

# THE INDUCTION OF MELANIZATION IN GOLDFISH SCALES WITH ACTH, *IN VITRO*

## Cellular and Subcellular Changes

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### ABSTRACT

The induction of melanization in xanthic goldfish scales with ACTH *in vitro* has been studied by light and electron microscopy utilizing ammoniated silver nitrate staining of premelanin and melanin. The melanized cells (melanophores and melanocytes) and the yellow pigmented cells (lipophores and the newly described lipocytes) were found to possess many similarities at the levels of cellular and subcellular structure. The latter cells contain characteristic cytoplasmic bodies which react positively to the premelanin stain. Changes accompanying ACTH stimulation of goldfish scales in tissue culture suggest that these bodies in the lipocytes and lipophores can become melanized. Electron micrographs illustrate the intermediate staining of newly formed melanin granules in an induced melanocyte and the appearance of a transitional melanolipophore. It is postulated that ACTH can promote the association of the enzyme tyrosinase with the preformed structure of unmelanized granules.

### INTRODUCTION

The studies of Dalton (1) and Birbeck, Mercer, and Barnicot (2) showed that melanin granules are derived from small vesicles of low density in the Golgi region of pigment cells. Wellings and Siegel (3) have described a progressive increase in the size and density of the vesicles as stages in the development of melanin granules in human malignant melanoma cells. Recently, the name "melanosome" has been proposed to apply to the precursor organelle of melanin granules (4). Birbeck and Barnicot (5), investigating the amelanotic melanocytes from hair bulbs of human albinos, found "ghost" granules, vesicles with low interior density having a size and distribution comparable to those of the dense mature melanin granules of the normal hair melanocyte. Thus, in the growth of melanin granules the formation of a

characteristic cytoplasmic structure and the deposition of melanin within it seem to be independent but normally coordinated processes.

A number of stimuli will cause the appearance *in vivo* of melanized cells in xanthic (amelanotic) goldfish scales where no such cells were previously found (6-9). Furthermore, Hu and Chavin (10) have demonstrated a similar induction of melanization *in vitro* in pieces of xanthic goldfish fin incubated in tissue culture with adrenocorticotrophic hormone (ACTH). The present investigation was undertaken to identify the precursor cell in which melanization is initiated by ACTH. In these experiments there are two groups of pigment cells which occur in the goldfish integument: (a) those containing melanin granules, the melanocytes and melanophores, and (b) those with an agranular

appearing yellow carotenoid pigment, the lipophores.

#### METHOD

Scales from black moor and xanthic goldfish (*Carassius auratus*, L.), fresh and incubated in tissue culture with ACTH, have been studied by light and electron microscopy using the ammoniated silver nitrate staining method for melanin and premelanin described previously (11). All xanthic scales were checked initially for the absence of melanized cells. For the *in vitro* induction of melanization in tissue culture, a medium composed of 90 per cent Difco 199 and 10 per cent bovine serum and containing 500  $\mu\text{g}/\text{ml}$  penicillin-streptomycin and 50  $\mu\text{g}/\text{ml}$  mycostatin was used. Scales freshly removed from the fish were rinsed twice in culture medium, cut in half longitudinally, and placed in sterile culture tubes with 3 ml of medium. ACTH (lot 146RS3, generously supplied by Dr. Joseph D. Fisher of the Armour Pharmaceutical Company, Kankakee, Illinois) was added to some tubes at a concentration of 3 International Units/ml. The tubes were incubated without agitation at 37°C for 46 hours.

Light microscopy of unstained scales was carried out with and without xylol treatment for the removal of the yellow pigments. Masson's ammoniated silver nitrate method for the selective staining of premelanin and melanin has been adapted for electron microscopy and is described in detail elsewhere (11). Briefly, the following steps are involved: (a) fresh scales were fixed 1 to 2 hours in cold isotonic buffered 5 per cent formalin and then rinsed twice in distilled water; (b) they were then incubated 26 minutes in a 10 per cent ammoniated silver nitrate solution at 58°C, followed by 15 minutes' rinsing in distilled water; (c) scales were then immersed 1 minute in a solution of gold chloride, sodium thiosulfate, and ammonium sulfocyanide, followed immediately by 4 minutes in a 6 per cent sodium thiosulfate solution and then by 10 minutes' washing in running water. Tissues to be examined by light microscopy were removed and mounted at this point.

