The pigmentary system of developing axolotls

III. An analysis of the albino phenotype

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SUMMARY

The albino 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 analyses of pteridine pigments were also carried out, and the pattern of pteridines in albino animals was found to be more complex than, and quantitatively enhanced (at all developmental stages examined) over, the pattern observed in comparable wild-type axolotls. The golden colour of albino axolotls is due primarily to sepiapterin (a yellow pteridine) and secondarily to riboflavin (and other flavins).

Coincident with enhanced levels of yellow pigments, xanthophore pigment organelles (pterinosomes) in albino skin reach a mature state earlier than they do in wild-type axolotl skin. This morphology is conserved throughout development in albino animals whereas it is gradually lost in the wild type. Unpigmented melanophores from albino axolotls are illustrated for the first time, and in larval albino axolotls the morphology of these cells is shown to be very similar to xanthophore morphology. In older albino animals xanthophores are easily distinguished from unpigmented melanophores. Iridophores seem to appear in albino skin at an earlier stage than they have been observed in wild-type skin. Morphologically, wild-type and albino iridophores are identical.

INTRODUCTION

Albinism is a pigment disorder that is characterized by the absence of melanin (Silvers, 1979). By definition albino animals have melanophores in their skin, but such cells are in some way impaired in their ability to synthesize melanin. In those cases that have been examined, this defect has usually been found to result from alterations in the structural gene for tyrosinase, the rate-limiting enzyme in the melanin biosynthetic pathway. In fact, in humans at least seven different types of 'albinism' have been identified based on analyses of tyrosinase activity; some forms were tyrosinase 'positive' and others were tyrosinase 'negative' (Pawelek & Korner, 1982). However, because melanization is a complex process, involving e.g. hormone–cell interactions, construction of elaborate pigment-containing organelles (melanosomes) and tyrosinase (a protein with a complex structure and

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regulatory properties), it is naïve to assume that only defective tyrosinase will result in an albinistic phenotype.

The *albino* gene was first introduced into laboratory stocks of axolotls ultimately by hybridization (*via* artificial insemination) between a female albino *Ambystoma tigrinum* and white *Ambystoma mexicanum* males (Humphrey, 1967). Because the viability of hybrid embryos was very low (only 2–3/700 eggs from the artificial insemination procedure survived beyond cleavage stages), Humphrey resorted to nuclear transfers and subsequently to gonad grafts in order to salvage animals that might be heterozygous for albinism (Humphrey, 1967). Eight animals survived gonad grafts from embryos that resulted from nuclear transfers. Of the remaining grafted animals, two (a male and a female) were mated and produced viable offspring. It was only after these offspring were raised to sexual maturity and subsequently bred to each other that the albino phenotype was recovered. Further details of this massive undertaking are described by Humphrey (1967); it is important to note that albino animals all carry at least some portion of the *A. tigrinum* genome.

Subsequent studies on albinism in axolotls have focused primarily on tyrosinase inactivation (Benjamin, 1970; Harsa-King, 1978, 1980) and secondarily on structural aspects of albino skin (Dunson, 1974). Benjamin (1970) stated that '... the *a* gene in the axolotl represents the least complicated mutation to analyse since only one type of pigment cell is affected; the melanophore'. Because no in-depth description of the pigments and pigment cells present in albino axolotl skin was available and because of our experience with albinos in the laboratory, we were sceptical of the generalization that *only* melanization is affected by the *a* gene.

Two morphological characteristics suggested that albino axolotls were not simply amelanotic versions of the wild-type phenotype: (1) the bright yellow pigmentation (presumably due to pteridines) and (2) the appearance of 'shiny' pigmented areas in albino skin (presumably due to the presence of iridophores). Our efforts were thus focused on examining when pigment cells appear in albino skin, what the structural features of those cells might be, and what types of bright-coloured pigments were present in albino skin that could account for the observed pigment pattern. The results are compared to similar observations made previously on the wild-type pigment pattern (Frost, Epp & Robinson, 1984b).

MATERIALS AND METHODS

Animals

Axolotls homozygous for the albino (*a*) 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.* (1984b).

Electron microscopy

Axolotl skin was prepared for transmission electron microscopy (TEM) as described previously (Frost *et al.* 1984b). The fixative used was 2·5 % glutaraldehyde in 0·1 M-cacodylate

buffer, pH 7·4, with postfixation in 2 % osmium tetroxide in the same buffer, and *en bloc* staining in 2 % aqueous uranyl acetate.

Pigment extraction

Soluble xanthophore pigments were extracted from albino axolotl skin and analysed by a combination of thin-layer (TLC) and column chromatographic techniques as described in detail in an earlier publication (Frost *et al.* 1984*b*). TLC plates were coated with a mixture of cellulose:silica gel G (1:1) and the solvent used for pteridine separations in one dimension was N-propanol:7 % ammonia (2:1, v/v) (Frost & Bagnara, 1978). Column chromatographic separations were carried out using a 1·5×40 cm glass column packed with Bio-Gel P-2 (200–400 mesh, BioRad). The column was prewashed extensively with distilled water and samples were eluted with distilled water. Pigments were identified by comparing their u.v.-fluorescent 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-hydroxypterin; 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

Three types of pigment cells are present in albino skin: melanophores (unmelanized), xanthophores, and iridophores. As in all amphibians we have studied, the chromatophores primarily responsible for the observed colour pattern are found in the dermal layer of the skin.

As described in wild-type animals (Frost *et al.* 1984*b*), each pigment cell type is distinguishable by its characteristic pigment-containing organelles, and, onto-genetically, each of the three cell types initially arises at a different time during development. Melanophores and xanthophores appear early in development, and iridophores are present somewhat later (in juveniles).

