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The pigmentary system of developing axolotls

II. An analysis of the melanoid phenotype

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SUMMARY

The melanoid mutant in the Mexican axolotl (*Ambystoma mexicanum*) is analysed with respect to the differentiation of pigment cells. Pigment cells were observed with the transmission electron microscope in order to determine any unusual structural characteristics and to determine what happens to each of the cell types as development proceeds. Chemical analysis of pteridine pigments was also carried out, and changes in pteridine biosynthesis were found to correlate well with changes in xanthophore morphology and number.

In melanoid axolotls, as development proceeds, melanophore numbers increase, xanthophores decrease, and iridophores fail to differentiate at all. This is considered to result from: (a) conversion of xanthophores (that are present in young larvae) to melanophores; (b) the gradual programming of the majority of chromatoblasts to become, exclusively, melanophores, and (c) the failure of some chromatoblasts (possibly iridoblasts) to differentiate altogether. The ultrastructural and chemical evidence presented in this study is compared to similar data for wild-type axolotls, and a mechanism regarding how the melanoid gene might act is suggested.

INTRODUCTION

The melanoid (*m*) gene in the axolotl (*Ambystoma mexicanum*) was first described by Humphrey & Bagnara (1967), as a simple recessive gene that affects pigment cell differentiation at the level of the neural crest precursor cells. Characteristics typical of the melanoid phenotype include: (1) a darker than normal background colour, due to an overabundance of black pigment cells (melanophores), (2) a loss of, or reduction in, the number of yellow (or bright-coloured) pigment cells (xanthophores), and (3) the absence of reflecting pigment cells (iridophores), which is most obvious in the eyes. Most of the previous work relating to the melanoid gene has been recently summarized by Frost & Malacinski (1980) and Frost, Briggs & Malacinski (1984*a*).

The purpose of this study is to describe some of the structural and biochemical features of melanoid axolotl skin, and to correlate changes in these features with other changes that occur during development. Previous attempts have been made to either biochemically analyse pigments (Benjamin, 1970; Dalton & Hoerter, 1974), or structurally analyse pigment cells (Dunson, 1974) from

melanoid axolotl skin. These earlier studies, however, involved an examination of larval melanoid axolotls only, and, for a variety of reasons, failed to demonstrate what the underlying mechanism of the melanoid gene might be.

As we have shown for wild-type axolotls (Frost, Epp & Robinson, 1984*b*), and as will be seen from the results presented herein, there are clear differences in both pigments and pigment cells that accompany growth and development in this animal. Furthermore, a comparison of the differences between melanoid and wild-type skin provides some insight into how the melanoid gene affects pigment cells. As our final objective, we compare the melanoid phenotype to the wild type.

MATERIALS AND METHODS

Animals

Axolotls homozygous for the melanoid (*m*) gene were obtained from the Indiana University Axolotl Colony, Bloomington, Indiana. Feeding, maintenance, and the categorization of axolotls into three arbitrary age-classes (larva, juvenile, adult) are described in Frost *et al.* (1984*a*).

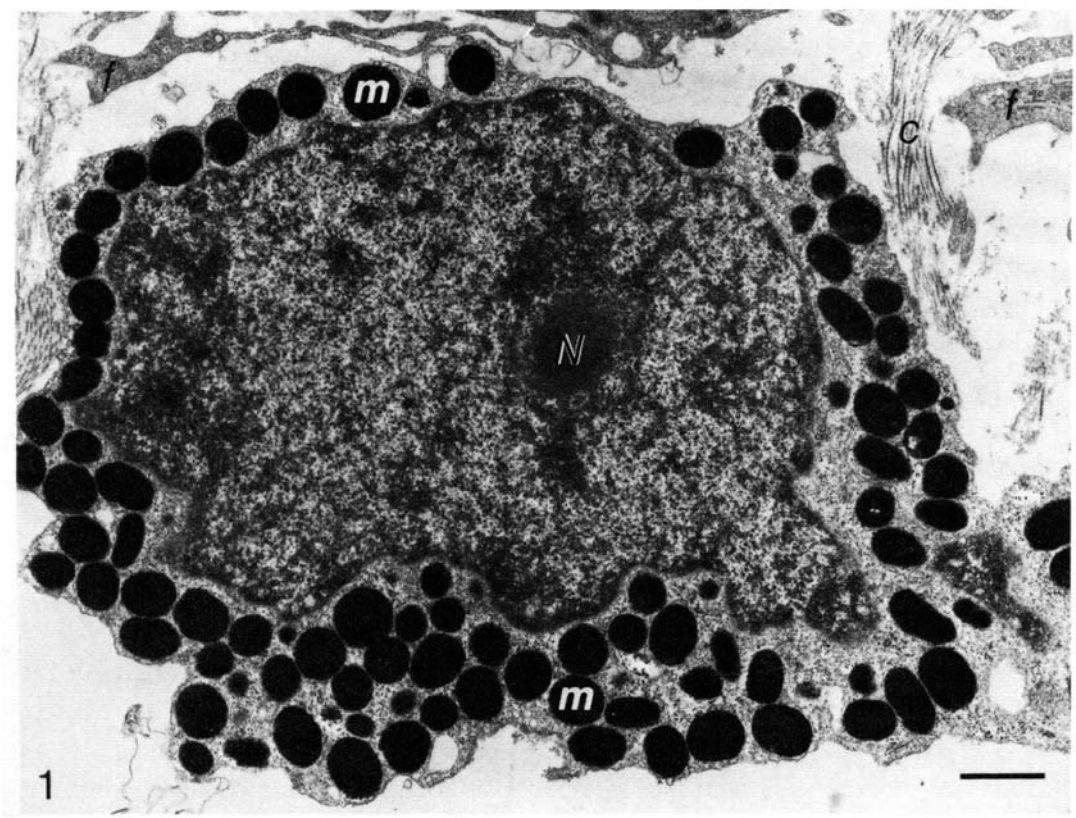

Fig. 1. Typical melanophore from young melanoid axolotl skin. Note the prominent nucleus (*N*), numerous melanosomes (*m*) and surrounding collagen (*c*) and fibroblast processes (*f*). Fixative B; bar = 1 μm.

