

# The pigmentary system of developing axolotls

## I. A biochemical and structural analysis of chromatophores in wild-type axolotls

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### SUMMARY

A biochemical and transmission electron microscopic description of the wild-type pigment phenotype in developing Mexican axolotls (*Ambystoma mexicanum*) is presented. There are three pigment cell types found in adult axolotl skin – melanophores, xanthophores and iridophores. Both pigments and pigment cells undergo specific developmental changes in axolotls. Melanophores are the predominant pigment cell type throughout development; xanthophores occur secondarily and in fewer numbers than melanophores; iridophores do not appear until well into the larval stage and remain thereafter as the least frequently encountered pigment cell type. Ultrastructural differences in xanthophore organelle (pterinosome) structure at different developmental stages correlate with changes in the pattern of pteridine biosynthesis. Sepiapterin, a yellow pteridine, is present in larval axolotl skin but not in adults. Riboflavin (also yellow) is present in minimal quantities in larval skin and large quantities in adult axolotl skin. Pterinosomes undergo a morphological “reversion” at some point prior to or shortly after axolotls attain sexual maturity. Correlated with the neotenic state of the axolotl, certain larval pigmentary features are retained throughout development. Notably, the pigment cells remain scattered in the dermis such that no two pigment cell bodies overlap, although cell processes may overlap. This study forms the basis for comparison of the wild type pigment phenotype to the three mutant phenotypes—melanoid, axanthic and albino—found in the axolotl.

### INTRODUCTION

Laboratory stocks of the Mexican axolotl (*Ambystoma mexicanum*) have been widely exploited for a variety of experimental purposes (e.g. see Malacinski & Brothers, 1974; Humphrey, 1975; American Zoologist, 1977). Of value to many researchers is that a number of pigment mutants exists in the axolotl (Frost & Malacinski, 1980) that provide obvious phenotypic markers useful in identifying the parentage of experimentally manipulated animals and animal tissues. Over and above the utility of pigment mutants to basic experimental work, the pigmentary system of the axolotl poses an interesting developmental and morphological problem. Moreover, in spite of a few attempts to *either* biochemically characterize the pigments of the axolotl (Benjamin, 1970; Lyerla & Dalton, 1971; Dalton & Hoerter, 1974) or to cytologically describe its pigment cells (Dunson, 1974), no coherent description of the axolotl pigmentary system exists.

In this and the accompanying reports (Frost, Epp & Robinson, 1984*a,b,c*), we describe the following: (1) the pigments and pigment cells present in wild-type and mutant axolotls, and (2) changes in the contents, distribution and arrangement of pigment cells that occur during axolotl development. Furthermore, correlating with the neotenic nature of axolotls (i.e. they do not normally metamorphose), changes in the pigmentary system that are known to accompany amphibian metamorphosis (see e.g., Smith-Gill & Carver, 1981) might not be expected to occur. Thus, the axolotl should provide a means for describing pigmentary changes that are presumably due to growth rather than those that result from metamorphosis.

#### MATERIALS AND METHODS

##### *Animals*

Axolotls were obtained from the Indiana University Axolotl Colony, Bloomington, Indiana. Animals were maintained in small finger bowls or aerated aquaria containing 50 % Holtfreter's solution (Rugh, 1962). Young animals were fed live brine shrimp every day; older animals were fed beef liver and/or earthworms every other day. Experimental animals were grouped into three developmental categories: larvae (ca. 2–4 cm in length; < 3 months old); juveniles (ca. 9–13 cm in length; not yet sexually mature), and adults (> 15 cm long; sexually mature). When fresh tissues were needed, animals were sacrificed either by decapitation or with an overdose of MS-222 (ethyl m-aminobenzoate, methanesulfonic acid salt, Aldrich Chem. Co.).

##### *Electron microscopy*

Skin samples were taken primarily from the head, mid-dorsal flank, and tail tip regions of axolotls. Axolotl skin was fixed for 2–4 h in either 2.5 % glutaraldehyde (fixative A) or 10 % acrolein (fixative B) in 0.1 M-cacodylate buffer, pH 7.4. Postfixation was carried out in 2 % aqueous osmium tetroxide for 1 h followed by dehydration in a graded ethanol series, and embedment in Epon. Sectioning was done using a diamond knife and a Sorvall MT-1 ultramicrotome. Sections were collected on Formvar-coated, carbon-stabilized grids, stained with uranyl acetate and lead citrate, and viewed in a Philips 300 transmission electron microscope.

##### *Pigment analysis*

Pigments were identified by their absorption spectra, R<sub>f</sub> values on thin layer chromatography (TLC) plates and/or ultraviolet (u.v.) fluorescent and absorption properties as compared to standards. Analysis for each category of development was carried out on at least three animals, each from a spawning of different parents. Throughout our studies, TLC data were supported by examination (not

reported here) of approximately 80 skin samples from axolotls of various ages, genotypes and spawnings. Thus, the results presented in this and the accompanying reports (Frost *et al.*, 1984a,b,c) represent "average" phenotypes. Among wild-type axolotls very little qualitative or quantitative variation in pigments analysed was observed within each age category.

Extraction procedures were carried out in darkness or under a red safe light to prevent photodegradation of labile pteridines (Taira, 1961). Skin (fresh frozen or from freshly killed animals) was weighed and then homogenized in a two times volume of 70 % ethanol. The homogenate was centrifuged at 700 g for 10 min and the supernatant saved. The remaining pellet was rehomogenized in a one times volume of 70 % ethanol, centrifuged as above, and the supernatants pooled. A five times volume of chloroform was then added to the supernatant. This was mixed well and centrifuged at 100 g for 10 min. The ethanol layer (yellow) was removed and re-extracted with chloroform until it was clear yellow, indicating the removal of most protein and lipid.

Approximately 0.5 ml of the resulting extract was saved for TLC analysis. The remainder of the sample was concentrated (by evaporation) to 2–4 ml total volume and applied to a column (1.5×40 cm) of Bio-Gel P-2 (200–400 mesh, BioRad) that had been extensively pre-washed with distilled water. The column was eluted with distilled water and 1 ml fractions were collected in a Buchler automatic fraction collector. Fractions were monitored for u.v. absorbance at 260 nm by passing the eluent through a Beckman DU spectrophotometer equipped with a flow cell. After collection, fractions were monitored for fluorescence in a Turner 111 fluorometer equipped with appropriate primary (excitation wavelength, 365 nm) and secondary (emission wavelength, 436 nm) filters. Following P-2 separation, fractions corresponding to fluorescent peaks were pooled and analysed by TLC for purity. Those samples that contained a single fluorescent compound were evaporated to dryness and resuspended in either 0.1 N NaOH or 0.1 N HCl, and a u.v. absorption spectrum was determined in a Beckman DU-8 scanning spectrophotometer.

Fractions that contained more than one fluorescent compound were further separated on either Ecteola (Sigma) or Cellex T (BioRad) columns (1.5×40 cm) eluted with 0.1 N NaOH. Prior to the column run, the Ecteola/Cellex T was extensively washed first with 0.1 N NaOH, then with 0.1 N HCl, and finally with 0.1 N NaOH. Fractions were collected and monitored as above. Fluorescent fractions were collected, pooled, concentrated, and then run through a P-2 column to remove the NaOH. The purity of the fractions was determined by TLC, and a wavelength scan was done to obtain an absorption spectrum for each of the purified compounds.