For electron microscopy the scales were fixed an additional 2 hours, after staining, in cold buffered 0.5 per cent potassium permanganate and embedded in the water-miscible resin fraction "aquon" (12). Unstained controls consisted of fresh scales fixed 2 hours in potassium permanganate fixative. Thin sections were cut on a Porter-Blum microtome and were examined with an R.C.A. electron microscope type EMU-2A. The light micrographs, Figs. 1 to 7, are views photographed through the thin dimension of the scales, whereas the electron micrographs, Figs. 8 to 17, are images of thin sections cut perpendicular to the plane of the scale.

#### RESULTS

The observations are briefly summarized in Table I.

##### *Light Microscopy*

Light microscopy of fresh xanthic goldfish scales shows dendritic lipophores but no cells containing visible mature melanin granules. On the other hand, black moor scales have numerous heavily pigmented melanophores interspersed among the yellow lipophores. Both the relatively small and finely dendritic micromelanophores and the large broadly dendritic macromelanophores (13) (Fig. 1) are seen. As well as staining the melanized cells, the ammoniated silver nitrate technic selectively stains the yellow pigmented cells in xanthic scales and has enabled the clear visualization of their form. Compared to the melanophores, the lipophores have a similar distribution of nearly identical forms. In the outer layer of the goldfish scale there are lipophores which, by their similarity of form to micromelanophores, might appropriately be called microlipophores (Fig. 2). In the deeper layer are large, broadly dendritic cells for which the name macrolipophore is most suitable (Fig. 3). Particularly significant to the present study is the observation of bipolar yellow pigmented cells in xanthic goldfish scales. When stained by ammoniated silver nitrate, these cells develop granular metallic deposits in their cytoplasm similar to those in lipophores (Fig. 4). The structure of these yellow cells is comparable to that of melanocytes and, therefore, it is suggested that the name lipocyte be applied to them.

Incubation of xanthic goldfish scales in tissue culture with ACTH for 46 hours resulted in the formation of a few bipolar or tripolar melanized cells (melanocytes), as observed previously in pieces of the tail fin by Hu and Chavin (10). The appearance of an induced melanocyte in an unstained preparation (Fig. 5) is strikingly similar to that of the lipocytes stained with ammoniated silver nitrate (Fig. 4). No melanophores were seen in incubated xanthic scales.

In the presence of ACTH a large number of the lipophores show an exaggerated stellate shape with extended slender dendrites. The cytoplasm of these lipophores has an increased affinity for the ammoniated silver nitrate stain (Fig. 6). Interspersed among these cells are unstimulated lipophores with short dendrites. In control incubations, without ACTH, neither stellate lipophores nor

TABLE I  
*Summary of Observations of Pigment Cell Structures Accompanying the Induction of Melanization*

