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Heterogeneous ultrastructure of melanosome formation in the goldfish induced by osmotic stress

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Summary. In this study, melanophore cyto-differentiation in the fins of xanthic goldfish that had been exposed to osmotic stress for 18 days was investigated. It was found that multi-vesicular bodies (MVB) are not the only type of premelanosome. Granules having a homogeneous matrix also function as premelanosomes.

The presence of acid phosphatase reaction product inside the melanin granules indicated that these organelles in this animal were also related to lysosomes. DOPA-oxidase of tyrosinase, the key enzyme in melanogenesis, was surprisingly not only detected in melanocytes but also in the Golgi stacks of dermal cells.

Due to the mechanisms of premelanosome formation it is evident that cytoplasmic material also serves as substrate for melanogenesis.

EDX microanalysis was performed to measure the ionic composition of the melanin granules. After aldehyde fixation the newly-formed melanin granules did not contain Na, but had accumulated Ca.

Key words: Electron microscopy, Lysosome, X-ray microanalysis, Melanin, Tyrosinase

Introduction

The goldfish *Carassius auratus* is very useful for studying factors causing melanogenesis. Any chromatic alteration may be readily detected in these fish normally lacking melanin the skin. Goldfish have been placed in saline solutions (Fukui, 1927; Graupner, 1933; Chavin, 1956; Turner et al., 1975), exposed to cold (Fukui, 1927), heat (Osterhage, 1932), injured mechanically in various ways (Smith, 1931; Osterhage, 1932), blinded (Rasquin, 1946), exposed to roentgen radiation (Smith, 1932; Davison and Ellinger, 1942) and to UV radiation (Goodrich and Trinkhaus, 1939), and injected with various chemicals and drugs (Oesterhage, 1932). All of these treatments have induced melano-genesis. Although it has been shown that the action of the pituitary-adrenal mechanism is involved (Chavin, 1956) the further basic mechanisms which elicit melanin synthesis have not been determined. The ultrastructural changes leading to melanin granule production induced by osmotic stress in this animal model have been described by Turner et al. (1975).

As a consequence of the growing body of evidence that melanosomes are special types of lysosomes (Toda and Fitzpatrick, 1972; Novikoff et al., 1979; Boissy et al., 1987; Vijayasarahi et al., 1991; Schraermeyer, 1992; Orlow et al., 1993; Smit et al., 1993; Winder et al., 1993; Zhou et al., 1993; Luo et al., 1994; Schraermeyer and Stieve, 1994; Diment et al., 1995; Orlow, 1995) melanogenesis in goldfish was reinvestigated involving cytochemistry for acid phosphatase, the marker enzyme of lysosomes.

Because it is known that melanin has a high affinity to various metal ionic species (White, 1958; Potts and Au, 1976) X-ray microanalysis was used to investigate whether the newly-formed melanin granules could accumulate the applied sodium ions.

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Materials and methods

Conventional electron microscopy

Xanthic goldfish *(Carassius auratus* L.), 5 cm in length and free of visible melanophores, were stressed by placing them in water containing 0.9% NaCl as described by Chavin (1956). The animals were maintained under a 12 hr light/12 hr dark photoperiod at 20-23 °C.

Four animals that had become pigmented were anaesthetized in a 1:2000 solution of 3-aminobenzoic acid ethyl ester (MS222) (Sigma Deisenhofen Germany) 18 days after having remained in the salt solution. Pigmented pieces of the fins were excised and fixed in 5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2, osmicated (1% OsO_4 for 2 h), block-stained with uranyl acetate, dehydrated in a graded ethanol series and embedded after overnight incubation in Spurr's resin.

Semithin sections were examined under a Leitz Orthoplan light microscope after staining with toluidine blue. Ultrathin sections were investigated under a Philips EM 300 microscope.

Localization of acid phosphatase activity

Pigmented pieces of fins were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) containing 0.05% DMSO (dimethyl sulphoxide) for 2 h. For ultrastructural localization of acid phosphatase (ACPase) Gomori's medium as modified by Barka and Anderson (1962) was used. Specimens were immersed in the solution containing 1 mM beta-glycerophosphate (Sigma) as substrate in 0.1M tris maleate buffer (pH 5) and 2.4 mM $Pb(NO_3)_2$ for 1 h on a shaker at room temperature. After two rinses in tris maleate buffer (pH 5, Sigma) fin pieces were postfixed in 1% OsO_4 in 0.1M sodium cacodylate buffer (pH 7.2) for 2 h and were dehydrated and embedded as described above. Controls were tested for non-specific reaction product by omitting the substrate, beta-glycerophosphate, from the medium. Ultrathin sections were examined both unstained and after poststaining with uranyl acetate and lead citrate.