Thin-layer chromatography was accomplished as outlined by Frost & Bagnara (1978). Aliquots (50 µl) of sample were run in one dimension (n-propanol: 7 % ammonia (2:1), solvent). The chromatogram was subsequently viewed and photographed under u.v. light (360 nm). TLC plates were then scanned in a

Turner fluorometer equipped with a TLC scanner. Commercially prepared standards of bipterin (BP), xanthopterin (XP), isoxanthopterin (IXP), pterin (2-amino-4-hydroxy-pterin, AHP), pterin-6-carboxylic acid (AHP-6-COOH), riboflavin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), guanine, guanosine, and guanosine triphosphate (GTP) were obtained from Sigma. Small amounts of D- and L-erythro-neopterin (NP) and sepiapterin (SP) were available from chemically synthesized laboratory stocks (Frost & Bagnara, 1979).

## RESULTS

### *Structural analysis of pigment cells*

Three types of pigment cells are routinely encountered in electron micrographs of axolotl skin – melanophores, xanthophores, and iridophores (Figs 1–3). Each of these cell types is easily identified by characteristic pigment-containing organelles – melanosomes, pterinosomes, and reflecting platelets, respectively.

All pigment cells observed in axolotls are best described as “differentiating.” For example, melanophores frequently contain well-developed Golgi with numerous associated vesicles, endoplasmic reticulum (ER), and a variety of different-sized and -shaped premelanosomes (Fig. 1B). These characteristics are observed in melanophores from larval, juvenile and adult axolotls and signs of differentiative activity are found both near the nucleus and in peripheral cell processes of melanophores (Figs 1 & 4). Premelanosomes are most often observed in melanophores of larval and juvenile axolotls, and less-frequently observed in adults.

Xanthophores of larval axolotls, like melanophores, also contain extensive Golgi and ER (Fig. 2). However, there are no structures in xanthophores identifiable as “pre-pterinosomes.” Instead, xanthophores are observed to contain numerous “empty” vesicles suspected to be pterinosomes in an early state of differentiation (Figs 2 & 5A) (a type I pterinosome as defined by Yasutomi & Hama, 1976). By the late-larval/early juvenile stages of development, pterinosome morphology begins to change. Although there are still “empty”-appearing pterinosomes at this stage, there are also vesicles containing fibrous material presumed to be pigment (Fig. 5A,B) (a type II pterinosome). In animals approaching sexual maturity (late juvenile/early adult), pterinosomes appear to contain considerably more fibrous material arranged in concentric whorls within the pigment vesicle (Fig. 5B) (a type III pterinosome). In yet older animals (i.e. in sexually mature, several-year-old axolotls), pterinosomes appear to “revert” back to a type I morphology, i.e., little or no fibrous material within the pterinosomes (Fig. 5C).

Iridophores are rarely encountered in young larval axolotls. These cells become more common in late-larval stages and are obvious throughout development thereafter. Cytoplasmic characteristics of iridophores from late-larval

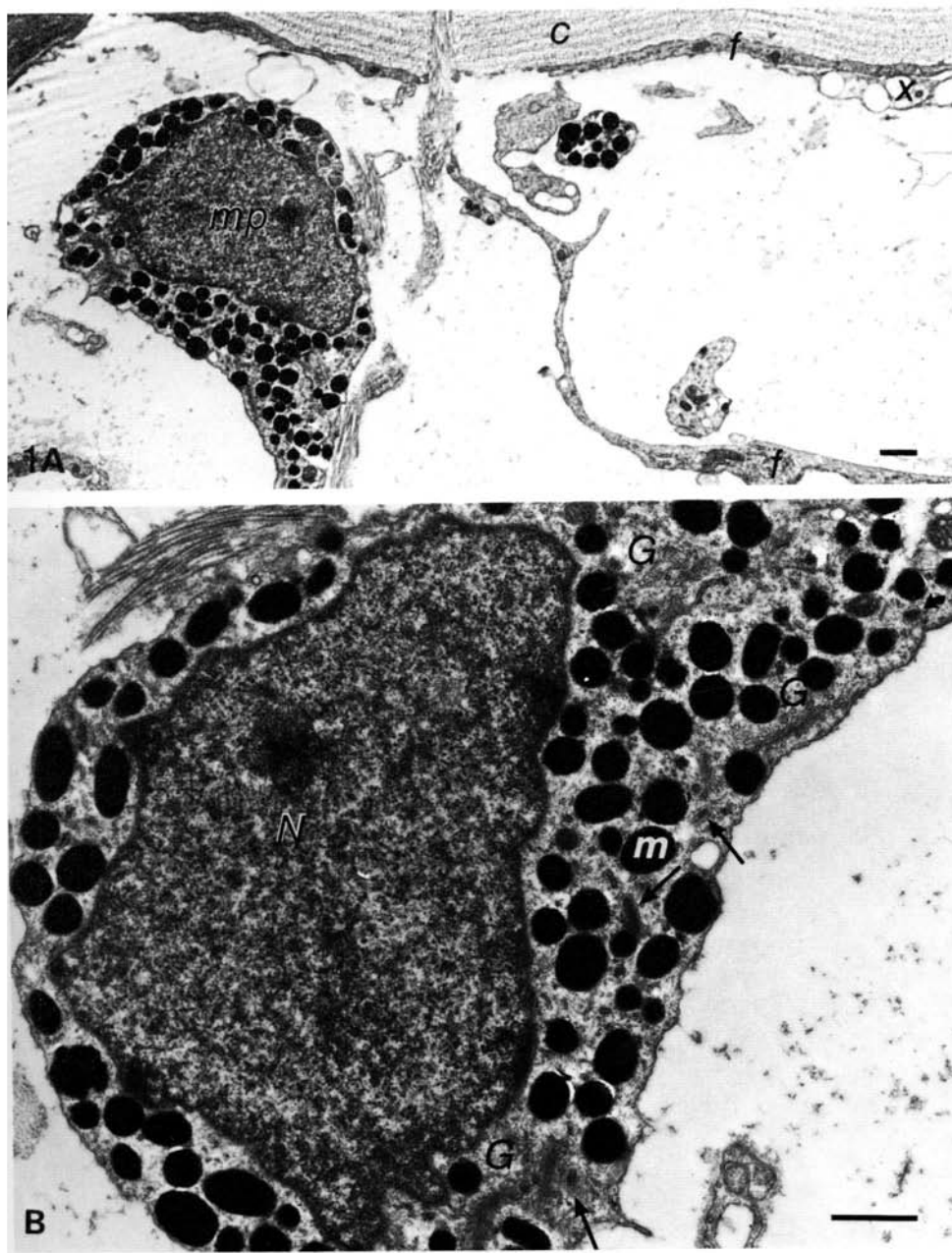
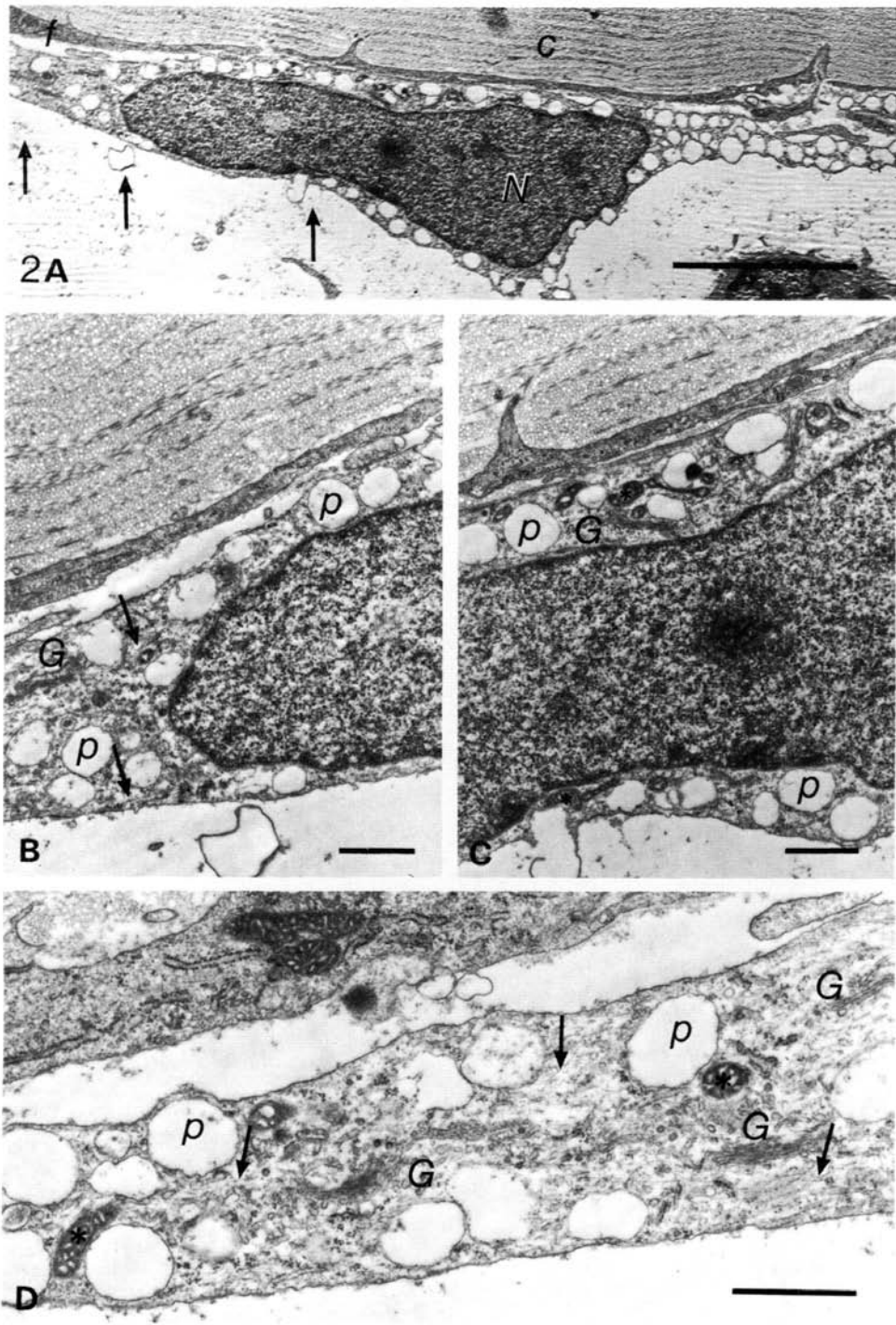