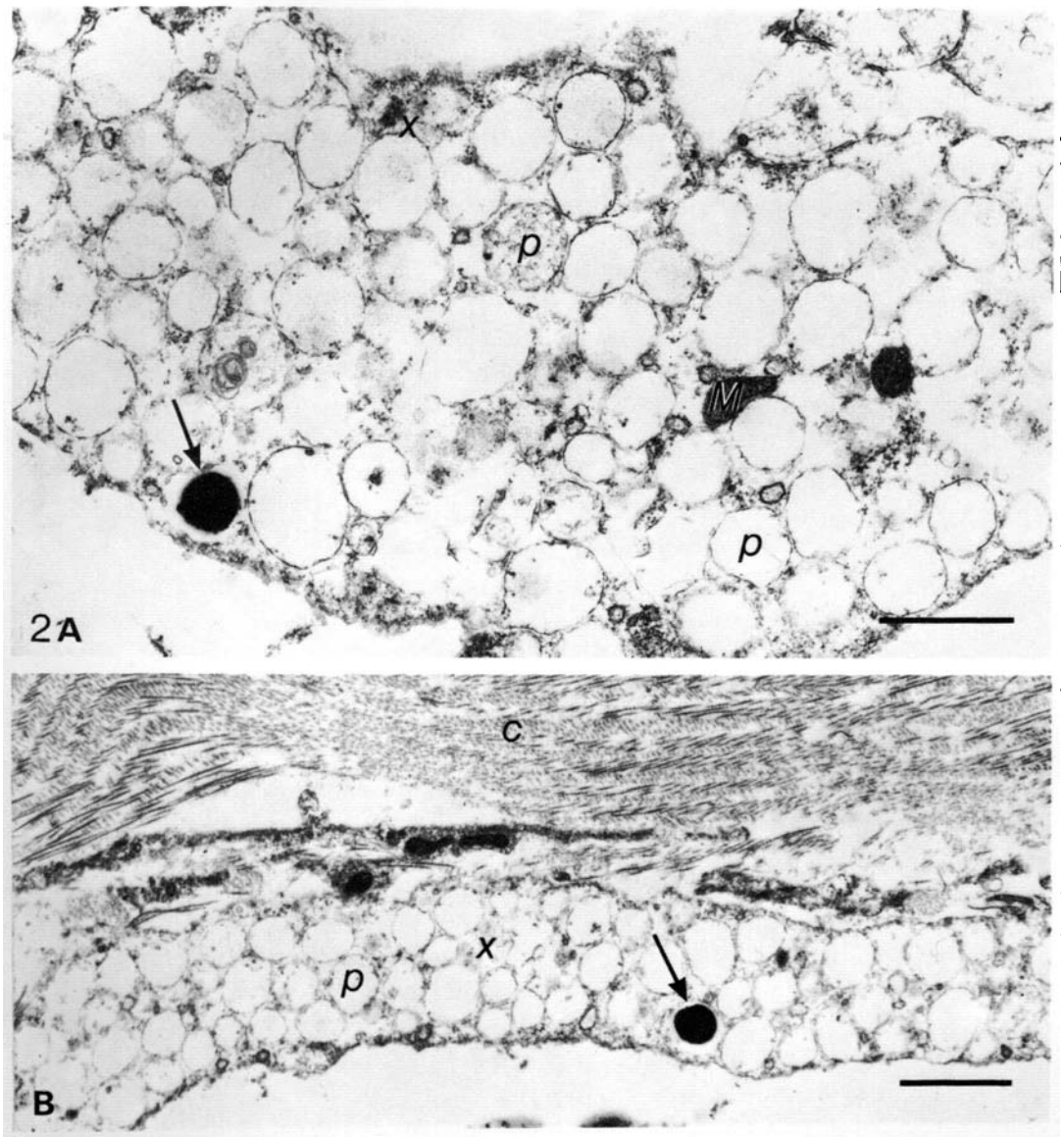

Fig. 2. (A,B) Xanthophore processes (*x*) from young melanoid skin. Xanthophores are infrequently observed in thin sections from melanoid skin. They are always located beneath the collagen matrix (*c*) and contain both type I pterinosomes (*p*; see text for explanation) and occasional melanized pterinosomes (arrows). *M* = mitochondrion. Fixative A; bar = 1 μm.

Electron microscopy

Axolotl skin was prepared for transmission electron microscopy (TEM) as described previously (Frost *et al.*, 1984*b*). The two fixatives used were (A) 2·5 % glutaraldehyde in 0·1 M-cacodylate buffer, pH 7·4, or (B) 10 % acrolein in 0·1 M-cacodylate, pH 7·4.

Pigment extraction

Soluble xanthophore pigments were extracted from melanoid axolotl skin and analysed by a combination of thin-layer (TLC) and column chromatographic techniques as described in detail elsewhere (Frost *et al.*, 1984*b*). Pigments were identified by comparing u.v.-fluorescent properties (colour), chromatographic mobility, and absorption spectra with similar data for commercially purchased standards. Pigment standards utilized in this study were: L- and D-erythroneopterin (NP), biopterin (BP), sepiapterin (SP), pterin (2-amino-4-hydroxy-pterin; AHP), pterin-6-carboxylic acid (AHP-6-COOH), xanthopterin (XP), isoxanthopterin (IXP), riboflavin, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).

RESULTS

Structural analysis of pigment cells

In melanoid axolotls the types of pigment cells encountered in skin vary depending on the age of animals. In young larval and early juvenile-stage melanoid axolotls, both melanophores (Fig. 1) and xanthophores (Fig. 2) are seen in thin sections of skin. In young animals, melanophores are located below the collagen matrix of the dermis (Fig. 3). These cells are characterized by numerous 'black' pigment granules (melanosomes), premelanosomes, Golgi, associated small vesicles, rough ER, mitochondria, microtubules, intermediate filaments, and a large prominent nucleus that is centrally located within the cell body (Figs 1, 3). Radiating out from the cell body are long cell processes that are usually filled with melanosomes.