Melanophores in albino axolotls contain three types of 'prepigment' organelles: (1) apparently empty vesicles, (2) vesicles containing a small amount of fibrous material and/or smaller vesicles, and (3) vesicles (premelanosomes) with varying degrees of the fibrous matrix typical of vertebrate premelanosomes (Bagnara & Hadley, 1973). In larval and young juvenile albinos, the first two types of melanophore organelles predominate (Fig. 1); in older albinos, the third type is most prevalent (Fig. 2).

Similarly, xanthophores in albino axolotls contain three basic types of pteridine-containing organelles (pterinosomes). Nearly twice the diameter of premelano-somes, the pterinosomes fit loosely into three categories: (1) apparently empty vesicles, (2) vesicles containing small quantities of fibrous material or smaller vesicles, and (3) vesicles containing varying degrees of fibrous material that appears to be concentrically arranged within the outer limiting membrane (a dense-matrix pterinosome). The first two types of pterinosomes are most evident in larval and young juvenile albinos (Fig. 3); the third type (dense-matrix pterinosome) predominates throughout the juvenile and adult stages of development (Fig. 4). We believe that the distinct types of organelle morphologies
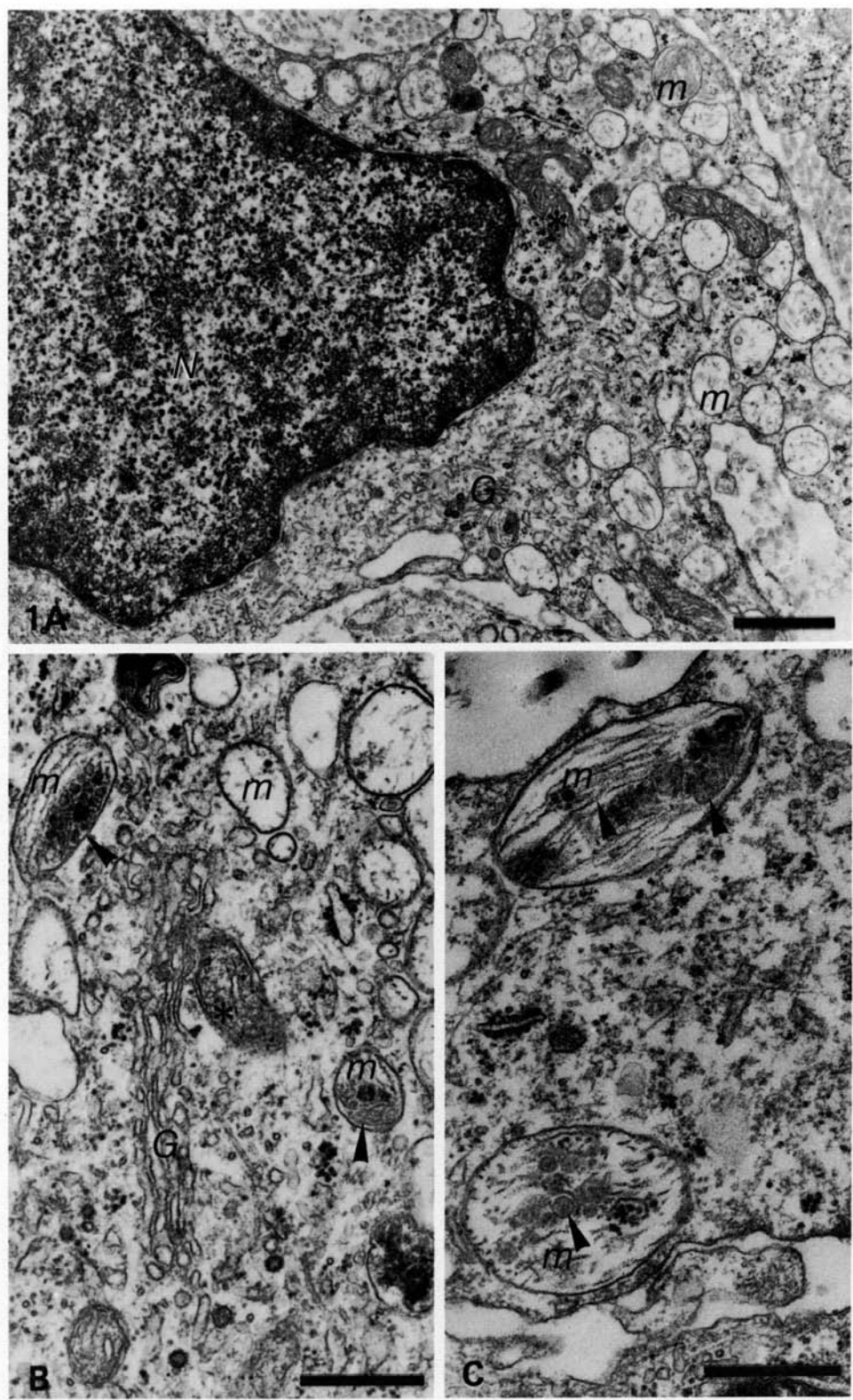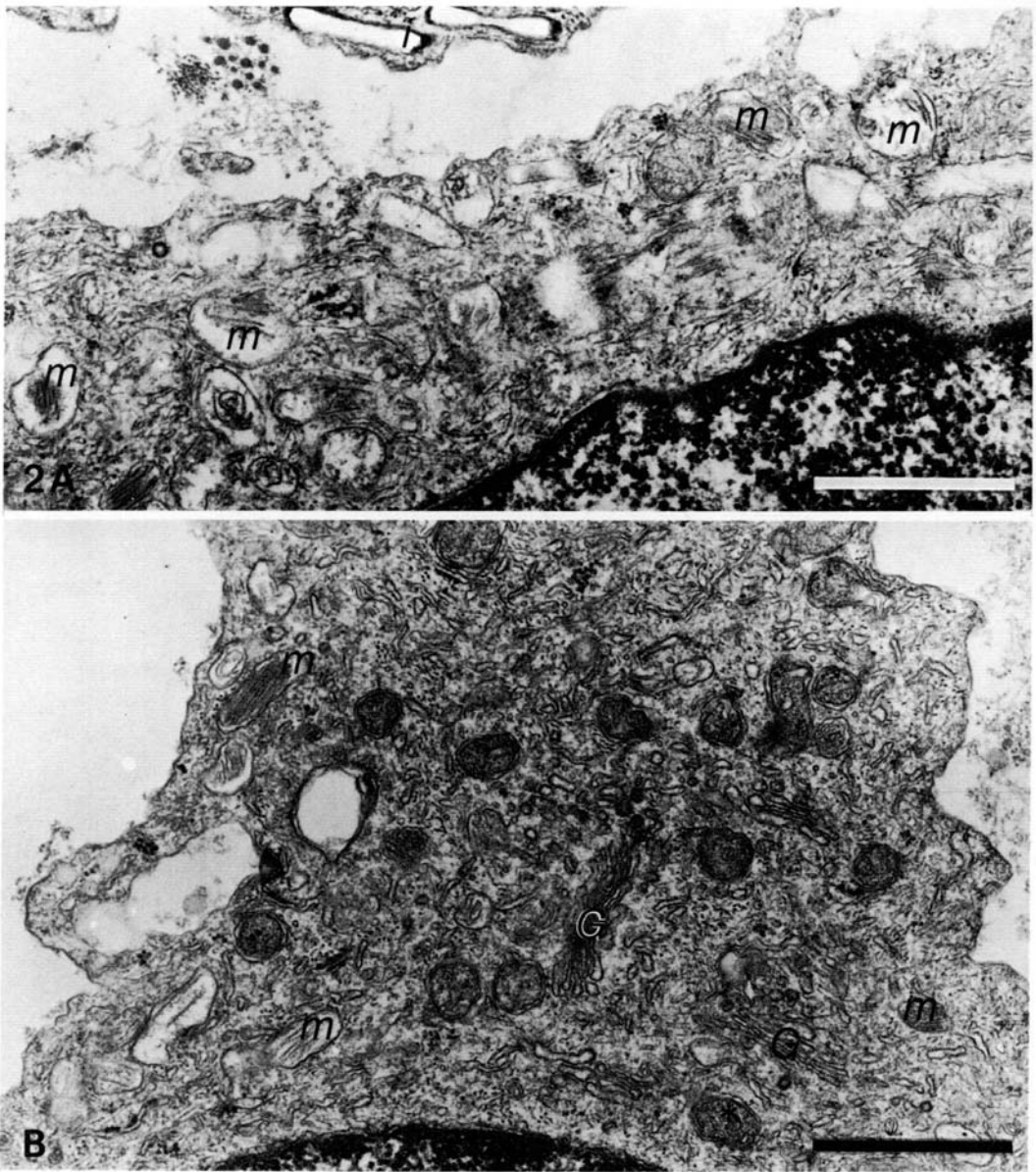
Fig. 2. (A,B) Cytoplasmic characteristics of melanophores from juvenile albino axolotl skin emphasizing the predominance of unmelanized, matrix-containing pre-melanosomes (*m*). Note the position of an iridophore process (*i*) above the melanophore in A. With the exception of increased numbers of matrix-containing premelanosomes, melanophores from older (juvenile/adult) animals, the appearance of these cells changes very little with development (compare Figs 1, 2). *G*, Golgi; *, mitochondrion. Bar, 1 μm.