Scale; Treatment	Light Microscopy		Electron Microscopy	
	Unstained	Premelanin Stain	KMnO <sub>4</sub> -Fixed	Premelanin Stain KMnO <sub>4</sub> -Fixed
Xanthic; Fresh	Micro- and macrolipophores and bipolar lipocytes seen	Micro- and macrolipophores and lipocytes stained selectively (Figs. 2 to 4)	Lipophore and lipocyte cytoplasmic bodies contain amorphous material (Fig. 10)	Lipophore and lipocyte cytoplasmic bodies showed fine silver deposits
Xanthic; Incub. 46 hrs. ACTH	Lipophores stellate, long fine dendrites. Several bi- and tripolar melanocytes (Fig. 5)	1) Lobate lipophores 2) Stellate cells darkly stained 3) Several melanocytes (Fig. 6)	Lipophore bodies mostly contain low density material (Fig. 13)	Lipophore bodies more heavily stained. Melanocyte granules with large and fine metallic deposits (Figs. 15 and 17)
Xanthic; Incub. 46 hrs. Control	Lipophores rounded, few short dendrites	Rounded lipophores lightly stained (Fig. 7)	Signs of degeneration. Lipophore bodies mostly empty. Dense vesicles aggregated (Fig. 12)	Empty large bodies. Sparse metallic deposits (Fig. 14)
Black Moor; Fresh	Lipophores with short rounded dendrites. Macro- and micro-melanophores and melanocytes observed (Fig. 1)	Lipophore granules stained. Melanophore cytoplasm completely blackened	Lipophore bodies mostly filled with amorphous material, melanophores with dense melanin granules (Figs. 8 and 9)	Lipophores with both light and heavy silver deposits in same cell. Melanin distinctively stained
Black Moor; Incub. 46 hrs. ACTH	Lipophores and melanophores more prominently dendritic	Degeneration of melanophores		Several melanin granules seen in cytoplasm with large bodies (Fig. 16). Enhanced staining of lipophore bodies. Melanophore granules unchanged
Black Moor; Incub. 46 hrs. Control	Degeneration of melanophores	Degenerating melanophores densely stained	Lipophore bodies mostly full. Membranes of melanin granules swollen (Fig. 11)	Sparse metallic deposits in lipophoric bodies. Melanin granules heavily stained

melanocytes were found and the ammoniated silver nitrate stain in the broadly dendritic lipophores was diminished and less granular (Fig. 7). The presence of ACTH in the tissue culture medium in which black moor goldfish scales were incubated resulted in the transformation of melanophores to a stellate configuration with slender dendrites similar to that observed in the lipophores. The induction of new melanocytes, however, could not be observed in the already heavily melanized scales. In all incubated scales there was evidence of tissue disintegration, migration of the pigment cells, and particularly the degeneration of melanophores in the absence of added ACTH.

### *Electron Microscopy*

At the epidermal-dermal junction of the goldfish scale there is a basement membrane 700 to 800  $m\mu$  thick with a dense zone on its epidermal side (Fig. 8). All pigment cells were found in the dermis with no evidence of penetration of their dendrites into the epidermal layer. The cytoplasm of melanophores in black moor goldfish is characterized by the presence of dense spherical membrane-bounded melanin granules 400 to 500  $m\mu$  in diameter, a few mitochondria with tubular type cristae, and some vesicles of endoplasmic reticulum (Fig. 9). No internal structures have been detected in mature melanin granules of the goldfish.

The lipophores of both xanthic and black moor goldfish contain mitochondria and sparse endoplasmic reticulum, similar to the melanophores (Fig. 10). In addition, the cytoplasm of lipophores has two distinctive components not found in

melanophores: (a) large bodies, 500 to 800  $m\mu$  in diameter, containing either amorphous material or structures resembling unorganized membranous fragments and (b) small vesicles, 40 to 50  $m\mu$  in size, having an unusually dense membrane and a low density interior. The latter component is frequently seen in heavy concentration. Although positive identification of a lipocyte in electron micrographs has been elusive, the cytoplasm of lipocytes must be presumed to be similar to that of the lipophores since, in the many scales examined, no cell suggesting the contrary has been observed.

The internal organization and fine structure of many cells showed considerable deterioration and "loosening" during 46 hours' incubation in tissue culture. The membranes surrounding melanin granules were more evident, often appearing swollen and separated from the dense interior (Fig. 11). Without ACTH in the medium the contents of the large bodies of lipophores were diminished, many were nearly empty, and much of the small vesicle component was missing from the cells or aggregated (Fig. 12). On the other hand, when ACTH was present in the tissue culture medium, the large bodies were consistently filled and the cells appeared less distorted (Fig. 13). In agreement with light microscopic findings, the ammoniated silver nitrate stain of xanthic scales incubated without ACTH was markedly reduced because of the loss of interior substance (Fig. 14). However, with ACTH many of the large bodies in the cytoplasm of yellow pigment cells showed heavy metallic deposits (Fig. 15).

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### FIGURES 1 TO 7

Light micrographs photographed through the thin dimension of the goldfish scales.