Also present in larval melanoid skin are xanthophores (yellow pigment cells) that are characterized by pterinosomes (pigment-containing organelles) in the cytoplasm. In melanoid axolotls, pterinosomes appear to be 'empty' or to contain small amounts of fibrous material (presumably pigment) (Fig. 2). This 'empty' appearance is typical of a type I pterinosome, which is, according to Yasutomi & Hama (1976), a pterinosome in an early state of differentiation.

Other cytoplasmic components of xanthophores are similar to those described above for melanophores. One unusual feature of melanoid xanthophores is the

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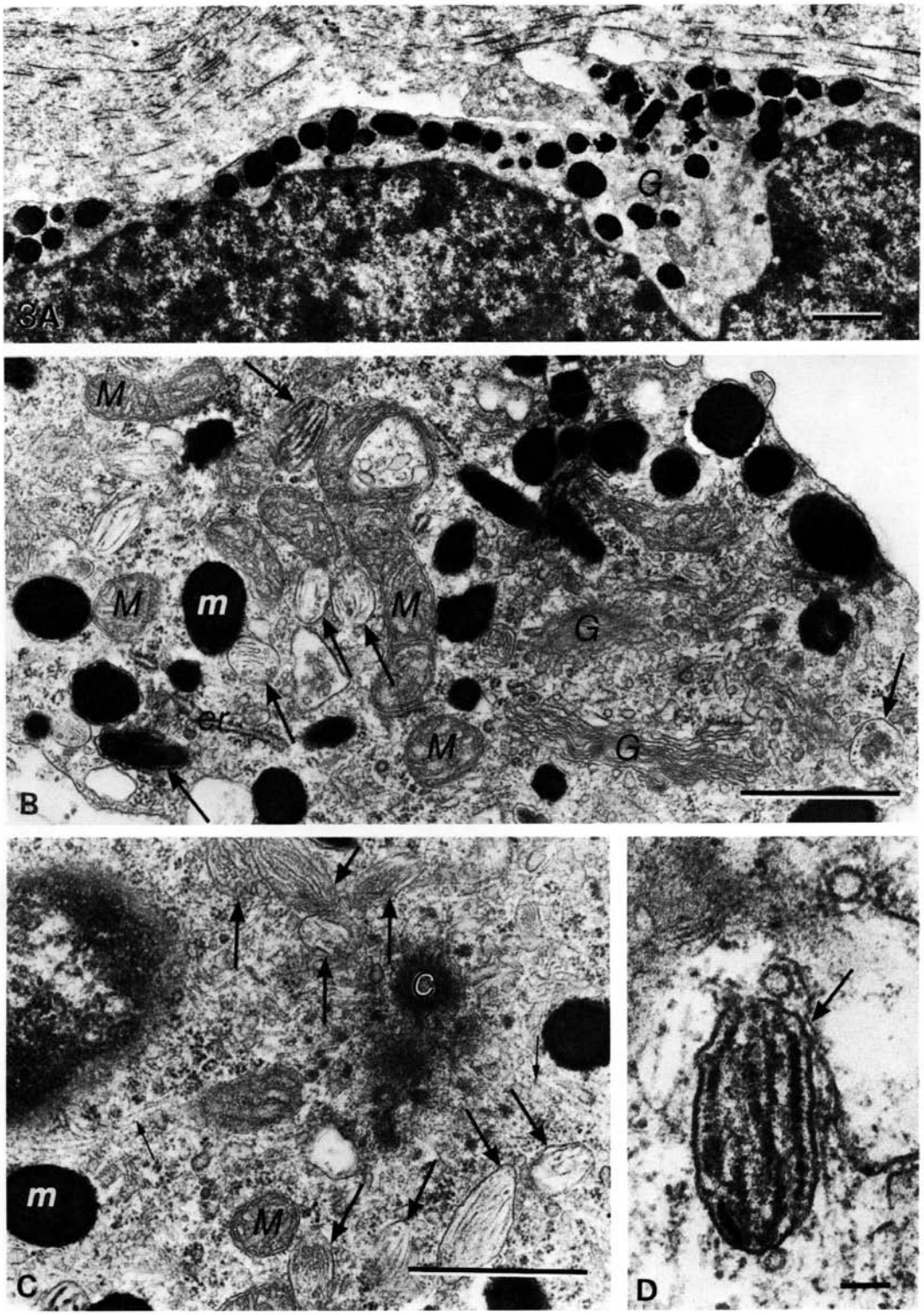
Fig. 3. Examples of cytoplasmic structures typical of melanophores from melanoid skin. (A) Low magnification of melanophore in the dermis of melanoid axolotl skin. Fixative B; bar = 1 μm. (B,C) Higher magnification views of melanophores illustrating an abundance of Golgi (*G*), premelanosomes and multivesicular bodies (large arrows), rough ER (*er*) and mitochondria (*M*). A centriole (*C*), related satellite elements and numerous intermediate filaments (small arrows) are also seen in (C). Fixative A; bar = 1 μm. (D) High magnification of a premelanosome (arrow) showing the typical 'internal lattice' characteristic of vertebrate premelanosomes. Fixative A; bar = 1 μm.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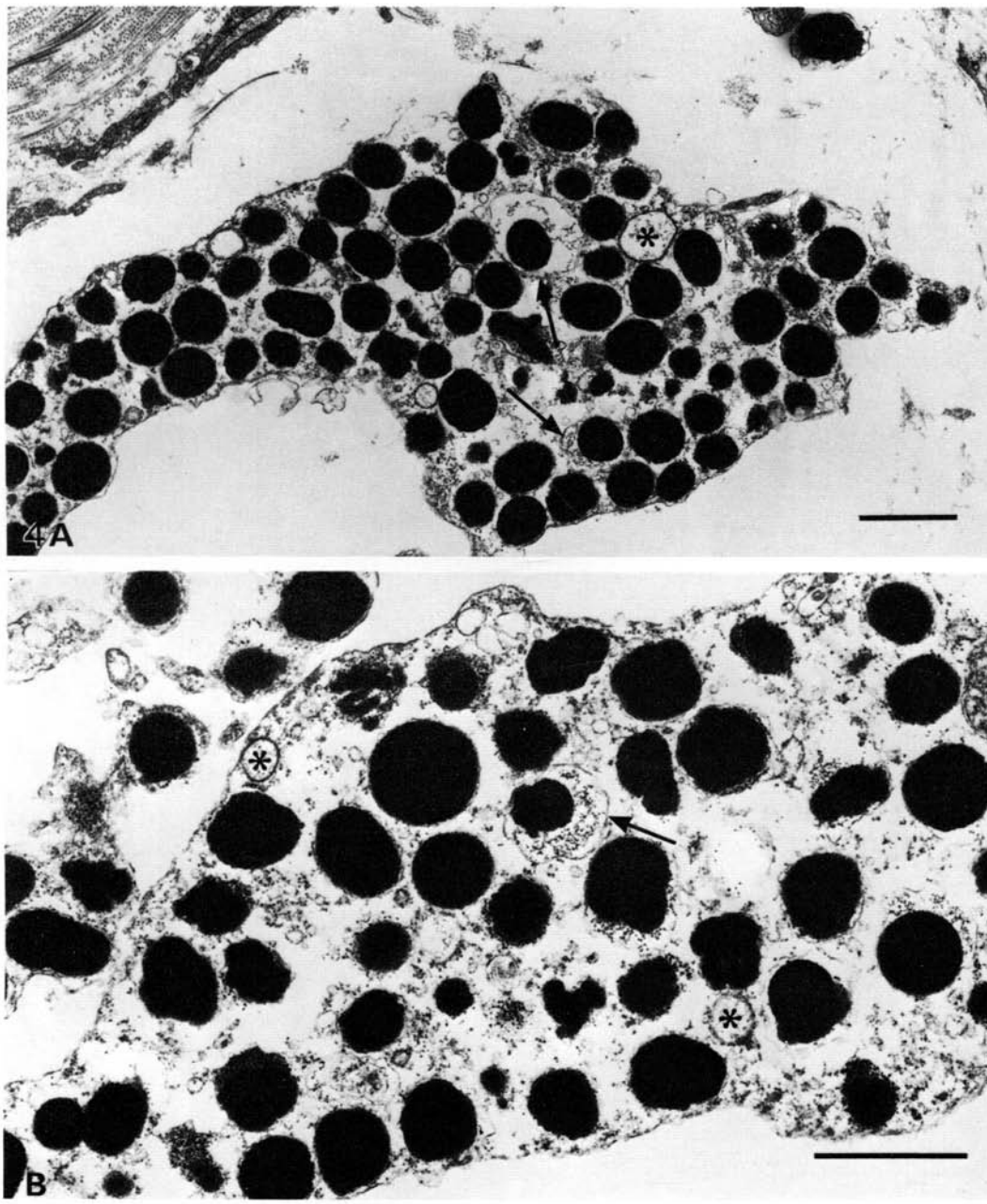