Fig. 1. (A) Melanophore from larval albino axolotl skin. Note the absence of any electron-dense (melanin) pigment in premelanosome (*m*) structures. Bar, 1 μm. (B,C) Examples of albino melanophore organelles illustrating the types of matrices that are observed (arrows). *N*, nucleus; *G*, Golgi; *, mitochondrion. Bar, 0·5 μm.
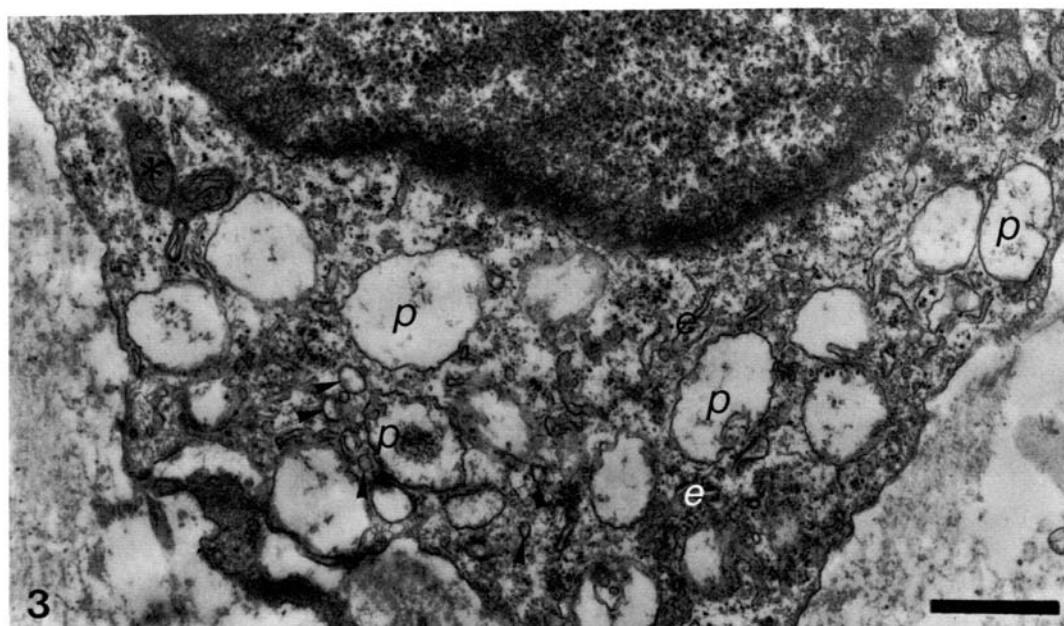
Fig. 3. Xanthophore from young larval albino axolotl skin. The large cytoplasmic vesicles that contain small quantities of fibrous-appearing material are pterinosomes (*p*). The cytoplasm also contains numerous small vesicles (arrows), extensive endoplasmic reticulum (*e*), mitochondria (*), and other structures. Bar, 1 μm.