Fig. 1. (A) Low-magnification electron micrograph of a melanophore (*mp*) in larval axolotl skin. Pigment cells in larval skin are always located beneath the collagen (*c*) layer. Processes of a fibroblast (*f*) and a xanthophore (*x*) are also observed. Fixative A; bar = 1  $\mu$ m.

(B) Higher magnification view of the melanophore shown in (A) (rotated 90°). *N* = nucleus; *m* = melanosome; *G* = Golgi; arrows = premelanosomes. Bar = 1  $\mu$ m.



axolotl skin include Golgi, ER, mitochondria, numerous small vesicles and multi-vesicular bodies, intermediate filaments, and occasional amorphous-appearing vesicles that are suspected to be either intact reflecting platelets or some type of pre-reflecting platelet (Fig. 3A). Iridophore cell bodies also contain sparse numbers of randomly oriented reflecting platelets identifiable by their characteristic oblong to rectangular morphology. Mature reflecting platelets frequently appear as "holes" on micrographs, presumably as a result of loss of contents (pigments) during tissue preparation (Menter, Obika, Tchen & Taylor, 1979). Within iridophore cell processes the arrangement of reflecting platelets is more organized, usually forming stacks oriented parallel to the overlying collagen layer and basement membrane (Fig. 3B,C). In older axolotls, there are no apparent changes in iridophore morphology, but because they are more frequently encountered during sectioning of "older" skin, we believe them to be present in greater numbers. There also appear to be more reflecting platelets present in both the cell bodies and processes of iridophores from "older" skin (e.g. compare Figs 3 & 6).

The only other cells that are encountered in axolotl dermis are fibroblasts. Fibroblasts are frequently observed to be closely associated with (in young animals) or embedded within (in older animals) the collagen matrix (Fig. 7). Such cells are often completely filled with extensive rough ER and are most likely responsible for growth of the collagen layer during development.

The three types of pigment cells are primarily located in the dermis of the skin, just beneath the collagen matrix (e.g. Figs 1–3). At no time during development are cell bodies of the three types observed to overlap. Only cell processes are seen to overlap each other, and serial thin sections suggest that there is a precise arrangement of the chromatophore types within the dermis of the dorsal head, flank and tail skin, such that melanophore processes overlap either xanthophore or iridophore processes, but xanthophore and iridophore processes do not overlap each other. This implies that melanophores outnumber both xanthophores and iridophores. Because we encountered xanthophores more often than iridophores, we believe that they, too, outnumbered iridophores.

The arrangement and number of pigment cell types encountered in any given region of skin does not change throughout development. The only notable morphological change in the dermis is the observed increase in the thickness of the

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Fig. 2. (A) Xanthophore in larval axolotl skin. Arrows denote the location of the higher magnification micrographs shown in (B–D). *c* = collagen layer; *f* = fibroblast; *N* = nucleus. Fixative A; bar = 10  $\mu$ m.

(B, C) Portion of a xanthophore cell body. Note the extensive Golgi (*G*) and associated small vesicles, intermediate filaments (arrows), pterinosomes (*p*), mitochondria (\*), and endoplasmic reticulum. Bar = 1  $\mu$ m.

(D) Portion of a xanthophore cell process. Abbreviations as in (B, C) Bar = 1  $\mu$ m.