Fig. 4. Melanophore processes from juvenile melanoid axolotl skin. Note the pterinosome-like melanosomes (arrows) and type I pterinosome-like organelles (*). Fixative A; bar = 1 μm.

occurrence of an occasional 'melanized' pterinosome (Fig. 2A,B). Melanized pterinosomes characteristically have a limiting membrane that is lifted away from the pigment granule and that resembles (in size and shape) the limiting membranes of nearby pterinosomes.

As aging progresses, xanthophores are less frequently encountered in thin sections of melanoid skin and melanophores are more frequent in occurrence. Melanophores in older (juvenile–adult stage) axolotls can be grouped into two categories: (1) those that contain only 'typical' melanosomes (e.g., as in larval melanoid skin, Fig. 1 or in wild-type skin), and (2) those that contain a mix of (a) 'typical' melanosomes, (b) melanosomes that resemble melanized pterinosomes (with limiting membranes that are elevated from the pigment granule; Fig. 4), and (c) occasional organelles that resemble type I pterinosomes (compare Figs 2 & 4). In pterinosome-like melanosomes, there are often wispy fibers of material within the limiting membrane and surrounding the electron-dense pigment granule of these organelles. The fibres are similar in appearance to those observed in the pterinosomes of xanthophores (compare Figs 2 & 4).

Pterinosome-like melanosomes have been observed in thin sections from a number of different melanoid skin samples and in tissue fixed by different methods. Furthermore, even when pterinosome-like melanosomes are observed in some melanophores, they are not present in all melanophores and such organelles have never been observed in melanophores from wild-type skin (Frost *et al.*, 1984*b*). These observations suggest that the expanded limiting membrane is probably not an artifact of our technique.

Two other cell types are present in the dermis of melanoid axolotl

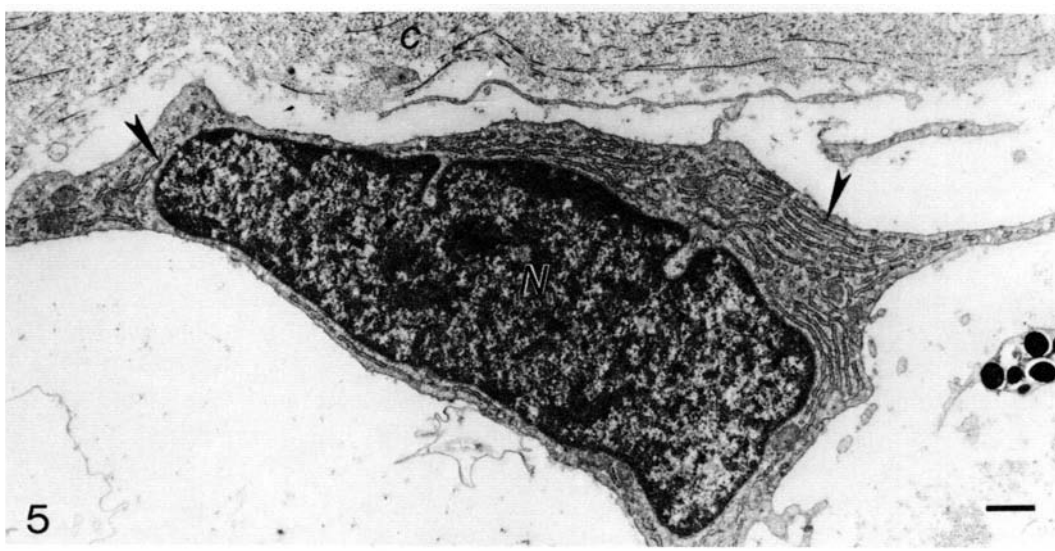

Fig. 5. Fibroblast present in melanoid skin. *N* = nucleus; arrowheads = rough ER; *c* = collagen. Fixative A; bar = 1 μm.

