The term "epidermal melanin unit" was used by Fitzpatrick (1965) to define a mammalian melanocyte surrounded by a constellation of Malpighian cells. He said that the two types of cell in this unit are functionally interdependent, melanin being produced in the melanocyte and delivered to the Malpighian cells. Although this term was new, the principle behind it was certainly not. Masson (1948) showed that mammalian melanocytes are secretory cells. He described them forming a horizontal network at the dermo-epidermal interface and establishing contact with the epidermal cells by cytoplasmic processes or "dendrites". He postulated a cytocrine process whereby the dendrites inject pigment granules into the surrounding basal epidermal cells.

Birbeck (1956) working on the pigmentation of the hair, said that cortical cells of the hair pinch off and digest parts of the dendritic processes of the melanocyte. The melanin granules are then liberated into and dispersed throughout the cytoplasm of the cortical cells. It was not clear whether melanosomes or only mature melanin granules were secreted into other cells. Alternatively, it has been suggested by Becker *et al* (1952) that the granules are released into intercellular spaces where they can be phagocytised by the Malpighian cells. Yet another mechanism of transfer was proposed by Fitzpatrick *et al* (1954). They suggested that soluble melanin precursors may be transferred from melanocytes to surrounding cells, and that these intermediate

compounds are then converted into melanin and deposited in granular form in the basal epidermal cells.

Millott and Lynn (1965) have photographed, in the skin of Rana pipiens, what appears to be transfer of melanin from a melanophore to an epidermal cell. It appears as if two granules are being transferred from a melanophore process that is in intimate contact with an epidermal cell.

Cruikshank and Harcourt (1966) by means of time-lapse cinemicrography have made detailed observations on the transfer of melanin granules from melanocytes in guinea pig and human skin. They have observed clumps of granules escape from the end of a dendrite of a melanocyte to be engulfed by an epithelial cell. They stressed that the epithelial cell is the active partner, because it removes pigment from the melanocyte.

It is almost certain that such "epidermal melanin units" are not confined to mammals, but are also found in amphibians. As long ago as 1885 Ehrmann described melanin granules in the epidermal cells of amphibians. Stearner (1946) working on Triturus torosus described the processes of dermal and epidermal melanophores extending for long distances between the ordinary epidermal cells. At their ends many of these processes were said to penetrate the epidermal cells and to pass over the outer side of the nucleus to form a cap. At the end of the melanophore process, he saw a fine line of granules passing into the epidermal cell. Another important observation he recorded was that the epidermal cells in the vicinity of an expanded melanophore contained more

pigment than those near a contracted one. This fact was stressed too by Hadley and Quevedo (1966) working on Rana pipiens and Xenopus laevis skin. However, they point to a further relationship between the distribution of melanin granules within melanophores, viz. the amount of pigment in the surrounding epidermal cells and the rate of melanogenesis. Thus they conclude that the dispersion of melanin granules within melanocytes is associated with a high rate of melanogenesis and the release of melanin granules into the surrounding epidermal cells. If this be so, then it might explain an observation made earlier that in a larvae of R. temporaria treated with phenylthiourea for seventeen days, beginning when nine days old, there was a marked decrease in the amount of epidermal melanin in both melanophores and general epidermal cells. This would be explained if the drug not only inhibited the formation of melanin in the epidermal melanophores, but also aggregated their pigment, so that the melanophore appeared round in shape with few processes. This might well decrease the opportunity for transfer of the granules to the epidermal cells.

My own limited observations on Rana temporaria larvae suggest that whatever the details of the process may be, transfer may occur between epidermal melanophores and surrounding cells. Fourteen days after the eggs have been laid, many melanophores are present in the larval skin. Many of them have fine branching processes which travel for considerable lengths just below the epidermal cells. Some of the processes come into intimate contact

with epidermal cells, and in nearly all cases, melanin granules appear to be coming off from the tips of the processes (figs. 31, 32). In fig. 20, the uppermost process of the central melanophore appears to have disintegrated liberating clumps of melanin granules into the epidermis.