#### FIGURE 1

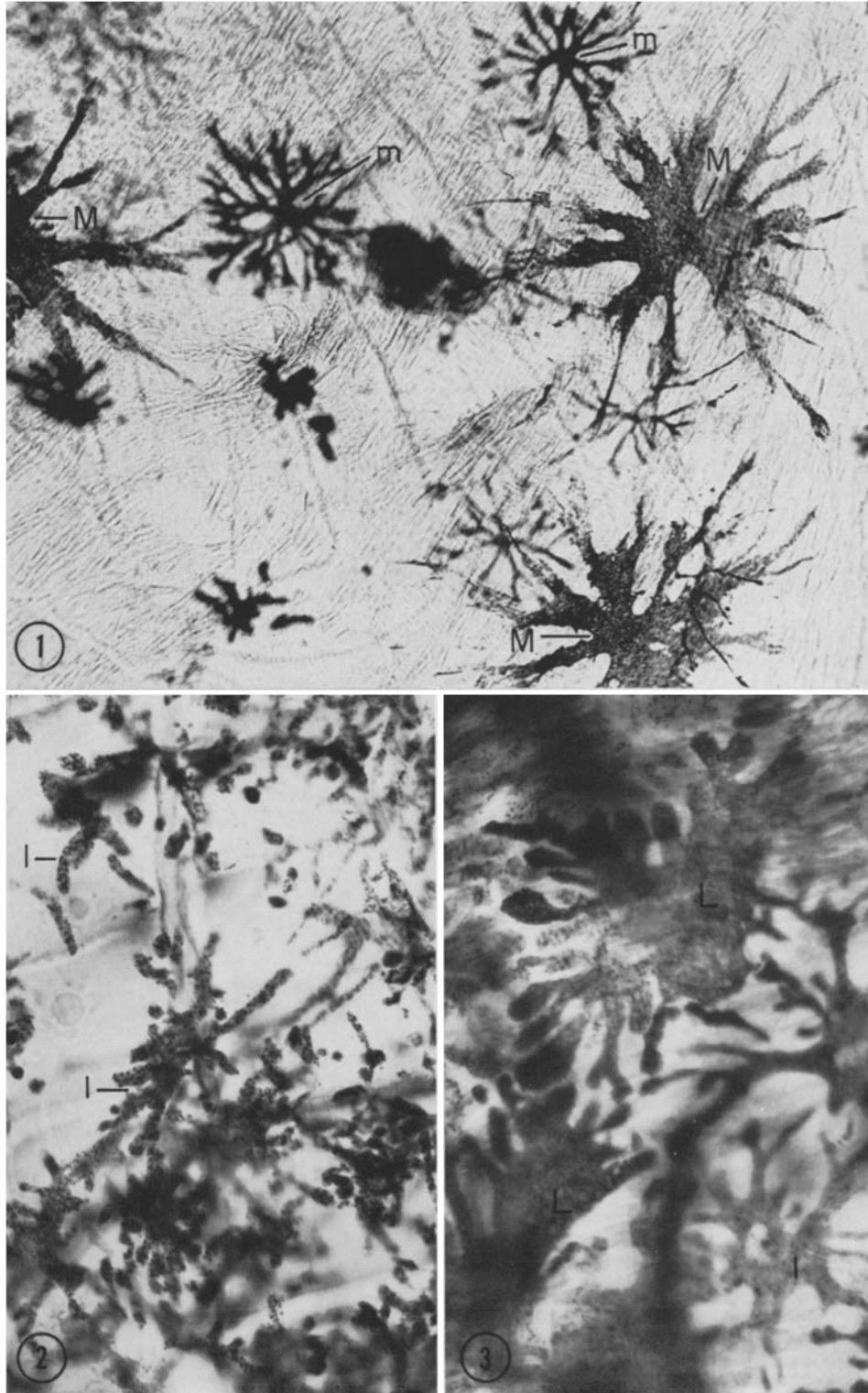
Black moor scale cleared with xylol to remove yellow pigment. The finely dendritic micromelanophores (*m*) and the large stellate macromelanophores (*M*) are seen.  $\times 180$ .

#### FIGURE 2

Microlipophores (*l*) in a xanthic scale stained with ammoniated silver nitrate. Numerous darkly stained granules are visible throughout the cytoplasm of these cells.  $\times 465$ .