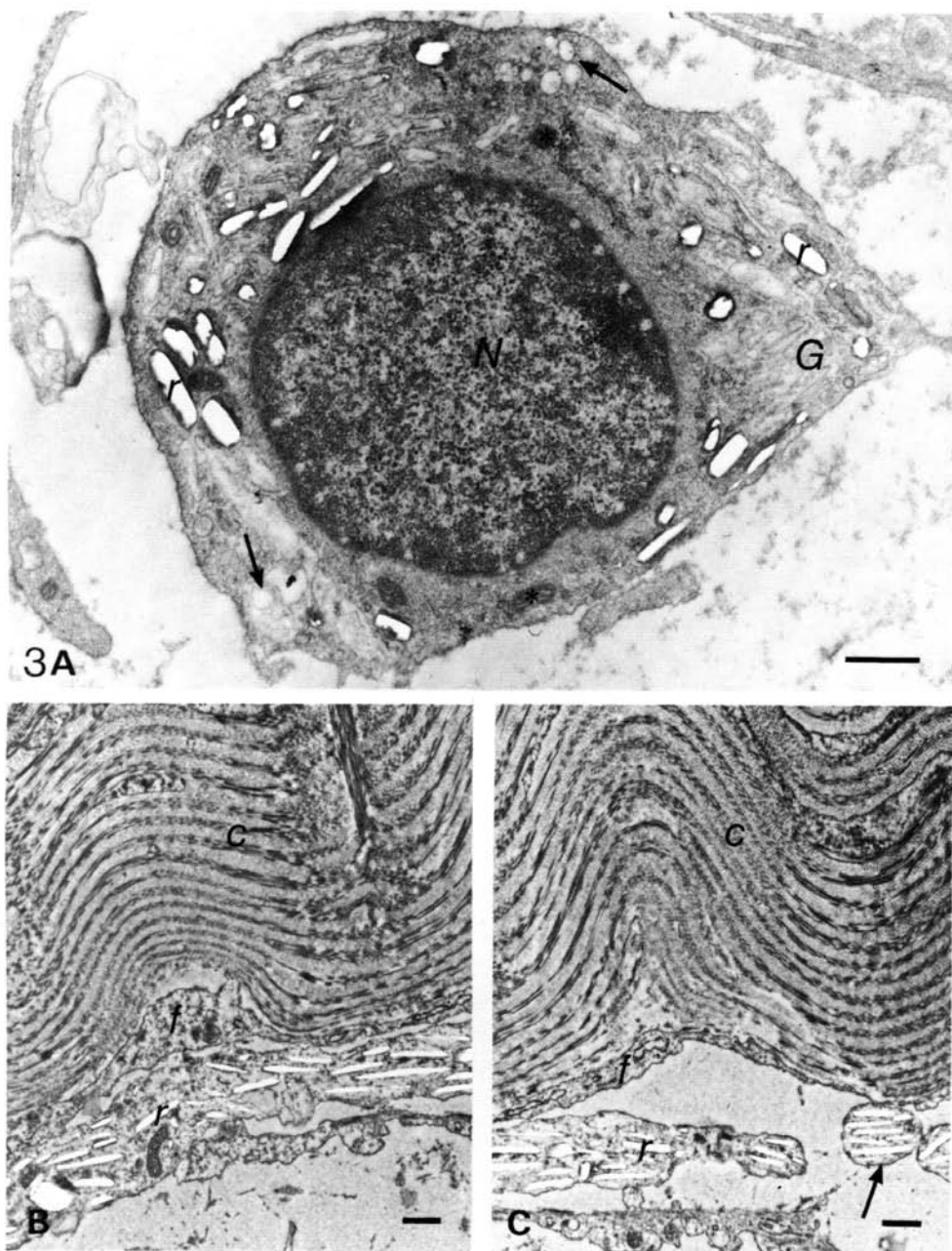


Fig. 3. (A) Iridophore in juvenile axolotl skin. This iridophore contains a nucleus (N), numerous reflecting platelets (r), Golgi (G), mitochondria (\*), and an assortment of different-sized and -shaped vesicles (arrows). Fixative A; bar =  $1\ \mu\text{m}$ .

(B,C) Iridophore processes in juvenile axolotl skin. The reflecting platelets (r) are more prominent in cell processes. These organelles are oriented parallel to the overlying collagen (c) and frequently appear to be stacked (arrow). Fixative B; bar =  $1\ \mu\text{m}$ .



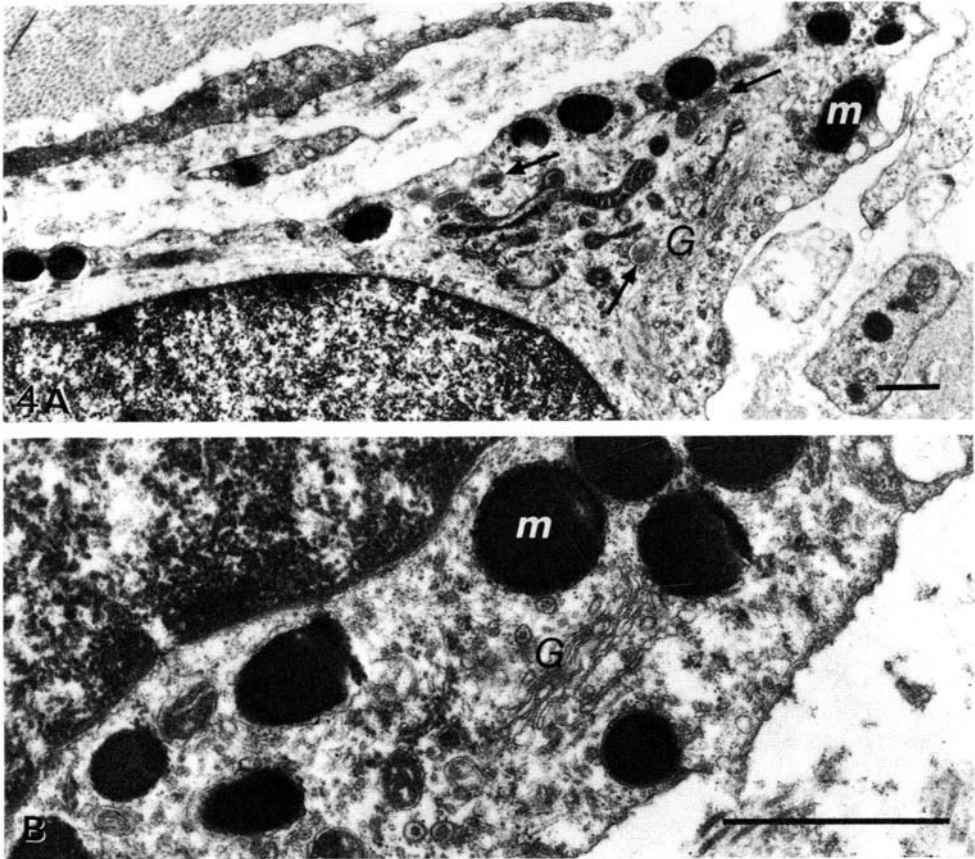


Fig. 4. (A) Melanophore in adult axolotl skin. Even in adult axolotls cytoplasmic characteristics typical of differentiating pigment cells are present (e.g. Golgi (G), small vesicles, and premelanosomes (arrows)). Fixative A; bar = 1  $\mu$ m.

(B) Higher magnification view of Golgi (G), melanosomes (m), and other cytoplasmic components of adult stage melanophores. Fixative A; bar = 1  $\mu$ m.

collagen layer, which in older animals becomes so thick that pigment cell processes and even entire cells are frequently observed to be “trapped” within the collagen matrix (compare Figs 1, 2 & 7).

### *Pigment analyses*

Each of the three chromatophore types found in axolotl skin contains a characteristic type of pigment(s): melanophores contain melanin, iridophores purines, and xanthophores pteridines (Obika, 1963; Bagnara & Hadley, 1973). We made no attempt to analyse melanin or purine pigments in the axolotl for reasons detailed in the Discussion. However, because obvious developmental changes in organelle morphology occur in pterinosomes we did analyse pterinosome pigments (pteridines).