Since the epidermal cells themselves contain no tyrosine or tyrosinase, it is unlikely a priori that melanin can form in them. It is therefore only reasonable to conjecture that it may have arisen elsewhere. After fourteen days, there is a positive indication of tyrosinase activity in the epidermal melanophores, so these would appear to be the sites of melanogenesis. It must be emphasized, however, that firm conclusions as to the details of melanin transfer cannot be given without carefully observing living material.

Fig. 31. Rana temporaria

Melanin granules apparently being released from a process (p) of a dermal melanophore as seen in a section through the skin of a larva, thirteen days old. Note that two granules are close to the process, but three (appearing pale) are further away and slightly out of focus.

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 100 μ

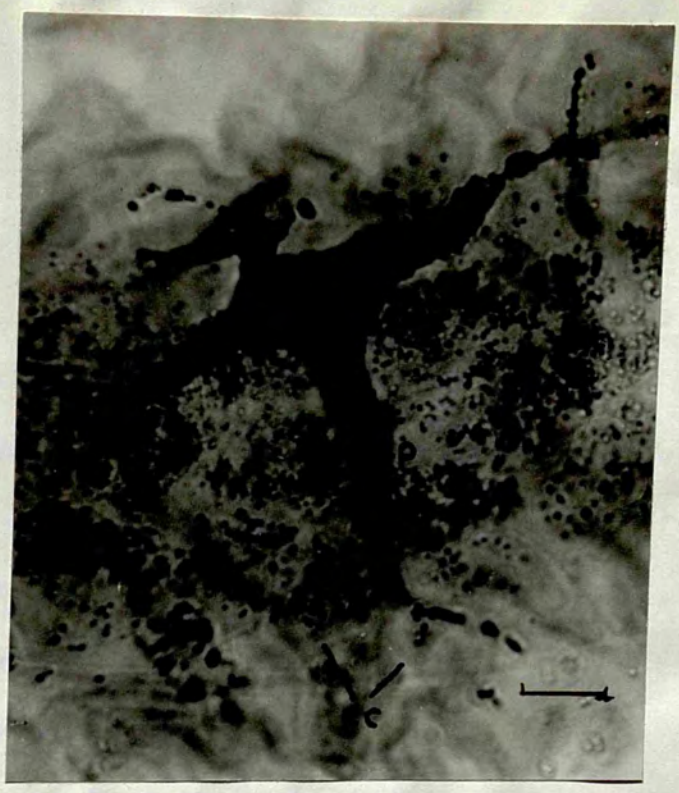


Fig. 32. Rana temporaria

An epidermal melanophore with long fine processes from a section through the skin of a larva, fourteen days old. The tip of the process (p) appears to be releasing pigment granules (g) into surrounding epidermal cells (ec).

Fixed: 10 per cent formol

Section: 6 μ

Stained: Mallory's triple

Scale: 100 μ

DISCUSSION

From the foregoing accounts it is obvious that melanin occurs widely in the two species of larval amphibians studied. The significance of its internal distribution is not always easy to see. Of the reasons given for its existence (page 7.) only excretion and a metabolic accident are comprehensible because the pigment is deep-seated. If excretion is its main function it is difficult to see why it is so widely distributed. For example, the picture presented by the melanin-laden peritoneal sheet around such important organs as the gut, liver, kidneys, heart, gonads and brain in some amphibian larvae, suggests that the melanin here is protective. But if the protection were against ultra-violet radiation, the hypothesis is invalid, because this is almost certainly absorbed by the skin. However, the possibility that melanin protects these organs from light cannot be overlooked.

It is possible that the melanophores around certain organs pass their melanin into the organs, where it can be accumulated or excreted. However, this is not always the case because in Rana temporaria melanin is found around but not in the gonads and likewise the pigment around the kidneys of Xenopus laevis is not found in them. Also there is evidence that the melanin in the liver and pancreas of Xenopus is synthesized there, and not merely accumulated.