represent stages in the development of pterinosomes (Frost *et al.* 1984*b*) as originally described by Yasutomi & Hama (1971).

The cytoplasm of melanophores and xanthophores (Figs 1, 3) from young larval albino axolotl skin is also characterized by an extensive Golgi system, numerous large and small vesicles scattered throughout, smooth and rough endoplasmic reticulum (ER), intermediate filaments, microtubules, mitochondria, and pigment organelles in various stages of development. With regard to albino melanophores from older animals (Fig. 2), these cytoplasmic characteristics persist throughout development and only changes in the proportions of pigment organelle types are observed. In mature xanthophores from older albino animals (Fig. 4) there is an increase in the number of dense-matrix pterinosomes, accompanied by increased amounts of cytoplasmic filaments and microtubules.

Iridophores appear during late larval development and, like xanthophores, 'newly differentiated' iridophores are easily distinguishable from mature, well-differentiated cells (compare Figs 5, 6). In larval animals iridophore cytoplasm (Fig. 5) contains extensive Golgi, numerous vesicles of varying shapes and sizes, smooth and rough ER, mitochondria, numerous intermediate filaments, microtubules, and pigment organelles (reflecting platelets) in various stages of differentiation. Prereflecting platelets in axolotls (Fig. 5) are presumed to derive initially from a pinching-off of the smooth ER to form a vesicle. These vesicles assume a variety of shapes and sizes; some are amorphous whereas others are more or less round or oval in appearance, and still others are elongate

structures that superficially resemble, in shape, the mature reflecting platelet. Mature reflecting platelets are rectangular cylinders that contain purines (e.g. guanine) as pigments. In electron micrographs, mature reflecting platelets appear to be 'empty holes' because the crystalline contents of these organelles generally fall out during processing.
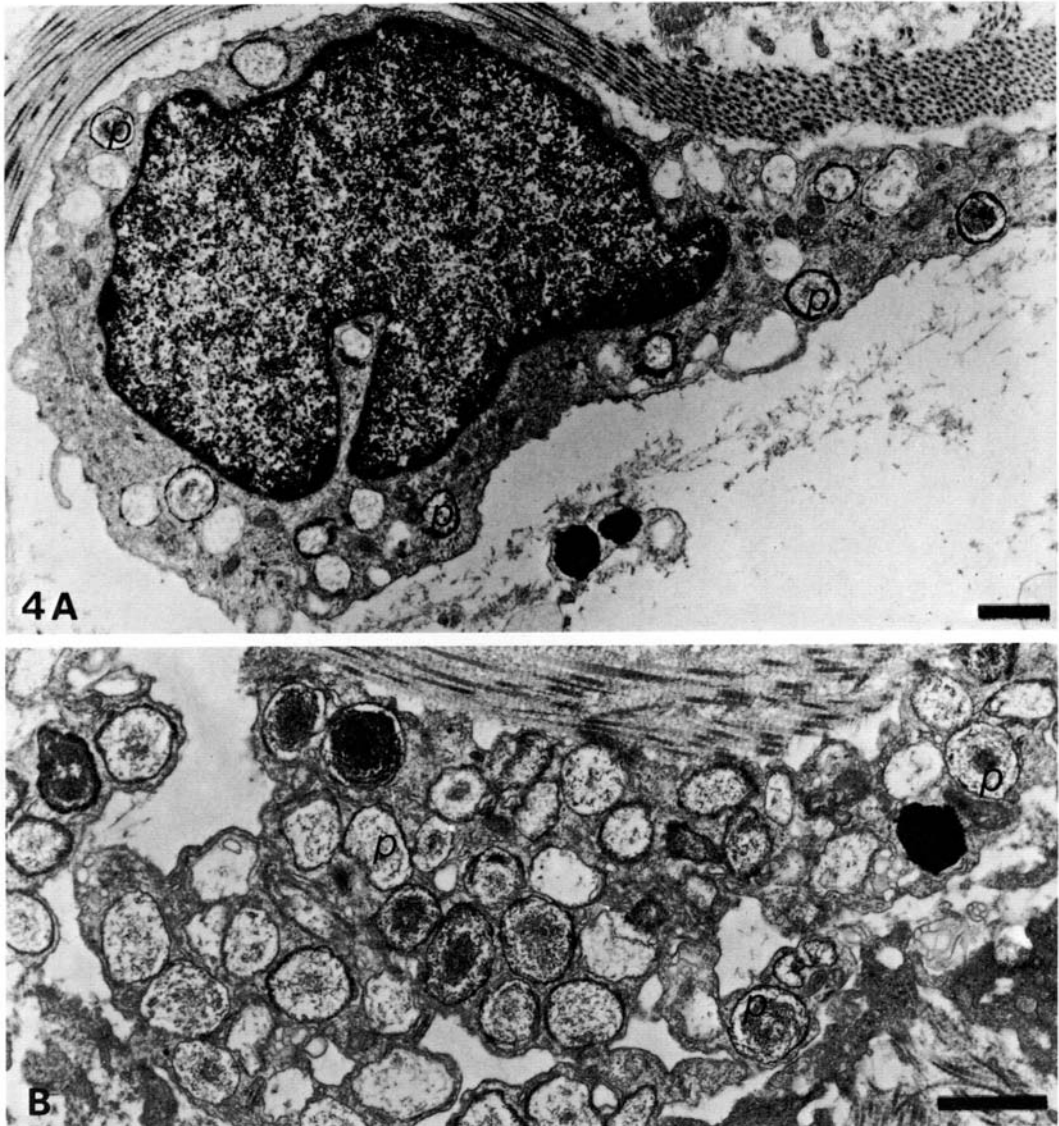