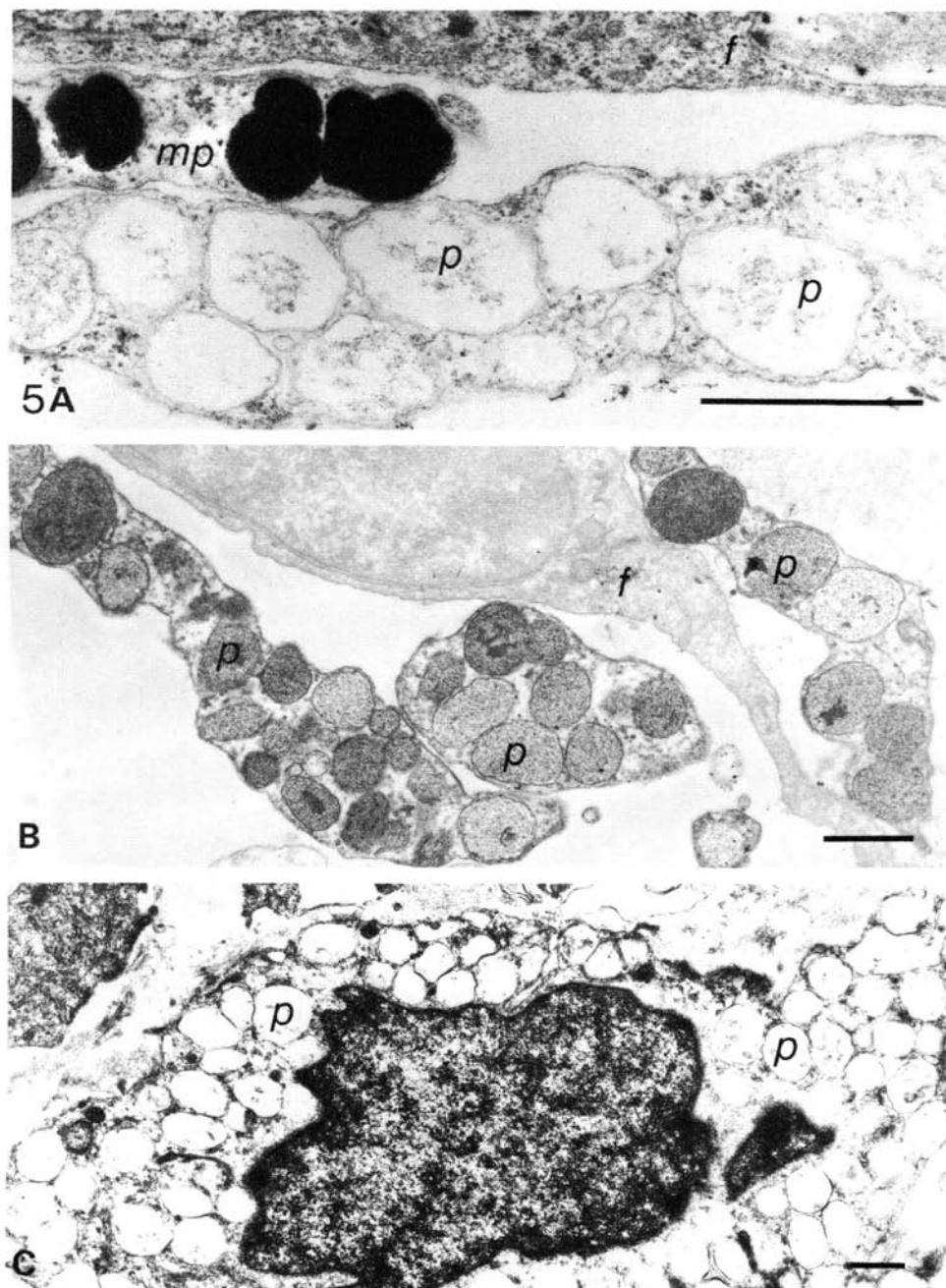


Fig. 5. (A) Pterinosomes (*p*) in a larval stage axolotl xanthophore process. Note also the melanophore process (*mp*) and fibroblast process (*f*). Fixative B; bar = 1  $\mu$ m.

(B) Pterinosomes (*p*) in juvenile stage axolotl xanthophore processes. *f* = fibroblast. Fixative B; bar = 1  $\mu$ m.

(C) Xanthophore in adult stage axolotl skin. Compare the pterinosomes (*p*) in (A), (B) and (C). Fixative A; bar = 1  $\mu$ m.

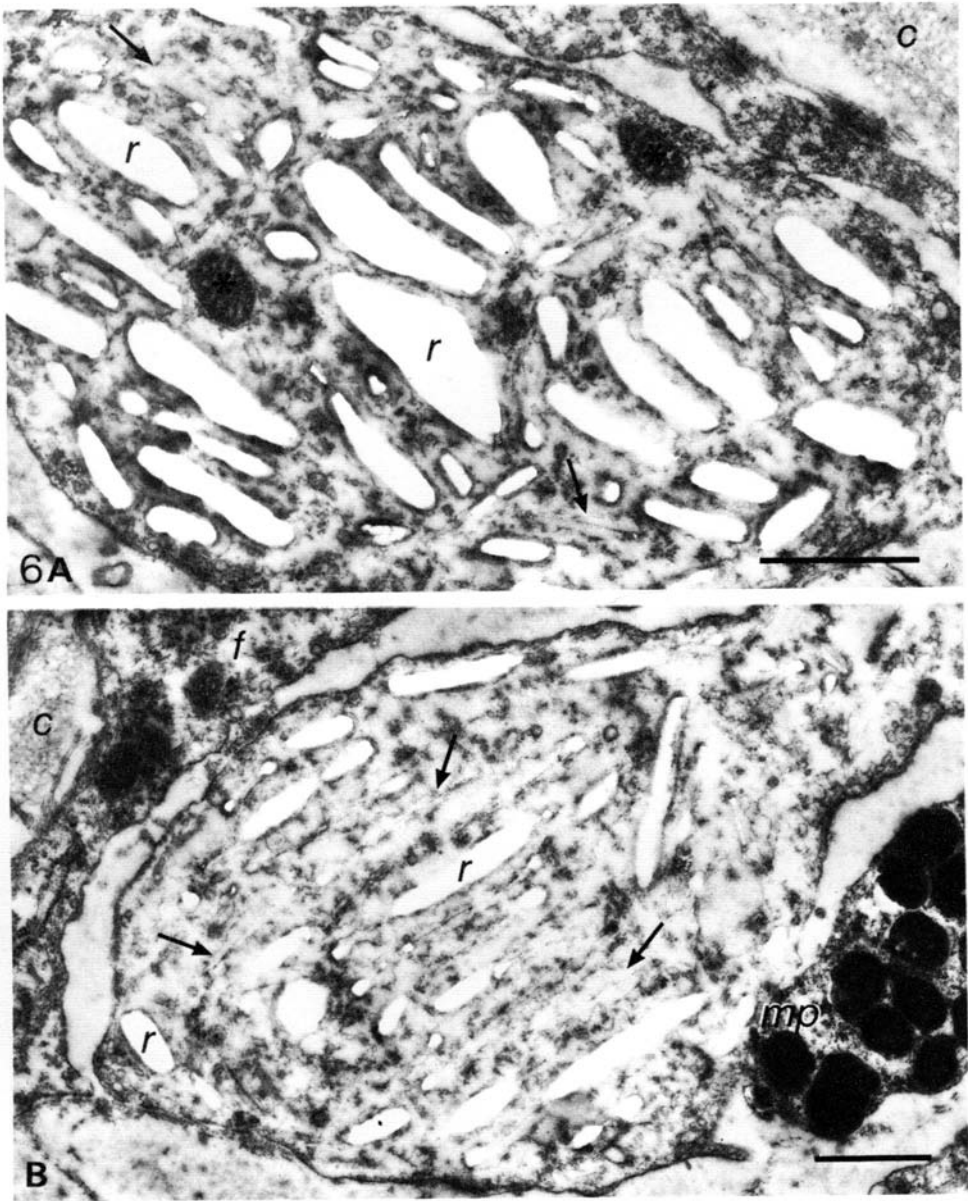


Fig. 6. (A,B) Iridophore processes in adult axolotl skin. The reflecting platelets (*r*) are stacked in a loosely organized fashion. Arrows denote platelets that have not lost their contents during tissue preparation. \* = mitochondria; *mp* = melanophore process; *f* = fibroblast process; *c* = collagen. Fixative A; bar = 1  $\mu$ m.