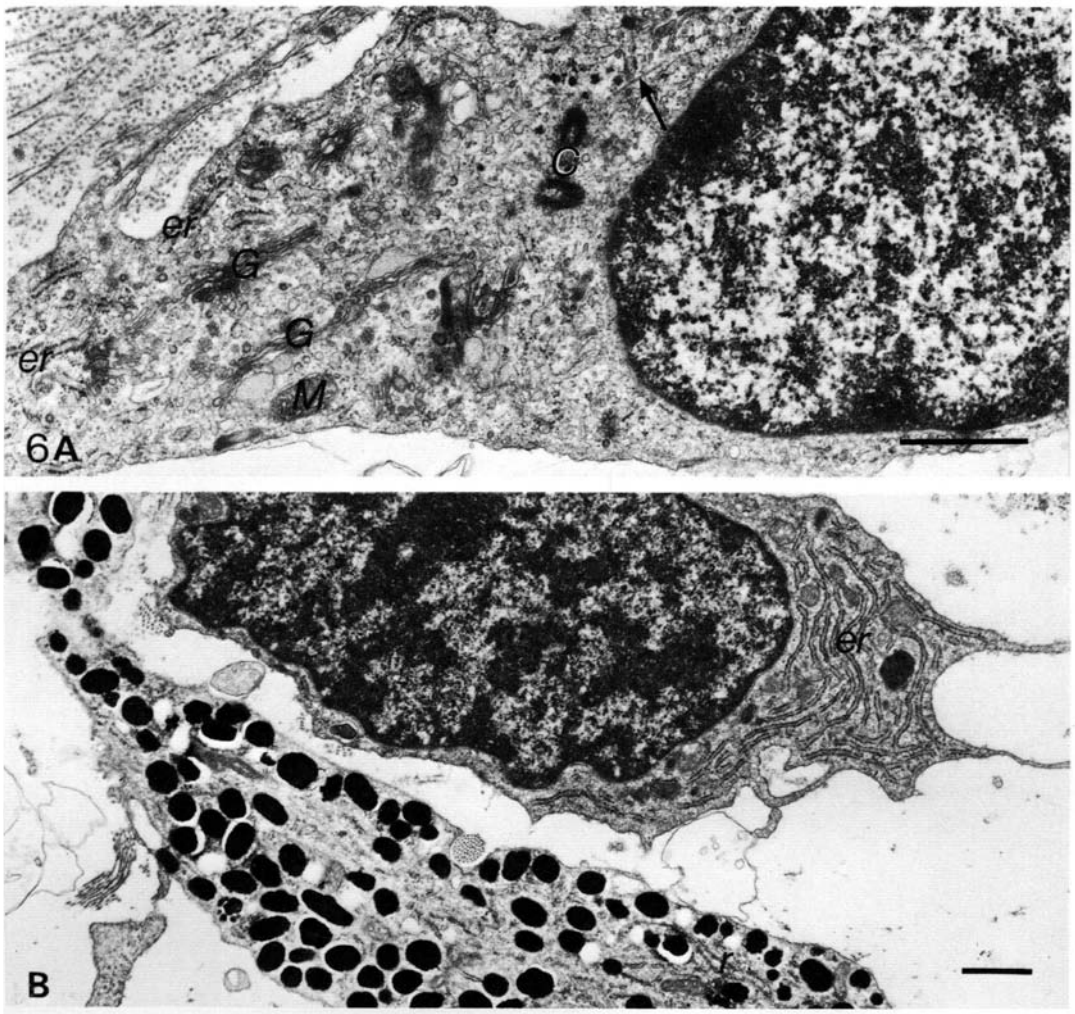

Fig. 6. Comparison of a presumptive chromatoblast (A) with a melanophore and a fibroblast (B). The general features of the chromatoblast cytoplasm are more similar to those of other pigment cells than they are to those of fibroblasts. *C* = centriole; *M* = mitochondrion; *G* = Golgi; *er* = rough ER; arrow = microtubules. Fixative A; bar = 1 μm.

skin–fibroblasts, the more numerous, and what may be a chromatoblast. Fibroblasts are always found in close association with the collagen matrix (Fig. 5). Presumed chromatoblasts have been observed primarily in juvenile (or older) stage animals, and occupy a position similar to that of other pigment cells, i.e., beneath but close to the collagen matrix of the dermis (Fig. 6). The cytoplasm of these cells is much more similar in appearance to that of other pigment cells (compare especially Figs 2, 4 & 6) than it is to that of a fibroblast. Fibroblast cytoplasm is generally very densely granular with an extensive network of rough ER (Fig. 5). Chromatoblast cytoplasm is much less granular with relatively little

rough ER (Fig. 6). Other characteristics typical of chromatoblasts include numerous small vesicles, Golgi, intermediate filaments, microtubules, and mitochondria. Like mature pigment cells, chromatoblasts have a centrally located cell nucleus and numerous long processes that radiate from the cell body. There are no structures in these cells that resemble any type of mature pigment organelle.