The clearest signs of excretion of melanin are to be found in the gut and brain walls in Xenopus and R. temporaria, and also in the kidney tubules of the latter. In these areas there are unmistakable signs of melanin being accumulated and discharged into the cavities of these organs. Other observed indications of melanin being excreted arise from the fact that phenylthiourea increases the amounts of melanin in these organs, and also there is no clear indication of tyrosinase activity in them. In accordance with this it was seen that during the period of rapid melanin synthesis in Xenopus, up to stage 47, large amounts of melanin are present in the gut and brain. These persist only as long as active synthesis persists in the larva generally, but thereafter the amounts decrease, and after stage 49 no melanin is present in these areas.

From Tables 1 and 2 it is apparent that phenylthiourea has the dual effects of inhibiting melanogenesis causing decreased pigmentation, and increasing pigmentation by causing increased melanin accumulation, and later perhaps, its excretion. In the last case where phenylthiourea may cause increased elimination of melanin this also leads to diminished pigmentation. In many cases, however, it was without effect.

Thus one can deduce that three processes can occur within the melanised areas of the two species of larvae studied. Melanin can be synthesized, accumulated and excreted, but the three processes do not necessarily occur together in any particular

pigmented site. There appears to be a balance between synthesis which depends largely on the availability of active tyrosinase and an appropriate substrate, and accumulation and excretion. Larvae do not become paler with age, and yet there are clear indications of melanin excretion taking place, so synthesis of melanin probably exceeds excretion in normal development.

In both Xenopus and Rana phenylthiourea decreases pigmentation in the dermis, peritoneum, meninges, eye, olfactory epithelium and sucker. In all these areas, too, tyrosine and tyrosinase are present, except that in the peritoneum and meninges of Xenopus no tyrosine could be demonstrated. Nevertheless these areas appear to be the sites of melanogenesis.

In other areas it is not so easy to explain the reason for the presence of melanin. In the connective tissue around the myotomes of Xenopus there is an indication of tyrosine and tyrosinase up to stage 49, and phenylthiourea slightly decreases the amount of melanin in this site from stage 45 onwards. However, in Rana in the same area no enzyme, substrate nor effect of phenylthiourea was demonstrated. This result is significant for it shows that although melanin may be present in the same area in Rana and Xenopus, the reason for its presence may not be the same in the two species.

In both Xenopus and Rana, melanin is found around the myotomes and heart. In Xenopus alone neither tyrosinase nor melanin is found within them, but in Rana melanin, yet no enzyme,

is found in the myotomes and heart. Since in Rana the effect of the drug is slightly to increase the pigmentation in these two areas, it is probable that melanin is accumulated here. Such an explanation may also apply in Rana to the connective tissue around the myotomes too, thus explaining the observation made above. As pigment is accumulated in the myotomes of Rana, it could later be cast out from them.

The pigmentation of the membranous labyrinth is noteworthy. In Xenopus there is very little melanin in this region, and phenylthiourea is without effect. This suggests that a little melanin may be accumulated at this site. However, in Rana much more melanin is seen in the walls of the membranous labyrinth and being discharged into the otic vesicle. Here, however, there are some indications of tyrosine and tyrosinase being present, and in accordance with this, phenylthiourea slightly decreases the pigmentation. One can only infer that here some melanin is being formed, accumulated, and also apparently excreted. This would imply that turn-over occurs. This possibility introduces a question over which there has been some disagreement. Sims (1961) came to the conclusion that in Xenopus there was no turn-over, but Millott and Lynn (1965) produced evidence from the same animal which would imply that active turn-over of melanin does occur. My findings agree with those of Millott and Lynn.

It has already been stated that in the liver and pancreas of Xenopus, melanin appears to be formed at least initially.

This could be associated with the fact that tyrosinase is clearly present around stage 49, although amounts of melanin found naturally in the liver at this stage are very small, and this could depend on factors affecting enzymatic activity. In Rana, however, there is no indication of tyrosinase in the liver, and phenylthiourea always increases the pigmentation. Thus, it appears that conditions in Xenopus and Rana are different for in the latter, melanin appears to be accumulated possibly after synthesis elsewhere. This may be pertinent in relation to remarks made by Niu and Twitty (1950) who described the transfer of melanin by "melanophages" from the skin of Triturus to the liver, though it was stated to occur only at metamorphosis. The effects of phenylthiourea are therefore somewhat complex. When it inhibits pigmentation it not only affects the density of melanin in the granules but also the shape of the melanophore, the distribution of the melanin and the number of granules. It is also possible that it breaks down the granular nature of the pigment and in some way causes it to be redistributed around the body.