An example of the array of u.v. fluorescent compounds extracted from larval and adult stage skin is shown in Fig. 8, which represents a side-by-side TLC comparison of larval and adult skin extracts. Pteridines appear as fluorescent

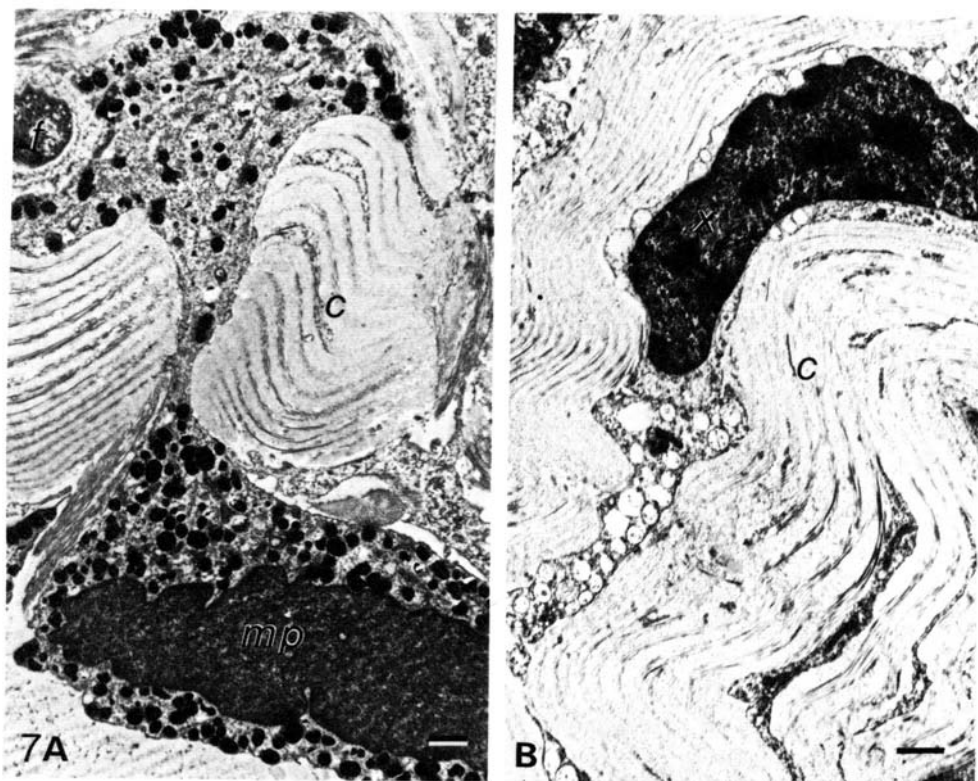


Fig. 7. (A) Melanophore (*mp*) in adult axolotl skin. Note that this cell is partially embedded within the collagen matrix (*c*) of the dermis. *f* = fibroblast. Fixative A; bar = 1  $\mu$ m.

(B) Xanthophore (*x*) in adult axolotl skin. This cell is completely embedded in the collagen matrix (*c*). Fixative A; bar = 1  $\mu$ m.

bands (white bands on a black background; Fig. 8A), each possessing a distinct fluorescent colour (e.g. yellow, green, blue-green, blue, or violet). When this chromatogram is scanned the profiles shown in Fig. 8B are obtained. Because initial skin extracts are prepared on a fresh-weight basis, the aliquots of sample applied to TLC plates should be roughly equivalent amounts. Therefore, the profiles shown in Fig. 8B represent both qualitative and approximate quantitative differences between pigments present at these two developmental stages. We realize that quantitative comparisons are, at best, estimates in this case. However, similar results were obtained each time an extract was prepared and at least three separate extracts were prepared for each age group suggesting that quantitative comparisons at least approximate real values.

The qualitative differences between larval and adult pteridine patterns are much more striking (Fig. 8B; summarized in Table 1). Seven pteridines and one flavin were identified from larval skin extracts: NP, AHP, IXP, XP, BP, SP, AHP-6-COOH, and (relatively) small quantities of riboflavin. Adult axolotl skin

extracts contain NP, AHP, IXP, XP, trace amounts of BP, and large quantities of riboflavin. There is no detectable SP or AHP-6-COOH in adult skin.

Representative examples of the initial P-2 column separation of these pteridines are illustrated in Fig. 9. Fig. 9 shows that four prominent fluorescent peaks are obtained from larval skin extracts. The first (and largest peak), when analysed by TLC, is found to contain AHP, IXP, NP, BP, and AHP-6-COOH. This peak can be subdivided into three regions based on the fluorescent colour of the fractions obtained. The first four to six fractions fluoresce blue-violet and

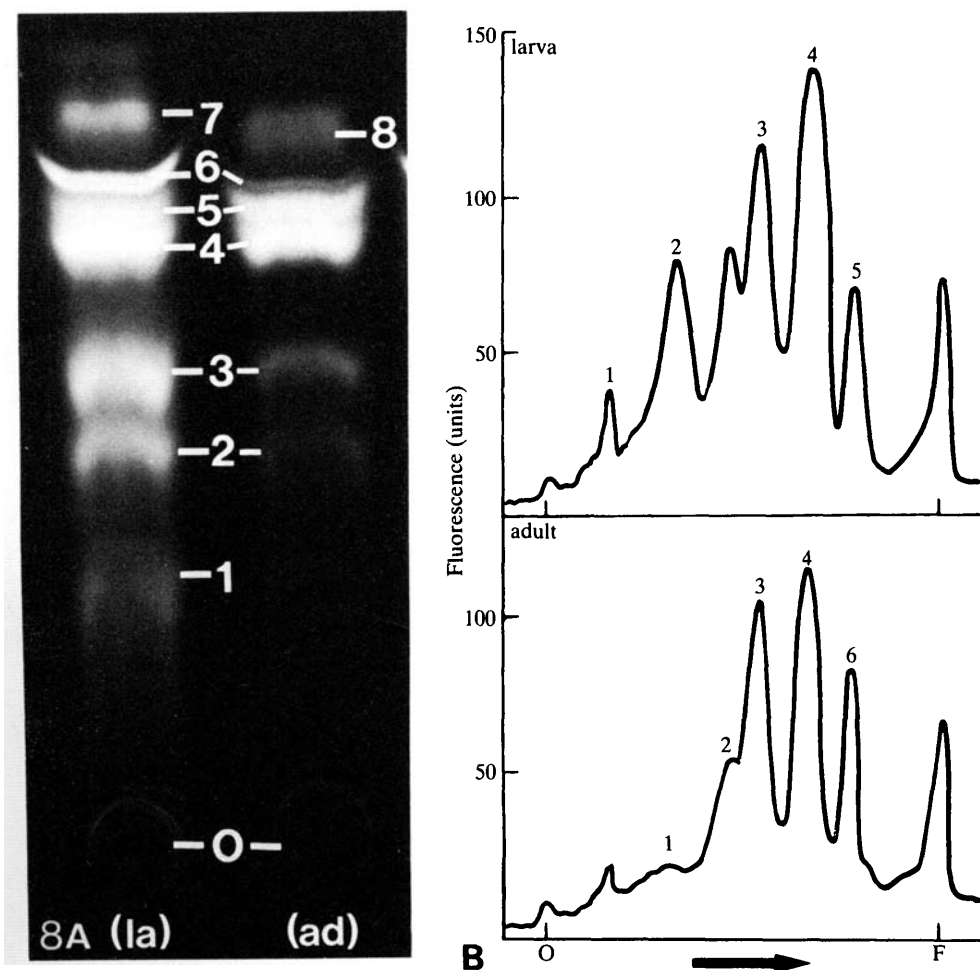


Fig. 8. (A) Photograph of a one-dimensional thin-layer chromatographic separation of ethanol-extracted pteridine pigments from larval (la) and adult (ad) stage axolotl skin. 0 = origin; 1 = AHP-6-COOH; 2 = XP; 3 = IXP; 4 = NP; 5 = AHP; 6 = BP; 7 = SP; 8 = riboflavin (see text for explanation of abbreviations).