Pigment analysis

Chemical extraction of xanthophore pigments from different stage axolotls may be correlated with the observed structural changes in xanthophores. A one-dimensional thin layer chromatogram of skin extracts from larval and adult

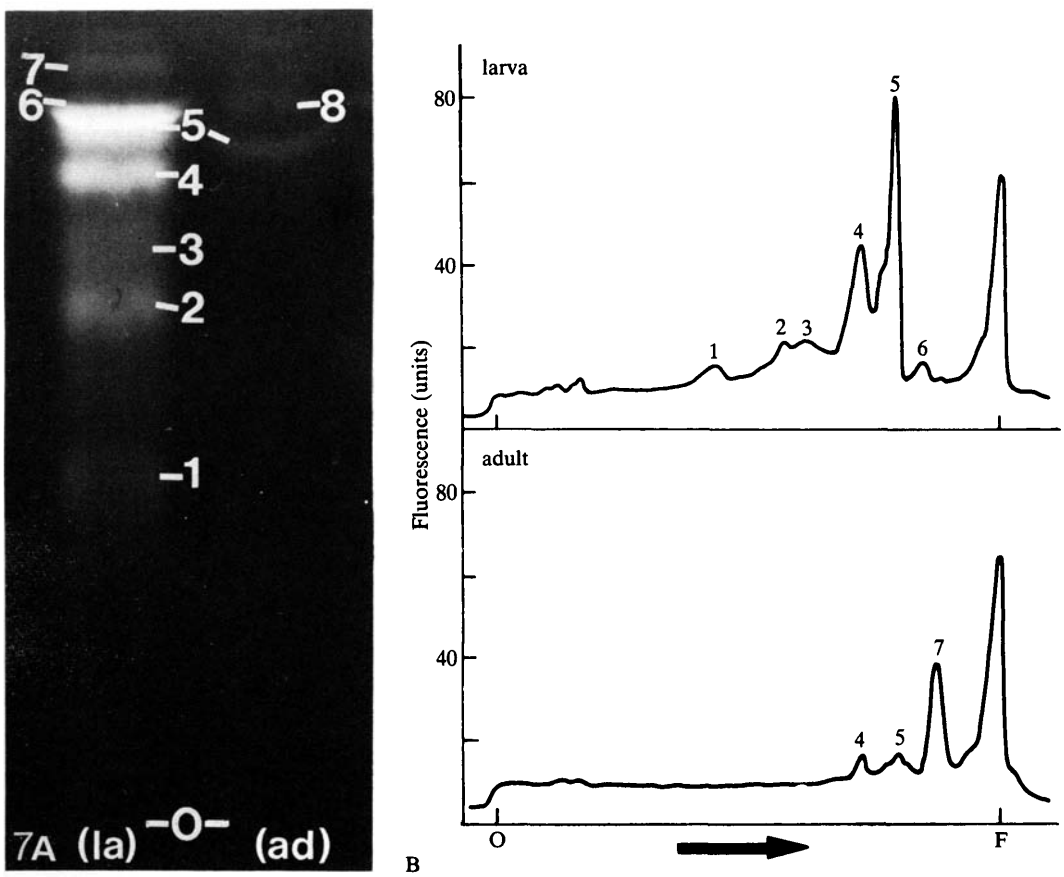

Fig. 7A. Photograph of a one-dimensional thin layer chromatographic separation of ethanol-extracted pteridine pigments from larval (la) and adult (ad) melanoid axolotl skin. 0 = origin; 1 = AHP-6-COOH; 2 = XP; 3 = IXP; 4 = NP; 5 = AHP; 6 = BP; 7 = SP; 8 = riboflavin (abbreviations are explained in the text).

Fig. 7B. Fluorometric scans of the chromatographic results shown in Fig. 7A. Numbered peaks contain the following pteridines: 1--AHP-6-COOH; 2--XP; 3--IXP; 4--NP; 5--AHP+BP (trace); 6--SP (trace); 7--riboflavin. O = origin; F = solvent front.

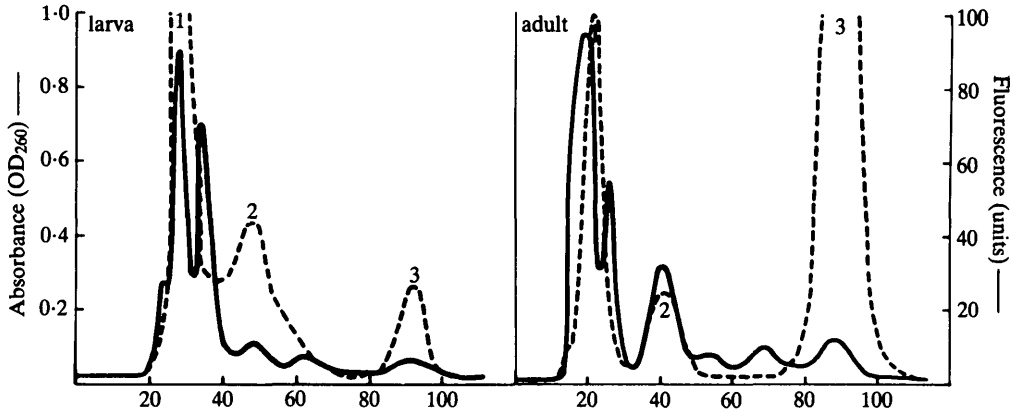

Fig. 8. Column chromatographic (Bio-Gel P2) separation of ethanol-extracted pigments from larval and adult melanoid skin. Fluorescent peaks contain the following pteridines. In the larva: 1--AHP, IXP, NP, BP; 2--XP; 3--riboflavin. In the adult: 1--AHP, NP (trace), flavin (FAD?), 2--flavin (FMN?); 3--riboflavin.

melanoid axolotls is shown in Fig. 7. Both the photograph of the chromatogram and the fluorometric scan illustrate very clearly that larval melanoid animals contain more (both quantitatively and qualitatively) pteridine pigments than do adults.

P-2 column separations of similar extracts are shown in Fig. 8. Fractionation of larval skin pigments yielded three fluorescent peaks (Fig. 8). The first peak can be divided into two regions based on u.v. fluorescent color of the fractions obtained. The first three fractions are intensely violet and contain primarily IXP. The latter four to six fractions fluoresce brilliant blue, and contain AHP, NP and trace amounts of both IXP and BP. The second peak fluoresces blue-green and contains primarily XP. The third (small) peak fluoresces a dull yellow colour and contains exclusively riboflavin.