#### FIGURE 3

Macrolipophores (*L*) in a xanthic scale stained with ammoniated silver nitrate. The normal thinly drawn out dendrites have contracted to a clubbed form during staining. In the fresh state their configuration is similar to the macromelanophores. A microlipophore is visible at lower right (*l*).  $\times 465$ .



As previously reported (11), the metallic deposits of the ammoniated silver nitrate stain are selectively localized in melanin granules as large spherical crystallites, and in the large bodies of yellow pigment cells as numerous small particles. Melanin pigment, itself electron opaque, is still visible between the large aggregates of metallic silver deposited in melanized cells (Fig. 16). This characteristic high density, possibly due to their very high physical density (14) or their content of copper and zinc (15, 16), is found in no other structure of the goldfish scale and positively identifies the stained bodies as melanin granules.

Fig. 16 shows the cytoplasm of an apparent yellow pigment cell in a black moor goldfish incubated 46 hours with ACTH. The characteristic large bodies are evident, having fine metallic particles of ammoniated silver nitrate stain. The small vesicular component is considerably diminished. Three typical melanin granules, identical in appearance to those in the adjacent melanophore, are also present in this cell. Several cells of this kind have been observed. The presence of melanin granules in the cytoplasm of these cells strongly suggests that melanization has occurred in the cytoplasm of the yellow pigment cell.

In a xanthic scale, previously checked for the absence of melanin and then incubated with ACTH, melanized granules have been observed

by electron microscopy (Fig. 17). The homogeneous dense background identifies these structures as melanin granules. Furthermore, these granules must be in the cytoplasm of a newly induced melanocyte since no other melanin-containing cells are present at this time. The ammoniated silver nitrate stain is not composed entirely of the large aggregates characteristic of mature melanin but still has many fine particles as usually found in the large bodies of yellow pigment cells.

#### DISCUSSION

The origin of melanocytes and melanophores induced in xanthic goldfish by various stimuli has remained uncertain. In studying this problem a preparation of ACTH has been utilized to effect the induction, as described by Hu and Chavin (10). Although the inductive agent is repeatedly referred to as ACTH, no tests of its purity have been done and it is recognized that some contaminant such as melanocyte-stimulating hormone may be present (17).

The two types of pigment cells in fish skin, melanized melanophores and melanocytes and yellow pigmented lipophores and lipocytes, have many characteristics in common. Both of these cell lines are derived embryologically from the neural crest (18). The lipophores are shown here to have cellular configurations, the microlipophore

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#### FIGURE 4

Lipocyte stained with ammoniated silver nitrate. The densely staining granules are distributed about the nucleus and in the arms of this bipolar cell. These granules are distinct from the yellow pigment substance.  $\times 600$ .