(B) Fluorometric scans of the chromatographic results shown in Fig. 8A. Numbered peaks contain the following pteridines. 1-AHP-6-COOH; 2-XP; 3-IXP; 4-NP, AHP, BP; 5-SP; 6-riboflavin. 0 = origin; F = solvent front.

Table 1. (A) *Relative quantitative estimates of the pteridines (and riboflavin) extracted from larval and adult axolotl skin, and (B) absorption maxima ( $\lambda_{\max}$ ) for each compound (see text for abbreviations).*

(A)	Pteridine							
	Age group	NP	AHP	IXP	XP	BP	SP	Riboflavin
	Larva	+++*	+++	+++	++	+++	+++	-/+
	Adult	+++	+++	++	+	+	-	+++
* Plus signs denote relative quantity of pigment extracted; minus signs denote no pigment detected (see chromatographic results in Fig. 8).								
(B)	$\lambda_{\max}$ (nm)							
	Solvent	NP	AHP	IXP	XP	BP	SP	Riboflavin
	0.1 N NaOH	354, 260, 220	360, 250, 221	—	—	—	430, 267, 215	438, 362, 260
	0.1 N HCl	—	—	345, 275, 217	354, 260, 227	—	—	—

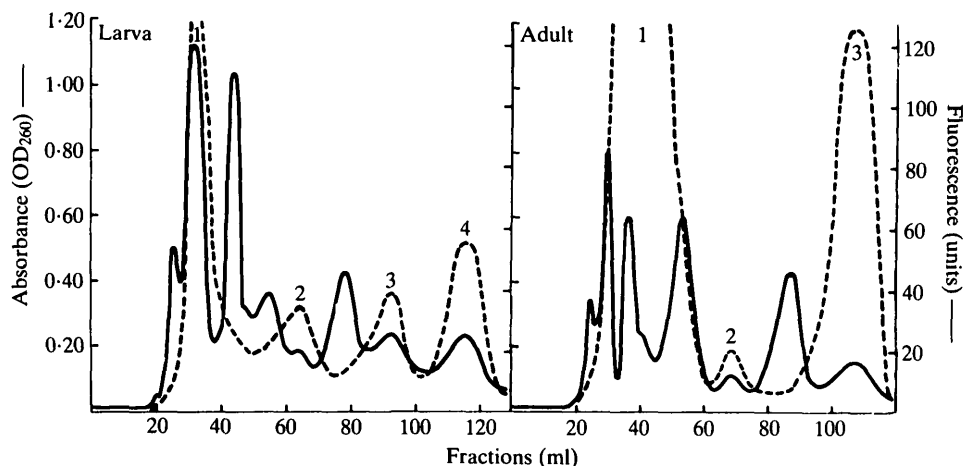


Fig. 9. Column chromatographic (Bio-Gel P-2) separation of ethanol-extracted pigments from larval and adult axolotl skin. Fluorescent peaks contain the following pteridines. In the larva: 1-AHP, IXP, NP, BP and AHP-6-COOH; 2-XP; 3-SP; 4-riboflavin. In the adult: 1-AHP, IXP, NP; 2-XP; 3-riboflavin. See text for explanation of abbreviations and experimental details.

have been determined by additional column separations and further TLC characterization (as described in the Methods) to contain IXP and AHP. The middle four to six fractions fluoresce blue-white, and contain BP, NP, AHP, AHP-6-COOH, and a strong u.v.-absorbing compound that co-migrates with GTP standards on TLC plates. Both NP and AHP can be separated from this peak in pure enough form to allow for identification by absorption spectra. However, both BP and AHP-6-COOH were identified only by TLC mobility and fluorescent properties, as neither of these two compounds could be separated well enough from contaminating GTP (and probably other u.v.-absorbing compounds, as well) to allow for unequivocal spectral measurements. The remaining four to six fractions of the peak contain primarily AHP and trace amounts of XP (blue-green fluorescence).

The second and third peaks run very close to each other but can be separated on the basis of u.v.-fluorescent colour. Green-fluorescing fractions contain primarily xanthopterin; yellow-fluorescing fractions contain sepiapterin. The final peak also fluoresces yellow under u.v. light, but much less intensely than the SP peak. This peak contains riboflavin exclusively. Another difference between SP and riboflavin is that SP, on exposure to either u.v. or visible light, degrades rapidly to a blue-fluorescing, colourless compound (AHP-6-COOH). Riboflavin also fades dramatically on exposure to light but does not change fluorescent colour.

Three fluorescent peaks are obtained from P-2 column separations of adult skin extracts. The first peak is very broad and can be separated into three regions (as in the larva, Fig. 9), fluorescing blue-violet, blue-white, and blue-green. By

further analysis, as above, these are found to contain IXP+AHP, AHP+NP, and AHP+XP, respectively. The second P-2 peak contains XP only. The third peak contains large quantities of yellow-fluorescing riboflavin.

#### DISCUSSION

Our studies of the axolotl pigmentary system did not involve chemical analysis of melanophores. At the present time there seems to be no single definitive structure for any of the melanins. Consequently, melanins have been grouped into two classes based on differential solubility, chemical composition, and colour (see Pawelek & Korner, 1981, for a review). The pigments of black or brown animals are eumelanins; those of yellow or red animals are phaeomelanins. Based on both ultrastructural and light microscopic observations, it seems clear that axolotl melanophores contain only eumelanins. Thus, quantitative measurements of melanin pigments would not likely be of much use other than to confirm the observation that as the number of melanophores increases with axolotl age and size, so too does the melanin content.

Similarly, because there are no observable structural changes in iridophore organelles that might suggest chemical pigment changes during development, and because of the relative scarcity of iridophores in axolotl skin, we made no specific attempts to isolate and characterize iridophore pigments (purines). Except for the purine derivative, GTP, other soluble purines were not identified by our methods, although significant amounts of u.v.-absorbing compounds, probably purines, are detectable in P-2 column separations (see Fig. 9). These frequently interfere with spectral measurements.

Dalton & Hoerter (1974) report that six u.v.-absorbing compounds, presumably purines, can be extracted from the iridophore-enriched peritoneum of wild-type axolotls. Our experiments involved extraction and structural analysis of dorsal skin, which we found to be sparsely populated with iridophores. We chose not to include an analysis of peritoneum in our studies for three reasons: (1) the features of dorsal skin are those that most prominently figure in animal colouration, (2) the dorsal skin provides the phenotypic characteristics that distinguish wild-type axolotls from axolotls carrying pigment mutations, and (3) it may be that development of pigmentation in peritoneum is not comparable to that in dorsal skin.

Dalton & Hoerter (1974) further suggest that age-specific differences in purine patterns do not occur. This may not be the case since it is unclear how many age groups they tested, and of the two that were mentioned (85 and 125 days), both would have been within a single age group based on our criteria (see Materials and Methods). Dalton & Hoerter also could not assign identities to most of their extracted compounds, and many of these may have been phosphorylated derivatives of purines. Thus, the determination of whether biochemical changes in purine pigments do occur during axolotl development awaits further study.