Localization of DOPA-oxydase activity

For ultrastructural localization of Dopa-oxidase activity of tyrosinase pigmented fin pieces were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 1 h. A routine procedure for ultrastructural localization of tyrosinase was used (Slominski et al., 1989). Specimens were washed twice in sodium cacodylate buffer and kept at 4 °C overnight in this buffer containing 5 mM L-3,4-dihydroxy-phenylalanine (L-DOPA) (Sigma). After this, these solutions were renewed, and the fin pieces were incubated for an additional 5 h at 37 °C. The reacted fin pieces were washed with sodium cacodylate buffer and immersed for 1 h at room temperature in the same buffer containing a mixture of osmium tetroxide (1%) and potassium ferrocyanide (1.5%). Finally, the specimens were dehdyrated and embedded for electron microscopy as described for acid phosphatase activity. Ultrathin sections were examined without and after poststaining with uranyl acetate and lead citrate.

X-ray microanalysis

For X-ray analysis unstained sections were used on nickel grids from blocks that had been embedded as described for routine microscope. X-rays were detected using a Si-Li drifted crystal detector connected to a JEM 2000 FX II electron microscope with an EDX Tractor TN 5502. The electron beam was focused on a 100 nm spot during measurement. Spectra were taken from the melanin matrix, the electron lucent part surrounding the melanin matrix and the pigment cell cytoplasm.

Results

Melanocytogenesis of all fish exposed to the 0.9% NaCl solution could be observed from twelve days on at the distal portions of the dorsal, pectoral and caudal fins.

Conventional electron microscopy

The formation of multivesicular bodies (MVB), which are precursors of melanin granules (Turner et al., 1975), was not only observed in the pigment cells but also in the dermal cells (Fig 1). In the extracellular space between dermal cells groups of vesicles were observed (Fig 2). The extracellular MVB lacked a common limiting membrane (Fig 2). Additionally, lamellar structures sometimes associated with vesicles were found in the extracellular space (Fig 3) in dermal and pigment cells (Fig 4). Melanin formation also took place in granules having a homogeneous matrix (Fig 5). Aggregates of 20 or more individual melanin granules were observed in melanocytes (Fig 6). Such accumulations of melanin were detected at the outer side of the fins (Fig 7) and seemed to be released into the water.

Ultrastructural localization of acid phosphatase activity

Electron dense reaction product of acid phosphatase was observed below the limiting membrane of melanin granules (Fig 8) and in the central melanin matrix, in Golgi stacks of dermal cells and melanocytes (not shown). Very strong activity was found in the cytoplasm of some dermal cells that were directly located to the external watery environment of the animals (Fig 9). After omitting the substrate we observed no electron

Fig. 1. A multivesicular body (arrow) which is a precursor of the melanin granule is also present in the dermal cells. The tissue has been incubated with DOPA. Electron dense reaction product of DOPA-oxidase is shown in the MVB, vesicles (V) and also in the Golgi apparatus (GO). This dermal cell is at the skin surface. The space where the water is normally found is indicated by an asterisk.

Fig. 2. In the extracellular space between dermal cells a group of vesicles (arrow) lacking a limiting membrane is observed by conventional electron microscopy.

Fig. 3. Additional lamellar structures (arrows) sometimes associated with vesicles (arrowhead) are found in the extracellular space between dermal cells by conventional electron microscopy.

Fig. 4. A large lamellar structure containing several smaller lamellar subunits (arrowheads) is present in a melanocyte shown by conventional electron microscopy

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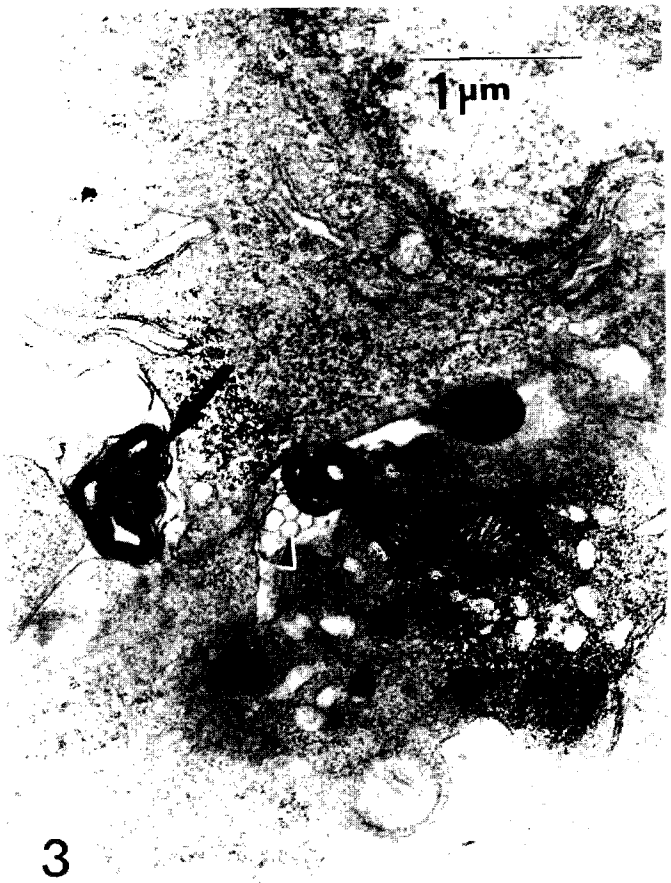