Fig. 4. (A) Xanthophore from juvenile albino axolotl skin. (B) Higher magnification of xanthophore organelles (pterinosomes, *p*) from older albino axolotl skin. Note the presence of an electron-dense fibrous matrix in many of the pterinosomes. (Compare xanthophores from larval albino skin [Fig. 3] with those from older albino skin [Fig. 4].) Bar, 1 μm.

Iridophores typical of juvenile and adult albino axolotl skin are shown in Fig. 6. Many of the organelles commonly associated with 'synthetic' activity (e.g. Golgi, ER, etc.) are no longer present in abundance in the cytoplasm. There are now
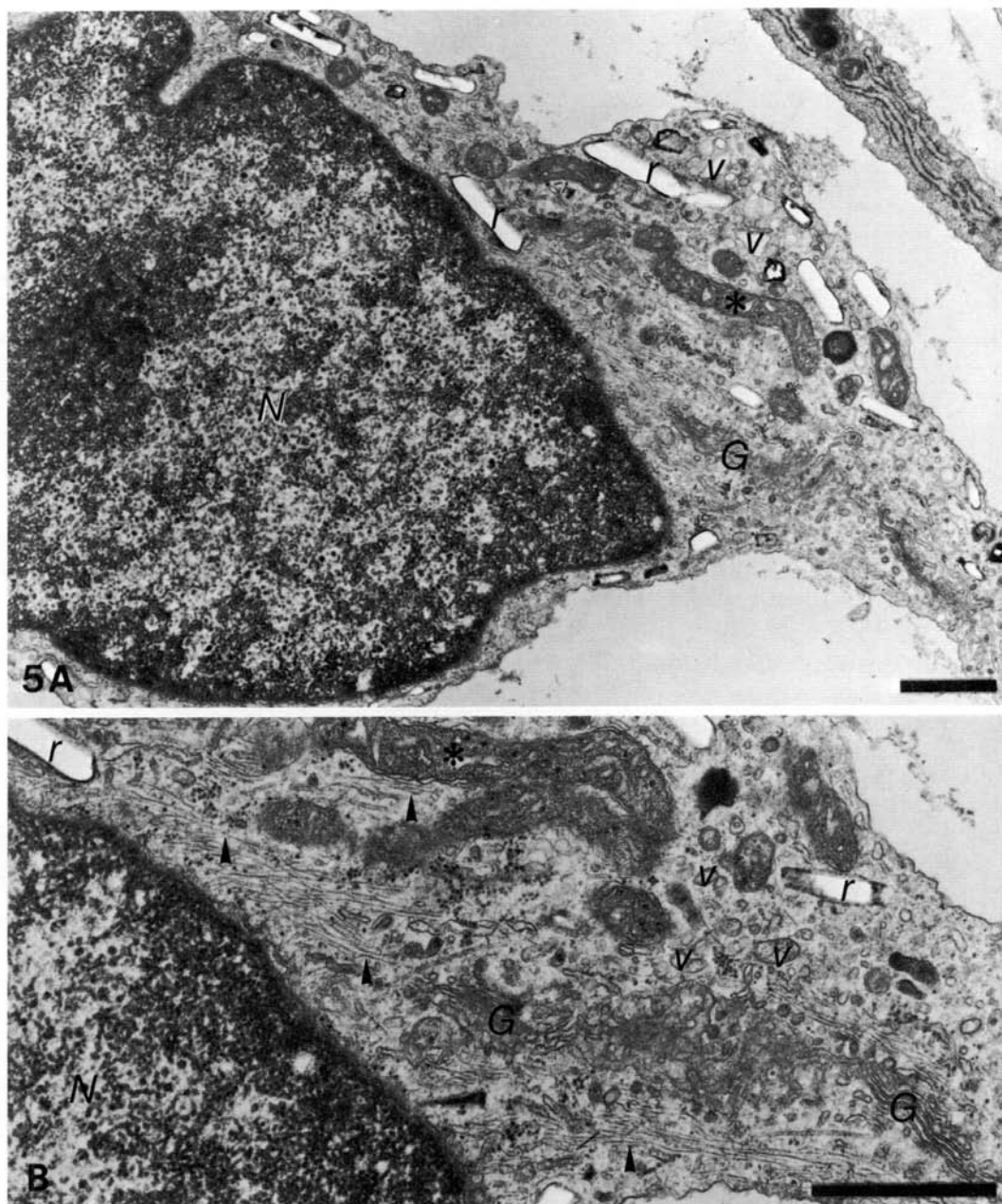

Fig. 5. Iridophore from late larval albino axolotl skin. Note the presence of reflecting platelets (r), Golgi (G), vesicles (v) that may function as prereflecting platelets, mitochondria (*), intermediate filaments (arrows) and the nucleus (N). These cytoplasmic characteristics are typical of pigment cells that are still actively differentiating. Bar, 1 μm.