Three fluorescent peaks are also obtained from P-2 separation of adult skin extracts, but the contents of these peaks are different from those of the larva. The first peak fluoresces grey-yellow, and was determined (by TLC) to contain trace amounts of NP, AHP and at least one flavin (probably FAD). The second peak fluoresces a pale yellow-green colour and contains trace amounts of XP and another flavin (probably FMN). The third, and in this case the largest, peak fluoresces yellow and contains riboflavin. The results of pigment extraction and identification are summarized in Table 1. Except for riboflavin, absorption spectra were not determined because of the small quantities of extractable material obtained.

DISCUSSION

The melanoid phenotype

Based on earlier studies of the melanoid gene (Humphrey & Bagnara, 1967; Bagnara, Frost & Matsumoto, 1978), it was clear that both xanthophores and

Table 1. *Relative quantitative estimates of the pteridines (and riboflavin) extracted from larval and adult melanoid axolotl skin (see text for abbreviations).*

	Pteridine							
Age group	NP	AHP	IXP	XP	BP	SP	AHP–6–COOH	Riboflavin
Larva	+++*	++++	++	++	+	+	+	−/+
Adult	+	++	−	−	−	−	−	++

* Plus signs denote relative quantity of pigment extracted; minus signs denote no pigment detected (see chromatographic results in Fig. 7).

melanophores would be present in larval melanoid axolotls, and that in adult skin, melanophores would predominate, perhaps exclusively. What happens to the pigment cells (xanthophores in particular) as aging progresses is a question that we attempted to address.

First, regarding the ontogeny and fate of pigment cells in aging melanoid axolotls, we suggest that as development proceeds, new xanthophores fail to differentiate from undifferentiated chromatoblasts, while melanophores continue to differentiate and proliferate in ever-increasing numbers. Based on both ultrastructural and chemical data, we further suggest that the fate of existing xanthophores is to convert to melanophores. The presence of 'melanized' pterinosomes in larval stage xanthophores and the observation of pterinosome-like melanosomes in some older stage melanophores, coupled with the disappearance of xanthophore pigments (pteridines) from the skin of older melanoid axolotls provided the evidence that led to this suggestion.

Other evidence that indirectly supports these suggestions is found in the literature. For example, increased numbers of melanophores in melanoid skin have been documented by cell counts (Hoerter, 1977). Moreover, it is known that total chromatophore numbers do not differ significantly between wild-type and melanoid skin (Sawada & Dalton, 1979), suggesting that the chromatoblast population remains stable (in terms of proportions) but that increasing proportions of chromatoblasts are programmed to become melanophores.

The notion that pigment cells can interconvert is one that has gained considerable support in recent years. Ide (1978, 1979) has documented the *in vitro* conversion of amphibian xanthophores to melanophores, iridophores to melanophores, and melanophores to iridophores. It is important to note that *in vitro*, xanthophores and iridophores both convert to melanophores. If, however, melanophores are cultured in the presence of 0·1 mM-guanosine (for example), melanosomes convert to reflecting platelets (Ide, 1978, 1979). The theoretical considerations of chromatoblast potential have been reviewed in detail by Bagnara *et al.* (1979), and we believe it is reasonable to speculate that xanthophores might convert to melanophores in melanoid axolotls.

In the one previously reported ultrastructural analysis of melanoid skin, Dunson (1974) reported that the block in iridophore development in melanoid axolotls must precede the formation of prepigment organelles, since there was no evidence of 'pre-reflecting platelets.' We also were unable to positively identify 'pre-reflecting platelets' and are uncertain what such structures might look like. However, we did observe cells that, based on their ultrastructural morphology and location in the skin, were presumed to be chromatoblasts. Furthermore, the appearance of these cells in late larval–juvenile stage animals and their relative scarcity overall suggests that these cells may be iridoblasts. If our presumption is correct, then the site of action of the melanoid gene on iridophore development is in fact prior to the formation of discrete pigment organelles. However, until more is known about iridophore differentiation, we

hesitate to conclude that the block precedes the formation of pre-pigment organelles.

Chemical data confirm that as development proceeds, pteridines gradually disappear from melanoid skin. NP and AHP are the last pteridines to disappear, and IXP is absent in adult melanoids and significantly reduced even in young larval melanoid axolotls. This pattern of reduced IXP and increased AHP is typical of the pteridine pattern observed in animals that have been treated with the xanthine dehydrogenase (XDH) inhibitor, allopurinol (Bagnara *et al.*, 1978; Frost & Bagnara, 1979). Like allopurinol, the melanoid gene also affects the synthesis of other pteridines (not just those produced by XDH activity). How this comes about is not clear based on information available regarding either this mutation or the effects of this drug.

Previously, both Benjamin (1970) and Lyerla & Dalton (1971) reported that pteridine patterns in melanoid animals were indistinguishable from the wild type. The results differ from ours first, because only larval animals were examined; second, because the paper chromatographic technique used failed to resolve pteridines as well as the TLC technique employed here, and third, because most of the presumed pteridines were not identified.

Similar chemical studies involving analysis of purines from melanoid skin (Dalton & Hoerter, 1974) revealed that both the number and kinds of u.v.-absorbing compounds found in melanoid skin differed from those present in wild-type skin. Unfortunately, the extracted compounds were again not identified, and thus no specific conclusions could be drawn regarding the effects of the *m* gene on purine biosynthesis.

In the future it should be possible to expand studies on the melanoid mutant based on earlier results demonstrating that melanoid phenocopies can be produced by feeding wild-type axolotls allopurinol (Bagnara *et al.*, 1978; Frost & Bagnara, 1978). Because the allopurinol-induced transition from wild-type phenotype to melanoid phenocopy is a gradual one, occurring over several months, it is suspected that xanthophore conversions, if they occur, can be carefully followed. Moreover, since XDH is known to participate in both purine and pteridine conversions (see Frost & Malacinski, 1980), a comparison of purine/pteridine biosynthesis in wild-type and mutant axolotls should provide information relevant to understanding the genetic defect.