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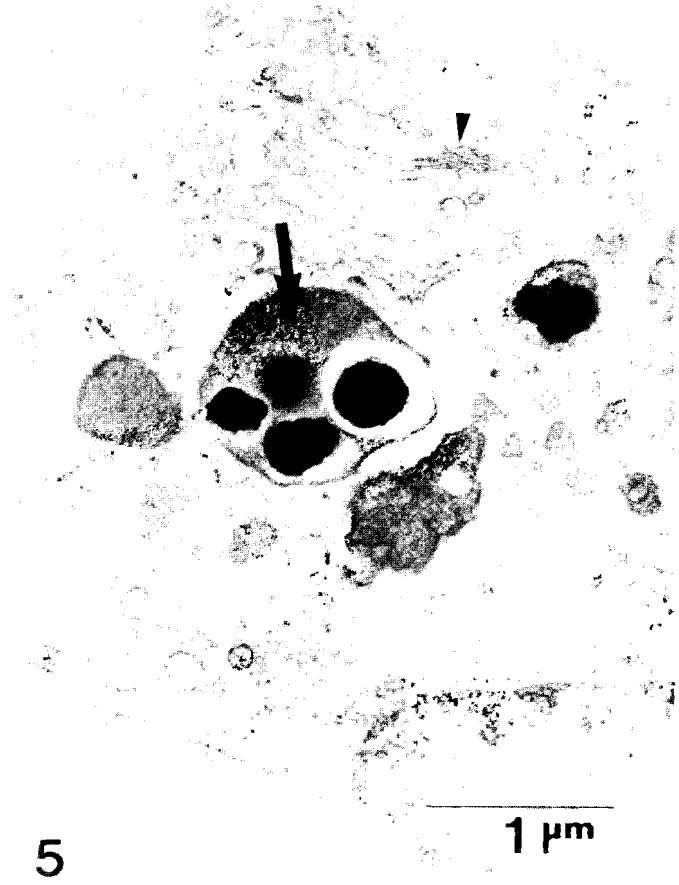

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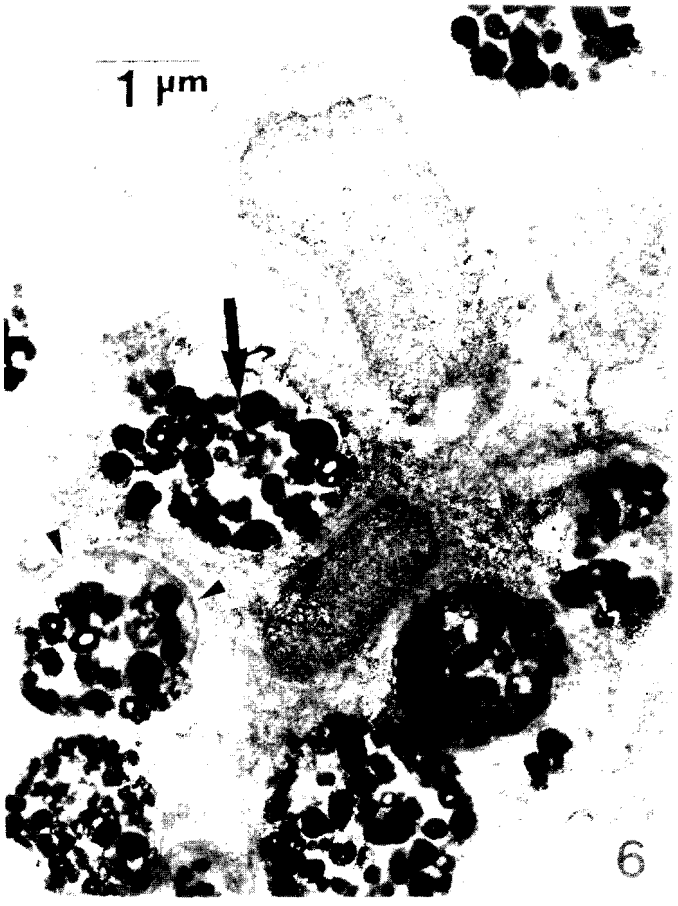