many more mature reflecting platelets spread throughout the iridophore cytoplasm and some of these organelles are very long (Fig. 6; ~3 μm). Moreover, many of the platelets are stacked and oriented parallel to the overlying basement membrane of the dermis. This is in contrast to iridophores from younger animals wherein there are relatively few mature reflecting platelets, most of these are
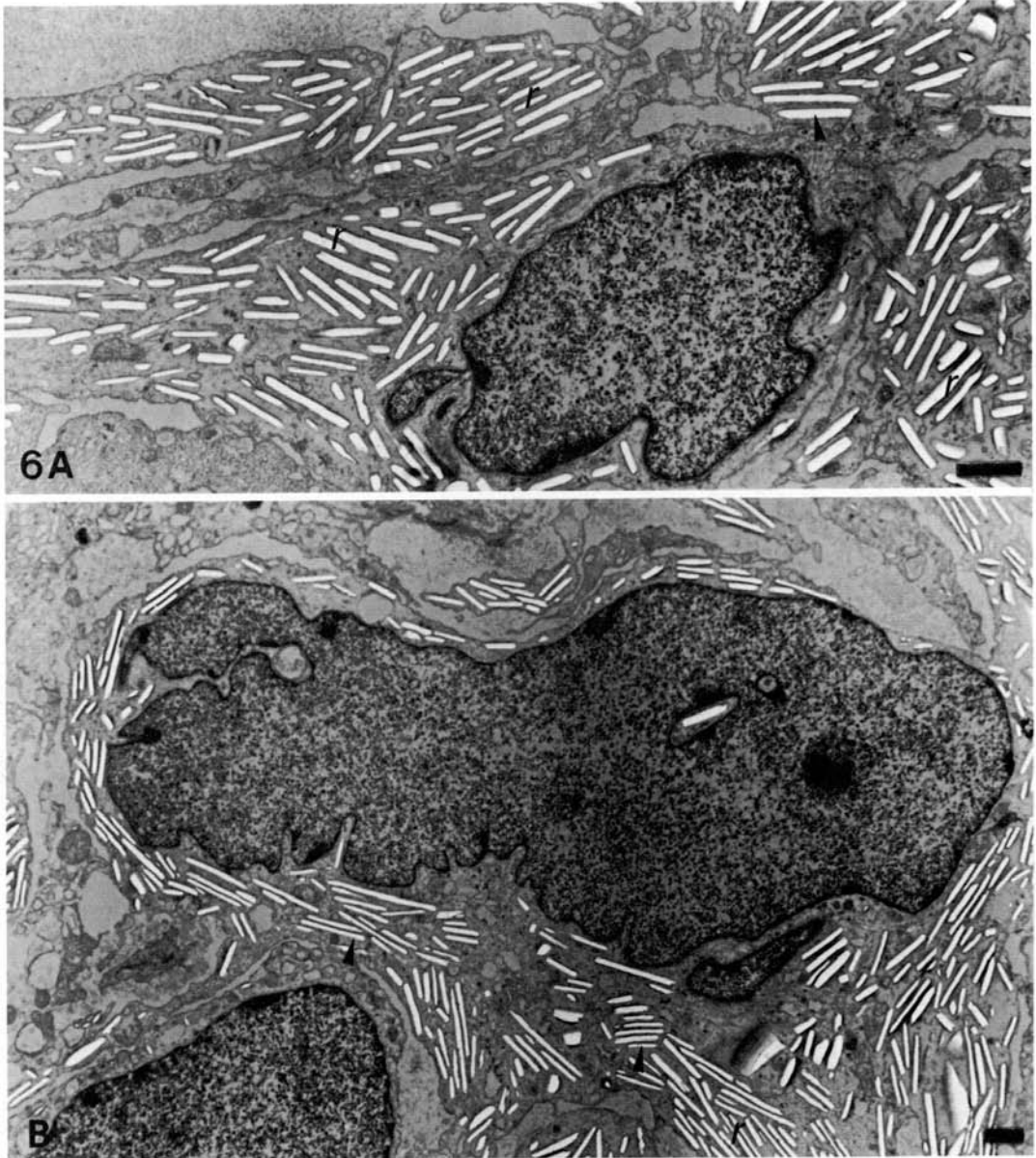

Fig. 6. Iridophores from juvenile/adult albino skin. Note the prevalence of mature reflecting platelets (*r*) scattered throughout the cytoplasm of the iridophores. Many of these organelles begin to show signs of stacking (arrows) and orientation parallel to the overlying basement membrane. Bar, 1 μm.

comparatively short in length, and all of these organelles appear to be randomly oriented in the cytoplasm (Fig. 5).

Melanophores and xanthophores are usually situated directly beneath the basement membrane in the dermis; they rarely overlap each other (except for cell processes). Iridophore cell bodies are primarily found below the xanthophore–melanophore layer, generally in conjunction with melanophore processes which may be observed between them and the basement membrane (e.g. Fig. 2). As noted previously in wild-type axolotls (Frost *et al.* 1984*b*), the only other cell type commonly found in association with dermal chromatophores is the fibroblast; this is also true in albino axolotl skin (fibroblasts not shown here; see examples in Frost *et al.* 1984*b*).

Pigment analyses

Pigment analysis was largely restricted to pterinosome pigments, i.e. pteridines and flavins (for reasons discussed in Frost *et al.* 1984*b*). In u.v. light these pigments are readily visualized as fluorescent spots of different colours (blue, purple, yellow, etc.) on TLC plates (Fig. 7). Fig. 7A is an example of a side-by-side comparison of the fluorescent compounds extracted from larval and adult albino skin; pigments appear as white (fluorescent) spots on a black (u.v. light) background. When this chromatogram is scanned in a fluorometer, the profiles shown in Fig. 7B are obtained. These profiles represent both a qualitative and an approximate quantitative comparison of xanthophore pigments between larval and adult albino animals.