Comparison of melanoid and wild-type phenotypes

The phenotypic differences between melanoid and wild-type axolotls are apparent even in very young larvae (Frost & Malacinski, 1980; Frost *et al.*, 1984*a*). Early in development, melanoid larvae are observed to have many more melanophores and far fewer xanthophores than do wild-type axolotls of a comparable age. In wild-type axolotls there is a hierarchy with respect to the numbers of pigment cell types present in skin such that melanophore numbers > xanthophores > iridophores (Frost *et al.*, 1984*b*). This pattern (of melanophores

predominating in numbers) persists throughout development. Melanoid axolotls carry this pattern to an extreme. In melanoids, not only do melanophores predominate throughout development, but in the adult, melanophores appear to be the *only* chromatophore type. Consequently, as melanoid axolotls grow, the yellow coloration provided by the xanthophores gradually disappears and the adult ends up a dark, velvety black colour. Wild-type adults are, by contrast, a mottled olive-green colour (Frost *et al.*, 1984*a*,*b*).

Melanophores and xanthophores observed in larval melanoid skin are very similar in morphology and location to melanophores and xanthophores in larval wild type skin. Chromatophores in melanoid skin are located in the dermis, beneath or within the collagen matrix, and they are arranged such that no two pigment cell bodies overlap, although pigment cell processes frequently overlap. This organization is identical to that described for wild-type chromatophores (Frost *et al.*, 1984*b*). Structurally, the cytoplasmic components of melanoid pigment cells are similar to those of wild-type pigment cells, except for the presence of an occasional 'melanized' pterinosome in larval melanoid xanthophores and the occurrence of 'pterinosome-like' melanosomes in later stage melanoid melanophores. The obvious structural changes in pterinosome morphology that occur during development of wild-type axolotls (Frost *et al.*, 1984*b*) do not occur in melanoids. Not surprisingly, the developmental changes that occur in melanoid pteridine patterns are significantly different from those observed in the wild-type (Frost *et al.*, 1984*a*,*b*). Specifically, the pteridine pattern observed in larval melanoid skin is more similar to that observed in adult wild-type skin (compare Fig. 7a with Fig. 8a in Frost *et al.*, 1984*b*) than it is to comparable aged wild-type skin.

CONCLUDING STATEMENTS

In order to understand the action of the melanoid gene it is necessary to discern how this mutation can (a) eliminate one cell type (iridophores) entirely, and (b) cause a gradual diminution and/or reprogramming of another cell type (xanthophores). We believe that we can now speculate about how this might come about.

Bagnara *et al.* (1979), in a synthesis of the available information on pigment cell differentiation, proposed a 'common origin' theory to explain how morphologically distinct pigment cells might be endowed with numerous physical, chemical and even structural similarities. The most significant question that remains to be answered concerns the mechanism by which undifferentiated chromatoblasts are determined to develop into either melanophores, xanthophores, or iridophores.

On the basis of our observations of melanoid axolotls we suggest that it may be the modulation in both purine and pteridine biosynthesis (via XDH activity perhaps) that results in the altered (i.e., melanoid) phenotype. XDH converts

the purines hypoxanthine to xanthine and xanthine to uric acid. It also converts the pteridines AHP to IXP, and possibly AHP to XP (see Frost & Malacinski, 1980). Chromatograms of pteridines from larval skin demonstrate that significantly more AHP is present in melanoid axolotls than in wild-type animals with concomitant decreases in IXP, XP and all other pteridines (with the possible exception of NP) as well. It may be significant that XDH requires a series of cofactors including molybdenum, NAD, FAD, and *a pteridine* (Johnson, Hainline & Rajagopalan, 1980). Perhaps the *m* gene results in the alteration or elimination of one of the regulatory molecules necessary for XDH activity. If the pteridine cofactor, for example, is gradually eliminated than xanthophores might be expected to develop in larvae, but because pteridine biosynthesis cannot be maintained, xanthophores would fail to either differentiate or be maintained in adults. As iridophores do not normally differentiate until later during development, and as XDH is necessary for certain purine conversions, reflecting platelets (and thus iridophores) might be expected to fail to differentiate altogether. This indeed is the case in melanoid axolotls.

There are also several ways in which xanthophores might be envisioned to convert to melanophores as is apparently the case in this mutant. Obika & Hama (1960) suggested that in the presence of pteridines, melanin synthesis (i.e., tyrosinase activity) is repressed. Tyrosinase, the rate-limiting enzyme in melanin synthesis, is known to be present in xanthophores (Yasutomi & Hama, 1976), and in pterinosomes in particular (Frost & Robinson, in prep.). Thus, in the absence of pteridine biosynthesis (or as pteridine synthesis declines), tyrosinase activity may be expressed such that melanization is promoted. This might explain how differentiated xanthophores could undergo a gradual conversion to melanophores, and how xanthoblasts might differentiate as melanophores.

What happens to iridophores in melanoid animals is more difficult to envision because we know so little about iridophore differentiation in general. These cells also contain tyrosinase (Frost & Robinson, in prep.). Thus, the enzymatic machinery for melanin synthesis is present but presumably inactive in these cells. We hesitate to speculate, however, that iridoblasts, like xanthoblasts, are reprogrammed to melanophores since we have observed cells that may be defective iridoblasts in melanoid skin. This view is not incompatible with the observation that total chromatophore numbers between wild type and melanoid axolotls are the same (Sawada & Dalton, 1978), since chromatophore counts were made only on young larval skin, probably well before significant numbers of iridophores would have differentiated in the first place.

As an alternative to the above hypothesis, it might be that the melanoid defect resides at the level of pigment substrate synthesis. Both iridophores and xanthophores require purines or a purine precursor (GTP; see Frost & Malacinski, 1980) for pigment synthesis. This alternative seems less likely than the previous suggestion because defects in purine metabolism would be expected to have serious consequences on all cells, not just chromatophores.

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