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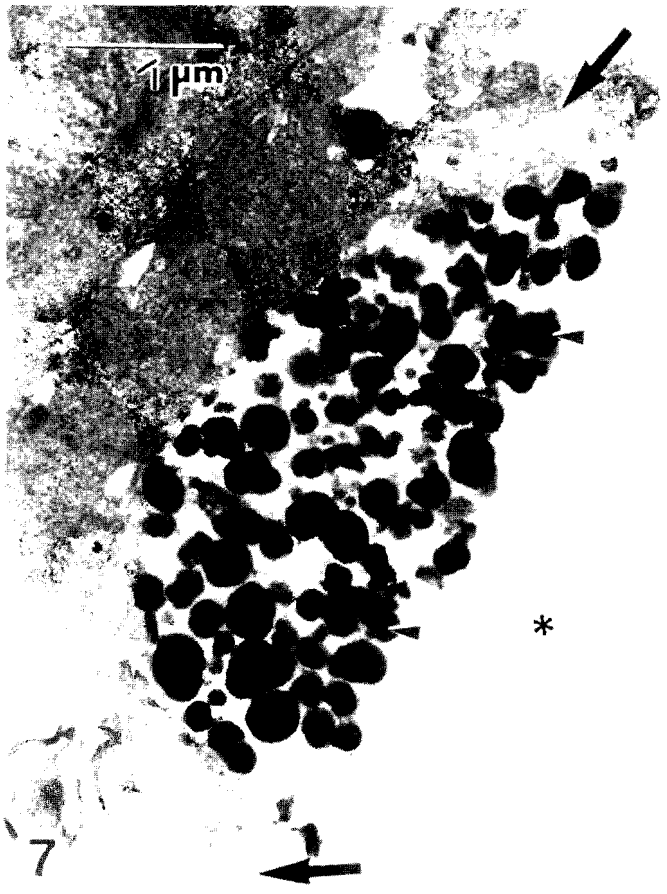

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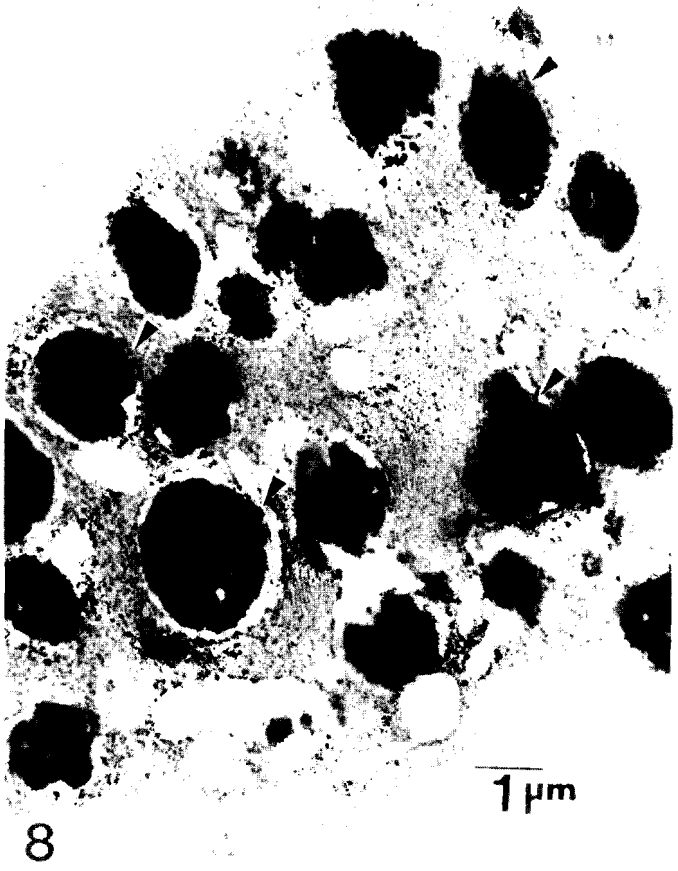

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Fig. 5. Melanin formation also takes place in melanocyte granules having a homogeneous matrix (arrow) of a melanocyte. Golgi-stacks shown by conventional electron microscopy are indicated by an arrowhead.

Fig. 6. Aggregates of 20 or more individual melanin granules with a common limiting membrane (arrowheads) or without such a membrane (arrow) are observed within the melanocytes by conventional electron microscopy.