Qualitatively, seven pteridines and two (or three) flavins were present in larval albino skin extracts: AHP-6-COOH, XP, FAD (FMN), IXP, NP, BP, AHP, SP, and riboflavin. [Details of how these spots were definitively identified, based on colour, chromatographic mobility, and spectral properties, and the reasons that these profiles also represent quantitative comparisons of skin pigments, are presented in Frost *et al.* (1984*b*).] Adult albino skin contains the same qualitative pattern of pteridines, but in quantities obviously reduced from those present in larval skin.

Separation of pigments on columns of Bio-Gel P-2 (Fig. 8) revealed a series of four fluorescent peaks. Peak 1 contains AHP, BP, NP, IXP, and AHP-6-COOH; peak 2 contains XP and a flavin(s); peak 3 contains SP; and peak 4 contains riboflavin. Again, the types of pigments present in skin extracts do not differ between larval and adult stages. Quantitatively, SP is present in significantly lower concentrations in adult albino skin than in larval albino skin, whereas the xanthopterin and riboflavin peaks are essentially the same at both developmental stages as is the heterogeneous peak no. 1.

Albino animals are a striking golden yellow colour throughout development (Frost, Briggs & Malacinski, 1984*a*). Early on, this colour is due primarily to yellow pigments present in xanthophores. Later in development, iridophores contribute to and enhance the yellow colour, making albino animals shiny golden in appearance.

DISCUSSION

This report is the first, to our knowledge, in which electron micrographs of melanophores from albino axolotl skin appear. Dunson (1974) figured a cell identified as a 'melanoblast' from an albino–axanthic larva. Because axanthic animals presumably lack xanthophores (Lyerla & Dalton, 1971), albino–axanthic animals might be expected to have *only* unpigmented melanophores. Dunson was apparently unable to identify unpigmented melanophores from pure albino animals, probably because she looked only at very young larvae, when xantho- phores and melanophores are very similar in appearance (e.g. compare the structural features of the cells in Figs 1, 3). Consequently, Dunson (1974) described albino–axanthic melanoblasts as having pigment granules with 'more electron dense material than pterinosomes and no completely empty granules ...'.
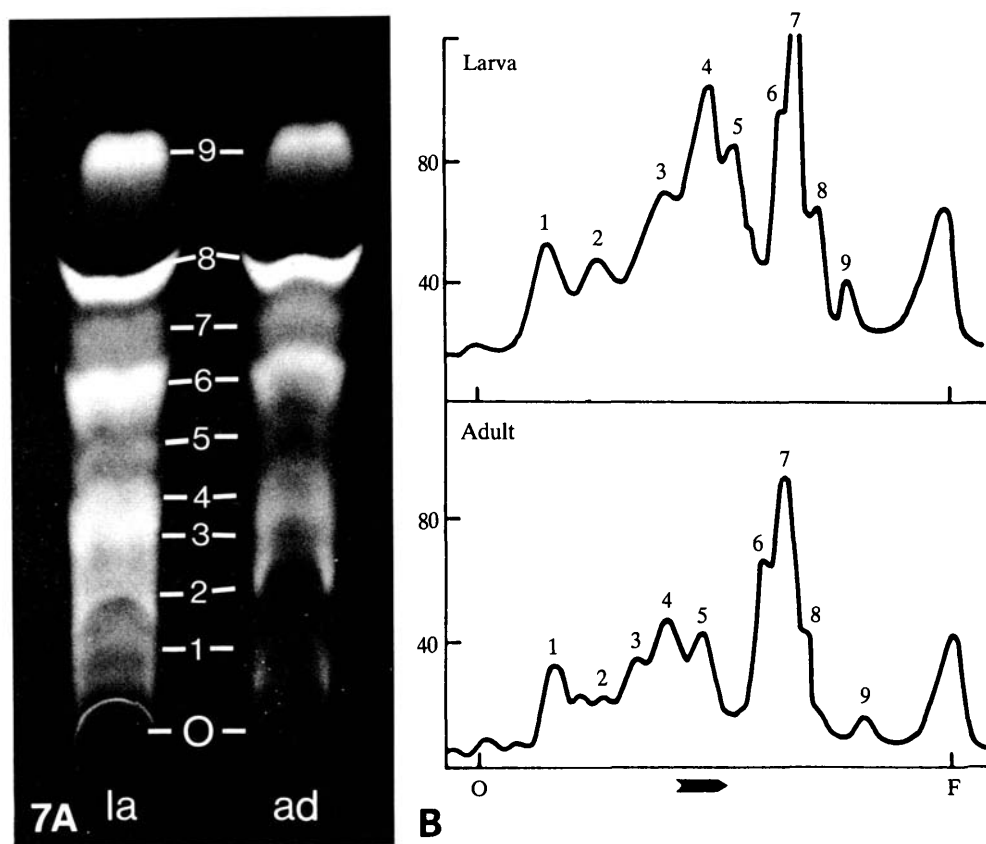