#### FIGURE 5

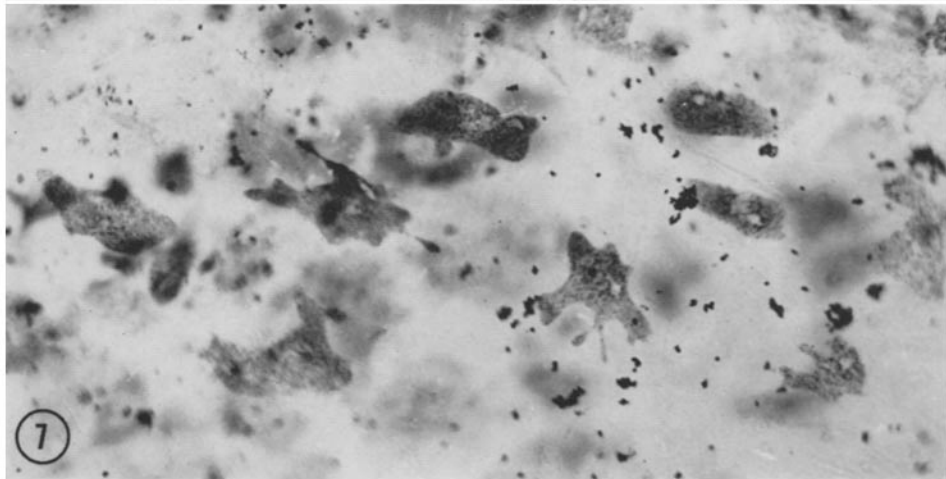
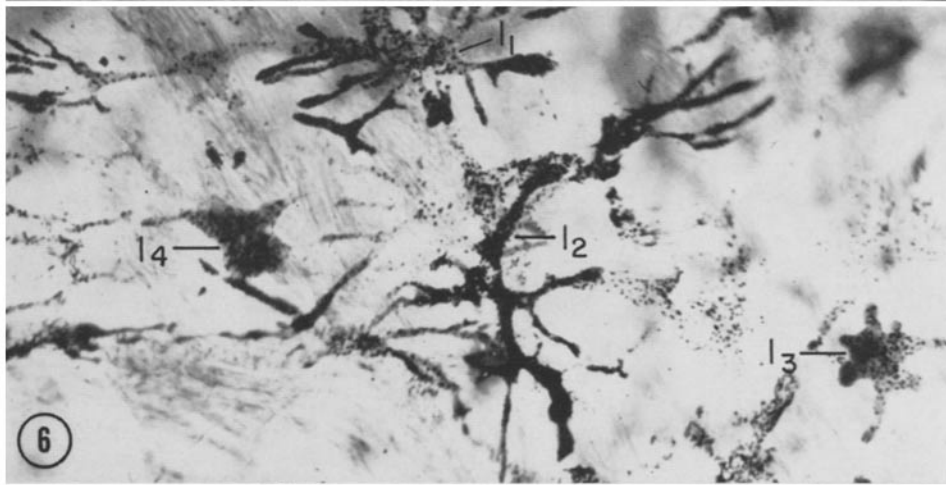
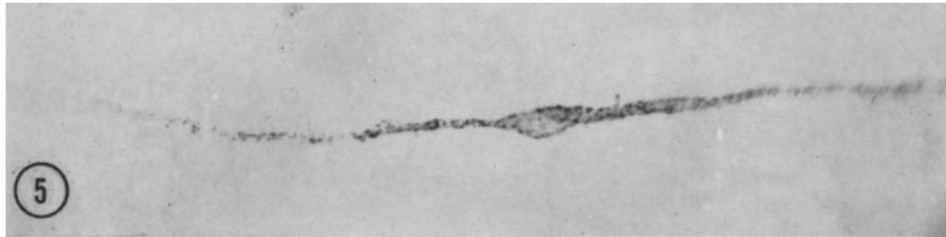
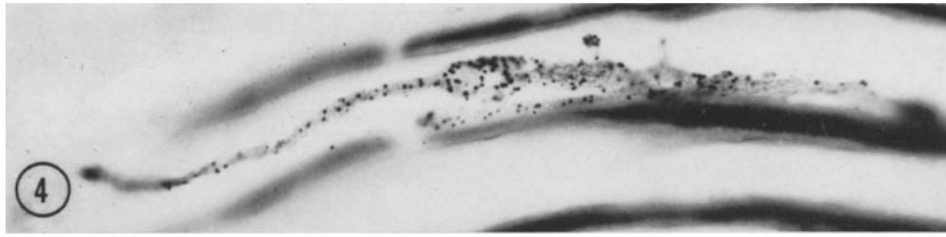
A bipolar melanocyte induced in a xanthic goldfish scale by incubation in tissue culture with ACTH. The tissue has been cleared with xylol. The size and shape of induced melanocytes and the distribution of their melanin granules are remarkably comparable to the appearance of the stained lipocyte in Fig. 4.  $\times 600$ .

#### FIGURE 6

Microlipophores in a xanthic scale incubated in tissue culture with ACTH and later stained with ammoniated silver nitrate. The majority of lipophores ( $l_1, l_2$ ) have become even more finely dendritic, as compared to those in Fig. 2, and the staining intensity has increased. In all incubated tissues some lipophores ( $l_3, l_4$ ) round up and show no enhancement of staining.  $\times 465$ .

#### FIGURE 7

Control preparation similar to Fig. 6 except that the tissue culture incubation fluid contained no ACTH. All lipophores have contracted and show a decrease affinity for the ammoniated silver nitrate stain.  $\times 465$ .