Fig. 7. Accumulations of melanin (arrowheads) are detected by conventional electron microscopy adhering to the outer side of the fins together with some cellular debris (arrows) and seem not to be released into the water by secretion but as a consequence of cell death. The place normally occupied by the aqueous environment of the fish is indicated by an asterisk.

Fig. 8. Electron dense reaction product of acid phosphatase is observed below the limiting membrane (arrowheads) and in the matrix of melanin granules within melanocytes.

dense reaction product of acid phosphatase.

Ultrastructural localization of DOPA oxidase activity

Electron dense reaction product of DOPA oxidase was observed in Golgi stacks, vesicles and melanin granules of dendritic melanocytes (Fig 10). Surprisingly, DOPA oxidase was also observed in Golgi stacks, vesicles (Figs. 11, 12) and MVB (Fig. 1) of epidermal cells.

X- ray microanalysis

In the matrix of the melanin granules large peaks of $P_{k\alpha}$ or/and $Os_{M\alpha}$ and $Ca_{k\alpha}$ were observed (Fig 13A). These elements were lacking in the electron-transparent portion of the melanin granules (Fig 13B). In the cytoplasm (Fig 13C) of the melanocytes counts for Ca were low, but high for P and/or Os. The spectra were recorded without any background substraction. Three almost identical spectra were recorded at the three different sites that were measured.

Discussion

In this study, pigmentation of the fins of all 4 xanthic goldfish was observable 12 days after introduction of the NaCl stress. The beginning of melanogenesis, therefore, was seen 7 days later than in the report of Turner et al. (1975). In better agreement to this study, Chavin (1956) observed pigmentation in 30 of 50 goldfish at day 10 after stress introduction.

Whereas in higher vertebrates the origin of melanosomes are lamellar or globular premelanosomes (for review see Hirobe, 1995; Schraermeyer, 1996) in goldfish the premelanosome was found to be an MVB (Turner et al., 1975). In this study, MVB were observed in only few melanocytes. This was probably caused by the fact that in most melanocytes melanogenesis was already completed, due to the long (18 days) exposure of osmotic stress.

MVB as premelanosomes were also observed in *Salamandra atra atra* (Trevisan et al., 1991). In xanthic sailfin mollies melanosome formation was described from MVB and alternatively from lamellar framework-type premelanosome (Blanchard et al., 1991). The latter result may indicate that melanization induced by osmotic stress is different from natural melanization.

The present study presents evidence that MVB are not the only type of premelanosome. Granules having a homogeneous matrix also function as premelanosomes, and therefore the morphology of the latter organelle is more heterogeneous than believed before. Multilamellar bodies, as shown in Fig. 4, may also function as matrix for melanin formation, although DOPA-oxidase was not observed in these organelles. The different organelles observed here resembled the premelanosomes observed in the ink gland of *Sepia officinalis* (Schraermeyer, 1994). The fact that accumulations of melanin were detected at the outer extracellular side of the fins indicates that the melanin is removed from the body of the fish, as in the skin of higher vertebrates, by its release into the water. The same observation was made by Chavin (1956) using light microscopy. Chavin stated that macrophages should transport and release the melanin granules. In this study the cell type responsible for transport and release could not be determined. In any case melanin in this experimental model is an excretory material which may help to remove toxic material from the body.

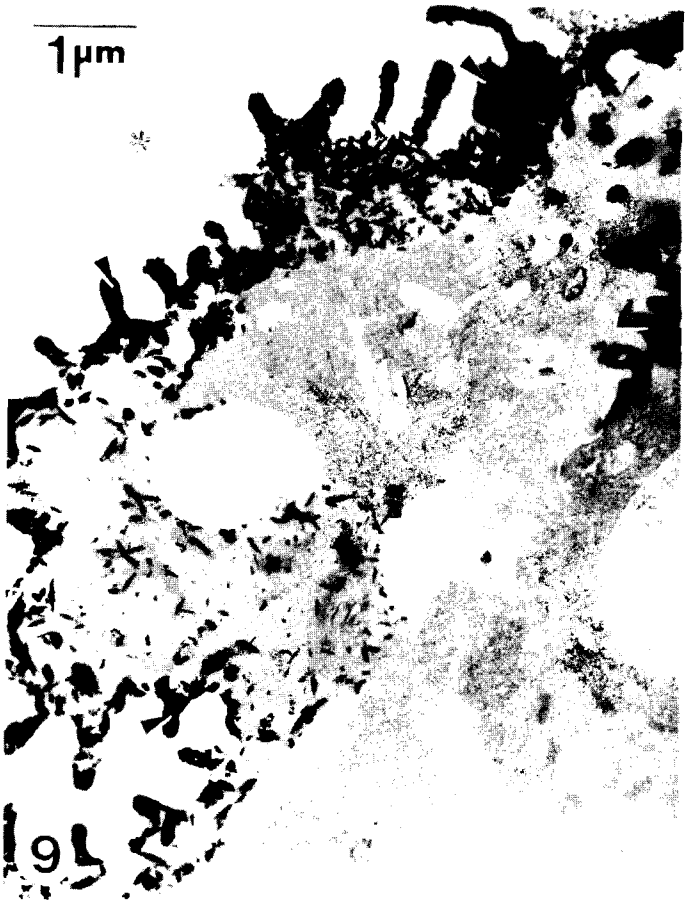
The reason for the high activity of acid phosphatase in some dermal cells is not clear. It may be induced by