Fig. 7. (A) Photograph of a one-dimensional thin-layer chromatographic separation of ethanol-extracted pteridine (and flavin) pigments from larval (*la*) and adult (*ad*) albino axolotl skin. O, origin; 1, AHP-6-COOH; 2, XP; 3, 4, flavins; 5, IXP; 6, NP; 7, AHP; 8, BP; 9, SP+riboflavin. (B) Fluorometric scans of the chromatographic results shown in Fig. 7A. Numbered peaks contain the following compounds: 1, AHP-6-COOH; 2, XP; 3, 4, flavins; 5, IXP; 6, NP; 7, AHP; 8, BP; 9, SP, riboflavin. O, origin; F, solvent front; x-axis, direction of scan; y-axis, fluorescence units.
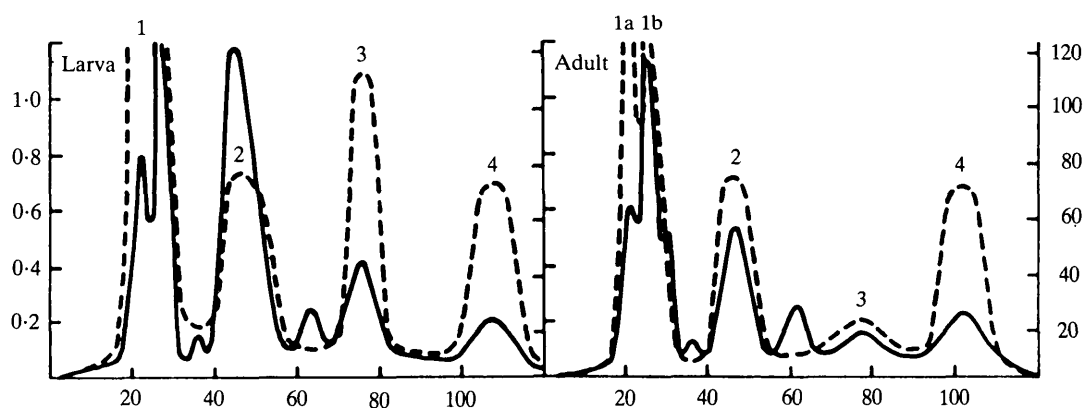
Fig. 8. Column chromatographic (Bio-Gel P-2) separation of ethanol-extracted pigments from larval and adult albino skin. Fluorescent peaks contain the following pteridines. In the larva: 1, AHP, IXP, NP, BP; 2, XP+flavins; 3, SP; 4, riboflavin. In the adult: 1a, b, AHP, IXP, NP, BP; 2, XP+flavins; 3, SP; 4, riboflavin. Axis labels are: x-axis, fraction numbers; y-axis (left), absorbance (OD_{260}) (——); y-axis (right), fluorescence units (---). (Note: Pteridines both absorb and fluoresce; fluorescence separates pteridines from non-fluorescent, u.v.-absorbing purine and purine-like compounds that are also present in axolotl skin extracts. We have illustrated both the fluorescing and absorbing profiles in this figure.)

We would generally concur with this description for albino animals but add that this morphology changes with development such that melanosomes gradually acquire the characteristic internal fibrillar matrix of a typical premelanosome. Furthermore, our results suggest that even in very young albino axolotls, matrix-containing organelles are present in melanophores, and as development progresses, melanophores contain proportionally more premelanosomes with matrices and fewer organelles with little or no matrix. The same is basically true of pterinosome development in xanthophores, although mature pterinosomes are distinctly different in appearance from premelanosomes (compare the structural features of the pigment organelles in Fig. 4B with those in Fig. 1B,C).

In a previous publication (Frost *et al.* 1984*b*) we described the pigment pattern in wild-type axolotls; several points worthy of note can be made when wild-type and albino axolotl pigment patterns are compared. Ontogenetically, pigment cells develop in the same order in both animals: melanophores first, followed by xanthophores, and lastly iridophores. However, iridophores appear in the dorsal skin of albinos at an age well before they are detectable in wild-type animals. Xanthophore organelles differentiate somewhat earlier in albinos as well, and there is apparently more yellow pigment in albino skin extracts than in wild-type extracts (at any developmental stage). Moreover, both xanthophores and iridophores were encountered more frequently in albino skin (during sectioning) than in comparable wild-type skin, suggesting that albino skin may contain more than the 'usual numbers' of these pigment cell types. Unfortunately, no cell counts have as yet been done to determine whether there are in fact significantly greater

numbers of xanthophores and iridophores in albino skin as compared to the wild type.

The greater amount of yellow pigmentation present in albino skin is a result of three factors: (1) the occurrence of sepiapterin and three flavins in albino axolotl skin *versus* sepiapterin and only one flavin in wild-type skin; (2) a quantitative increase in *all* pteridines in albino skin (which may simply be indicative of more xanthophores in albino skin) as compared to wild type; and (3) the persistence of yellow xanthophore pigments throughout the life of albino axolotls. With regard to this latter point, we reported that in older, wild-type axolotls, xanthophore morphology 'reverts' to a more larval-like state in which the pterinosomes appear to be mostly 'empty' and the amount of pteridine pigment is decreased substantially over what was present in larvae (Frost *et al.* 1984*b*). In adult albino axolotls, the amount of pteridine pigment present also decreases somewhat with age, but this reduction results in pteridine levels that are still greater than or equal to those found in larval wild-type axolotl skin.

We recognize that any of the observed differences between albino and wild-type axolotls, e.g. the earlier differentiation of iridophores, the only slight reduction in yellow pigmentation during development, or the additional flavin(s) in albinos, could be either due to an actual *a* gene effect, or to the influence of the *A. tigrinum* genome. We have not yet examined wild-type or albino *A. tigrinum* larvae to see if the pteridine pattern is the same as that observed in larval albino *A. mexicanum*.

Whatever the underlying cause of these apparent differences between wild-type and albino pigment cell differentiation, it seems clear that an albino axolotl is not simply an animal lacking melanin pigment. The fact that all three pigment cells are affected by the *a* gene in various ways further supports the interrelatedness of chromatophores in general (a hypothesis put forward in Bagnara *et al.* 1978) and suggests, as we have emphasized in earlier publications (Frost *et al.* 1984*a,b,c*; Frost & Malacinski, 1980), that metabolic perturbations in one pigment cell type may lead to unexpected changes in either the development of and/or metabolism in other cell types.

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