Perhaps the best approach to examining purine synthesis in axolotl iridophores is to examine axolotls that are believed to overproliferate iridophores (a suspected iridophore mutant; see Frost & Malacinski, 1980).

The obvious structural changes in pterinosomes from "young vs. old" axolotls suggests that there might be either qualitative and/or quantitative differences in xanthophore pigments. Our data on pteridines substantiate both ideas. Most importantly, we found that adult axolotls contain two fewer pteridines – SP and AHP-6-COOH – than do larvae. Sepiapterin, a yellow pigment, is the only visibly coloured pteridine (the others are colourless) present in axolotl skin. This pigment is most likely responsible for the obvious yellow colour of larval xanthophores and also for the greenish colour that is observed in older axolotl skin.

Pterin-6-COOH is believed to arise as a degradation product of SP (Taira, 1961). Thus the absence of this compound from adult skin is not unexpected and its presence in larvae suggests that even greater quantities of SP exist *in vivo* than our analyses detected. The missing pteridines in adult axolotls suggest that a particular branch in the pteridine biosynthetic pathway has somehow been altered. Current thinking concerning pteridine biosynthesis is that all pteridines derive from GTP as a common precursor. GTP is enzymatically converted to neopterin-triphosphate (NP-PPP). Here the pathway splits. One branch leads to the synthesis of BP, SP, and the drosopterins (these latter [red] pigments are not present in the axolotl). Another branch leads to the dephosphorylation of NP-PPP to NP and its subsequent conversion to other unconjugated pteridines such as AHP, IXP and XP (see Frost & Malacinski, 1980, for a brief discussion of pteridine biosynthesis). Adult axolotls apparently convert only small amounts of NP-PPP to BP and none to SP. Larval axolotls synthesize both compounds in significant quantities.

It is also notable that of all the u.v.-absorbing purines (and purine-like compounds) that are likely to be present in skin, we found only GTP to be consistently present in all skin extracts. This suggests that GTP pools may be greater than might be expected for its normal metabolic roles, and that "excess" GTP might in fact be localized in xanthophores for pteridine biosynthesis.

The observed morphological changes in pterinosomes correlate well with the chemical data. Pterinosomes in very young larvae are typically "empty" and resemble "type I" pterinosomes as described by Yasutomi & Hama (1976). Pterinosome differentiation proceeds to "types II and III" concomitant with increases in BP and SP synthesis in late larval and juvenile animals. Pterinosomes revert to a "type I" morphology in adults, coincidentally with reduced BP levels and the elimination of SP. It may be that the absence of SP in adult xanthophores accounts for the absence of a well-organized internal structure in pterinosomes.

Yasutomi & Hama (1976) suggest that their pterinosome "types" are equivalent to stages in pterinosome differentiation. However, an alternative

explanation of differences in pterinosome morphology may be more accurately reflected by the pigment composition of the organelles, rather than solely by their "state of differentiation." Clearly, these two phenomena, pterinosome differentiation and pigment composition, may be so closely interrelated in most animals that they cannot be separated. The axolotl, by virtue of its unusual life history, may be a unique exception.

One final notable feature of adult axolotl skin is that it contains much greater quantities of extractable riboflavin than larval skin. The significance of this is, however, not clear. Although flavins are not unusual components of skin (Bagnara & Hadley, 1973), it is not known where they may be located in skin. Riboflavin may be in xanthophores, melanophores (Obika & Negishi, 1972; Obika, 1976), or perhaps in all cells of the skin. In any case, the drab green colour of aging axolotls must be due to a yellow pigment somewhere in the skin, and because SP is absent in adult axolotl skin, riboflavin would be the logical candidate for this colour-producing role.

Our ultrastructural studies demonstrate that certain larval characteristics of skin are retained throughout the life of this neotene. This is especially true regarding the arrangement of pigment cells. During metamorphosis in many amphibians, the pigment cells of the dermis are reorganized into a physiologically responsive, colour-producing dermal chromatophore unit (Bagnara Taylor & Hadley, 1968). The stacked arrangement of pigment cells, together with the pigments present in such cells are ultimately responsible for the observed colour of the animal (Bagnara *et al.*, 1968; Frost & Robinson, 1984).

The axolotl never displays any tendency toward reorganizing chromatophores into discrete dermal units. Instead, the pigment cells of axolotls remain more or less randomly scattered throughout the dermis. As aging progresses, pigment cells frequently come to lie wholly or partially embedded within layers of collagen (e.g. see Fig. 7). We view this inability of chromatophores to rearrange within the dermis to be the retention of a larval feature. This idea is supported by similar studies on the developmental cytology of newt skin (Forbes, Zaccaria & Dent, 1973) wherein pigment cell types were observed to reorganize and intermingle in the dermis of both older larvae and, following metamorphosis, in adults.

Other characteristics of the axolotl chromatophore system that may represent persistent larval features include the apparent retention in the adult of cellular characteristics that indicate continued differentiative capabilities (e.g., well-developed and extensive Golgi and prepigment organelles such as premelanosomes), and an apparent numerical hierarchy with respect to the numbers of chromatophores present in skin (i.e., melanophores > xanthophores > iridophores). The latter point is perhaps a reflection of the well-known ontogenetic sequence of chromatophore development in amphibians (Bagnara & Hadley, 1973; Forbes *et al.*, 1973), wherein melanophores appear earliest and clearly predominate during larval development. Later, xanthophores, and then iridophores, appear. After metamorphosis and the establishment of an adult

pigment pattern, differences in numbers of chromatophore types may become indiscernible, especially in areas of skin where particular colour patterns become established (see e.g. Forbes *et al.*, 1973). This is not the case in the axolotl, and it seems that increases in chromatophore number are simply to keep pace with growth.

#### CONCLUDING STATEMENTS

The aspects of the pigmentary system of the axolotl discussed in this study are, like the life history of this animal, unusual. Most of the unusual features of this system may result either directly or indirectly from an inability, under normal circumstances, to metamorphose. For example, axolotls are, by our experience, relatively "slow developers." This perceived "slowness to develop" is enhanced by the neotenic nature of these animals, and by the ontogenetic sequence that is maintained by chromatophores throughout development. This ontogenetic sequence of pigment cell appearance dictates that in many amphibians, numbers of one pigment cell type (melanophores) clearly predominate, at least for a time, during larval development. In the axolotl, this condition seems to be maintained throughout development. There are never great numbers of pigment cells present in any small region of skin. The chromatophores that are present fail to organize themselves into functional units as is seen subsequent to metamorphosis in many amphibians (Bagnara *et al.*, 1968).

The scarcity of iridophores in general, the apparent "reversion" of pterinosomes and "loss" of SP in adult xanthophores, and the ever-darkening colour of aging axolotls further suggest that the maintenance (and perhaps normal proliferation) of iridophores and xanthophores may depend to some extent on the process of metamorphosis. Whether this assumption is correct awaits further experimentation that must include an assessment of what happens to pigment cells when axolotls are induced to metamorphose.

Obviously, many questions remain to be answered concerning the development of pigmentary systems in general. Because the axolotl provides both genetic mutants (see Frost & Malacinski, 1980, and the accompanying reports, Frost *et al.*, 1984*a,b,c*) and a system that can be artificially controlled with respect to metamorphosis (e.g. Prahlad & DeLanney, 1965), we anticipate that the axolotl pigmentary system may provide important clues to both the normal and abnormal function and development of pigments and pigment cells.

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