Fig. 9. Very strong activity of acid phosphatase indicated by arrowheads is found in the cytoplasm of some dermal cells that are located close to the border of the aqueous environment of the fish (asterisk).

Fig. 10. Electron dense reaction product of DOPA oxidase is observed in Golgi stacks (GO), vesicles (arrows) and melanin granules (arrowheads) of dendritic melanocytes.

Fig. 11. Strong DOPA-oxidase activity is observed in Golgi stacks (GO) and vesicles (arrowheads) of an epidermal cell. The place normally occupied by the aqueous environment of the fish is indicated by an asterisk.

Fig. 12. DOPA-oxidase activity is observed in Golgi stacks (GO) and vesicles (arrowheads) of an epidermal cell. A lamellar body (L) can be seen in a dermal cell. The place normally occupied by the aqueous environment of the fish is indicated by an asterisk.



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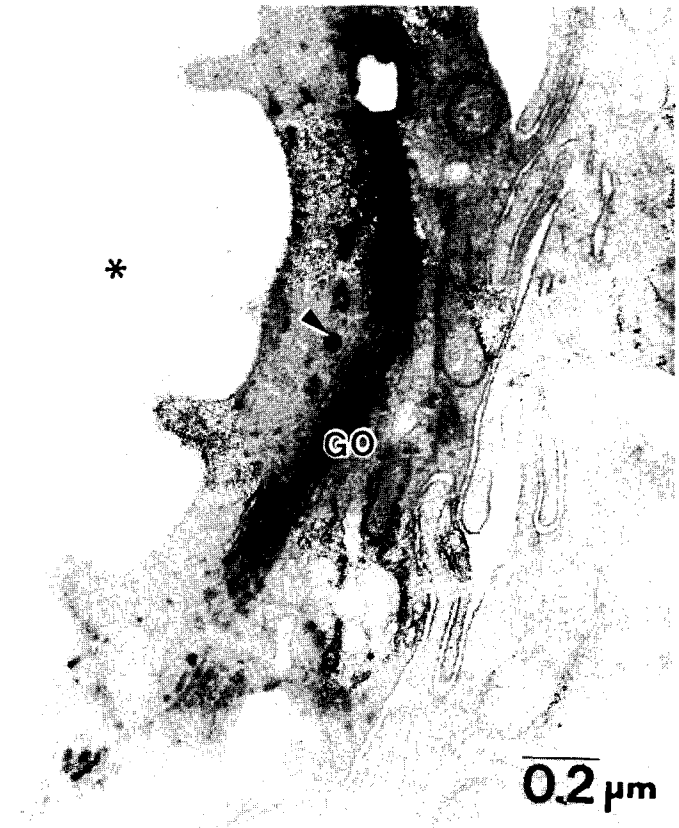

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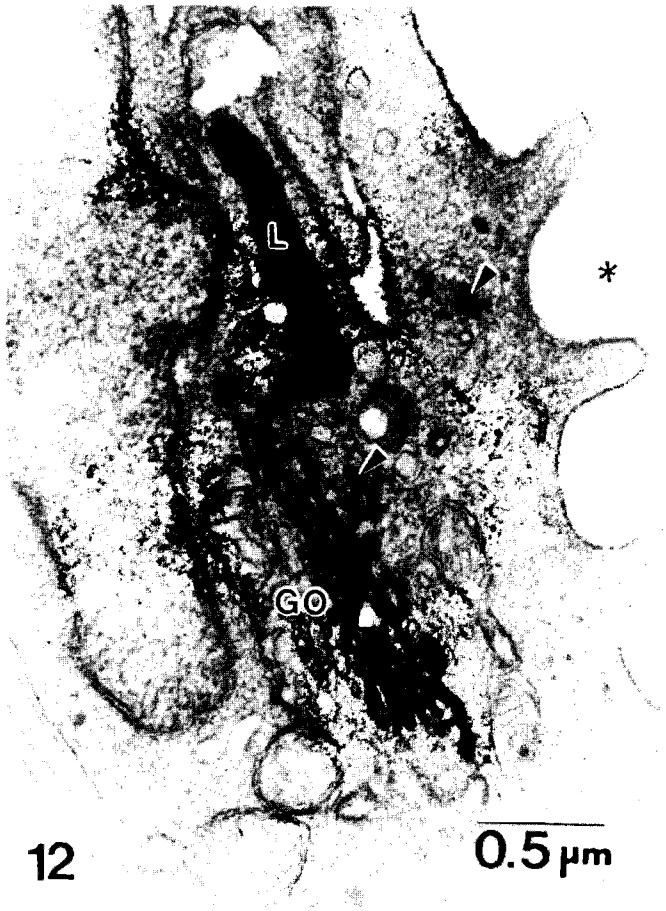