Although from the evidence given in Section 9 it is not clear whether mitochondria are directly concerned in the formation of melanin, because they are well known to be sites of enzymatic activity, yet the possibility of their participation in synthesis of the pigment or the matrix on which it is carried, is a

reasonable one to bear in mind. However, it is important to remember that when Du Buy et al (1949) derived melanin granules from mitochondria, they based their conclusions entirely on work done on certain mouse melanomas. They found that the melanotic cells had few mitochondria, but contained cytoplasmic granules in varying stages of melanization. These granules were said to have a property that is characteristic of granular mitochondria, namely, that they stained under appropriate conditions with Janus green B. It was on these grounds, coupled with their enzymatic properties that Du Buy believed melanised granules to be altered mitochondria, that have become melanised. However, these inferences are not necessarily applicable here because Du Buy based his conclusions on a study of malignant cells in mice.

In the absence of electron microscope and biochemical studies on amphibian mitochondria and melanin granules it is impossible to draw any definite conclusions about their relationship.

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SUMMARY

1. The widely distributed black and brown pigments in adult Rana pipiens, Rana temporaria, Hyla versicolor, Hyla arborea, Xenopus laevis, Triturus cristatus, and Triturus vulgaris have been investigated. In both larvae and adults they have been identified histochemically as melanin.

2. Living and fixed melanophores from the peritoneum of Xenopus laevis and Triturus vulgaris have been studied, and the effects of the fixatives recorded.

3. The development of pigmentation in Xenopus laevis larvae has been studied and described from stages 23 to 52. This was compared with the formation of melanin in Rana temporaria larvae of equivalent stages.

4. The effect of 0.01 and 0.001 per cent solutions of phenylthiourea on pigmentation in Xenopus and Rana larvae of the same stages as those noted above has been studied and described. In some areas of the larvae, the drug reduces pigmentation, in others it has no effect or actually increases pigmentation. The differing responses of Xenopus and Rana larvae to phenylthiourea have been compared.

5. Evidence for the presence of tyrosine and tyrosinase, in the body tissues of both Xenopus and Rana larvae of the

same stages as those already noted, was sought by histochemical means. The information from these tests, from the effect of phenylthiourea and from the distribution of melanin is presented in Tables 1 and 2, and the findings are discussed.

6. Epidermal mucous cells from Xenopus larvae have been stained, and their positive reaction to incubation with a DOPA solution described.

7. Mitochondria have been stained and their relationship with melanin granules examined in peritoneal melanophores and liver from Xenopus larvae. There is no consistent spatial relationship between the two, and there is as yet no clear evidence of any association between them.

8. In Rana temporaria larvae, epidermal and dermal melanophores appear to discharge melanin granules into the surrounding epidermal cells.

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Fig.17. Melanin in muscles of R. temporaria, thirteen days old.

Fig.18. Melanin in liver of R. temporaria, fourteen days old.

Fig.19. Melanin in liver of R. temporaria after treatment with phenylthiourea.

Fig.20. Melanin in skin of R. temporaria, fourteen days old.

Fig.21. Melanin in skin of R. temporaria, after treatment with phenylthiourea.

Fig.22. Melanin in brain of R. temporaria, thirteen days old.

Fig.23. Melanin in brain of R. temporaria, after treatment with phenylthiourea.

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Fig.27.A Melanin and mitochondria in peritoneum of X. laevis, stage 56.

Fig.27.B Melanin and mitochondria in peritoneum of X. laevis, stage 56.

Fig.28. Melanin and mitochondria in liver of X. laevis, stage 56.

Fig.29. Melanin and mitochondria in liver of X. laevis, after bleaching.

Fig.30. Melanin and mitochondria in liver of X. laevis, after bleaching.

Fig.31. Release of melanin from a dermal melanophore in R. temporaria.

Fig.32. Epidermal melanophore from R. temporaria.

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