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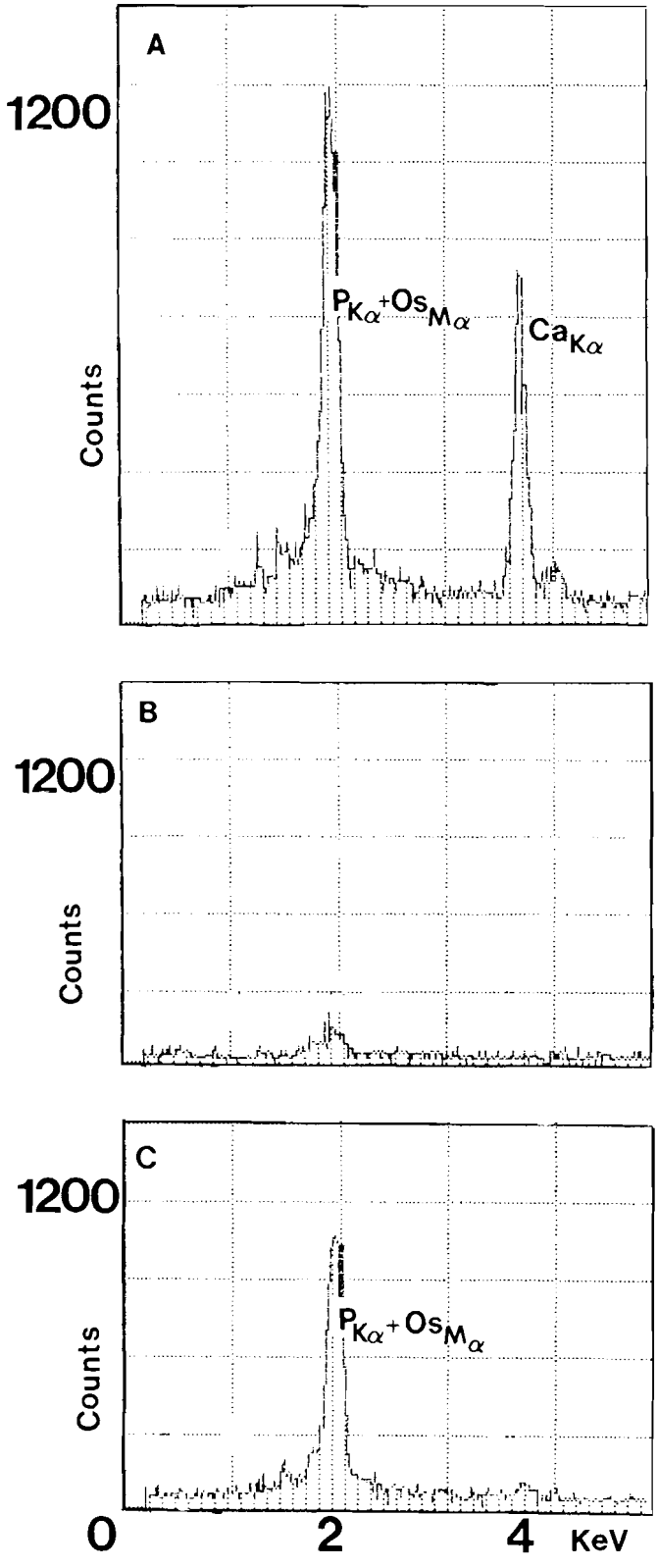
osmotic damage and may indicate the beginning of cell death.

The presence of acid phosphatase reaction product inside the melanin granules further adds to the evidence that melanosomes in this animal model are a special types of lysosome (Toda and Fitzpatrick, 1972; Novikoff et al., 1979; Boissy et al., 1987; Vijayasarahi et al., 1991; Schraermeyer, 1992; Orlow et al., 1993; Smit et al., 1993; Winder et al., 1993; Zhou et al., 1993; Luo et al., 1994; Schraermeyer and Stieve, 1994; Diment et al., 1995; Orlow, 1995).



Whereas the Dopa-oxidase activity within the pigment cells corresponds to the observations of Turner et al. (1975), the finding of this enzyme within dermal cells is new and unexpected. The difference between earlier results and these may originate from the different duration of the osmotic stress. It was reported that tyrosinase also has ion-exchange properties (White, 1958). So one explanation is that the enzyme may be unspecifically upregulated by an enhanced intracellular sodium concentration. Together with the fact that DOPA-oxidase containing MVB were also present, melanin formation is possible in the latter cell type, although mature melanin granules were not detected.

As observed by Turner et al. (1975) the plasmalemma of the melanophores shows numerous pinocytotic vesicles. As observed in this study such vesicles were also shed into the extracellular space. This may be an attempt by the cells to remove some of the invaded ions which may be secreted together with the vesicles. From the morphological point of view it cannot be ruled out that pinocytotic vesicles are also incorporated into the MVB, serving also like Golgi-derived vesicles as a structural component for melanogenesis. Because endocytotic vesicles are generally transported to lysosomes the latter view also coincides with the lysosomal nature of the melanosome (see below). The fact that a lot of very different stress factors could induce melanogenesis (see Introduction) in goldfish may be based on the same cause. All the stressors produce tissue damage. The damaged tissues or proteins have to be removed by the lysosomal pathway. The tyrosine residues of the proteins to be degraded may provide the structural base for melanin formation during lysosomal degradation. Although this hypothesis is attractive it remains to be investigated further. Nevertheless, it has been shown that melanization takes place in lysosomes (Winder et al., 1993; Schraermeyer and Stieve, 1994).

The mechanism of melanization in the MVB is not understood. According to Turner et al. (1975) Golgi-derived tyrosinase-containing vesicles fuse with larger rough endoplasmic reticulum-derived vesicles. Then small vesicles invert and are incorporated into the larger ones which now expose tyrosinase at their outer side.

Fig. 13. The spectra of X-ray microanalysis obtained from aldehyde-fixed plastic embedded sections are shown. In the matrix of the melanin granules large peaks of $P_{K\alpha}$ or/and $Os_{M\alpha}$ and $Ca_{K\alpha}$ are observed (**A**). These elements are lacking in the electron lucent portion of the melanin granules (**B**). In the cytoplasm (**C**) of a melanocyte, counts for Ca are low, but high for P and/or Os. The spectra are recorded without any background substraction.

Melanin is synthesized around the periphery of the inverted vesicles and fills the inter vesicular spaces and finally the internal vesicles (Turner et al., 1975). This mechanism, however, includes one important aspect not yet regarded. During invagination of vesicles into the lumen of MVB cytoplasm is also incorporated into the internal vesicles. Because the content of the internal vesicles is also converted into melanin cytoplasmic material consequently also serves as substrate for melanogenesis (see Fig. 12 in Turner et al., 1975). It is at present hypothetical that this mechanism is an attempt to sequester and bind toxic material to melanin, in this case unphysiological high sodium, but at least it removes it from the cells.

To clarify this point EDX analysis was performed to measure the ionic composition of the melanin granules. From earlier studies it is known that melanin binds metal ions very strongly so that glutaraldehyde fixation only causes a small loss (10-20%) of zinc, iron, calcium and copper when compared with cryofixation (Samuelson et al., 1990). Unfortunately, in the latter study glutaraldehyde fixation caused a complete loss of potassium, the concentration of which was as high as calcium after cryofixation. From the results of this study it cannot be concluded whether the large peak in the matrix of the melanin granules is induced by $P_{k\alpha}$ or/and $Os_{M\alpha}$. The second peak in Fig. 13A and C may originate from $Ca_{k\alpha}$ or $K_{K\beta}$. However, due to the fact that K was completely lost form melanin after aldehyde-fixation followed by plastic embedding (Samuelson et al., 1990) we conclude that the second peak originates from Ca alone.

The high level of calcium in melanin is in agreement with earlier studies (Panessa and Zadunaisky, 1981). It has already been shown that melanin has the capacity to act as cation-exchange material of considerable activity and can bind sodium (White, 1958). Thus, as a consequence of the experimental design it cannot be ruled out that in the living state the melanin granules had incorporated sodium lost during aldehyde fixation. Therefore, further studies are underway involving cryotechniques to clear up the original question as to whether melanin formation in the osmotically-stressed goldfish provides a mechanism to bind and remove unphysiological high sodium.

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