(Fig. 2) and the macrolipophore (Fig. 3), analogous to those of melanophores (13), and to exhibit the presumed immature form of a bipolar lipocyte (Fig. 4). Chavin (8) has demonstrated that following hypophysectomy or injection of epinephrine the pigments in both melanophores and lipophores become centrally aggregated. In the present study it was observed that both pigment cells responded similarly to ACTH *in vitro* by assuming a more finely dendritic and stellate shape. On the cytoplasmic level the pigment cells are characterized by the presence of membrane-bounded bodies, either melanin granules or the large bodies of lipophores, which are similar in size and distribution. Both of these cytoplasmic organelles possess a specific affinity for the ammoniated silver nitrate staining reaction which was used previously (11) to demonstrate the presence of premelanin (19) in the granules of the yellow pigment cells.

Incubation of scales from xanthic goldfish with ACTH leads to the induction of melanocytes and to the intensification of the ammoniated silver nitrate staining reaction in the large bodies of the yellow pigment cells. By light microscopy the num-

ber of melanocytes induced by ACTH approximates the population of lipocytes observable in control xanthic scales. These observations taken together with the similarities mentioned above suggest that the lipocyte is the source of the induced melanocyte and that the large bodies are unmelanized precursors of the melanin granules. Electron microscopy of xanthic scales incubated with ACTH and stained with ammoniated silver nitrate (Fig. 17) shows that the staining characteristic of early melanin granules in the melanocyte is intermediate between the fine metallic deposits seen in large bodies and the coarse aggregates typical of mature melanin granules. These findings are not incompatible with the recent suggestion (20, 21) that mitosis of propigment cells may precede the induction of melanocytes from unmelanized precursor cells. However, neither mitotic figures nor melanoblasts have yet been seen in either *in vivo* (9) or the present *in vitro* studies.

A clearer view of the relationships among the pigment cells of the goldfish integument is now possible. Gordon (13) has described the normal growth of melanophores to be a maturation of

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#### FIGURES 8 TO 17

Electron micrographs of thin sections cut perpendicular to the plane of the goldfish scales and, hence, presenting views perpendicular to those in the preceding light micrographs.

#### FIGURE 8

Cross-section of the dermal region of a scale from a black moor goldfish. A small portion of two epidermal cells can be seen at the upper left margin just above the basement membrane (*B*). At the lower right is seen the edge of the dense bony support in the center of the scale. The dermis is occupied mostly by the cross-sections of intertwined dendrites of lipophores and melanophores. The latter are characterized by the dense spherical melanin granules (*mg*) several of which have been torn from the section during cutting. The cytoplasm of lipophores contain large bodies (*lb*) slightly bigger than melanin granules but with a low density interior, and numerous small dense vesicles (*v*).  $\times 6500$ .

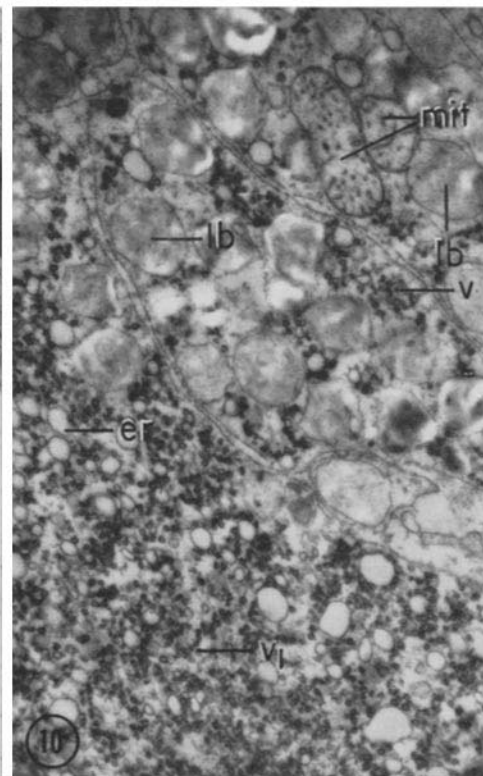
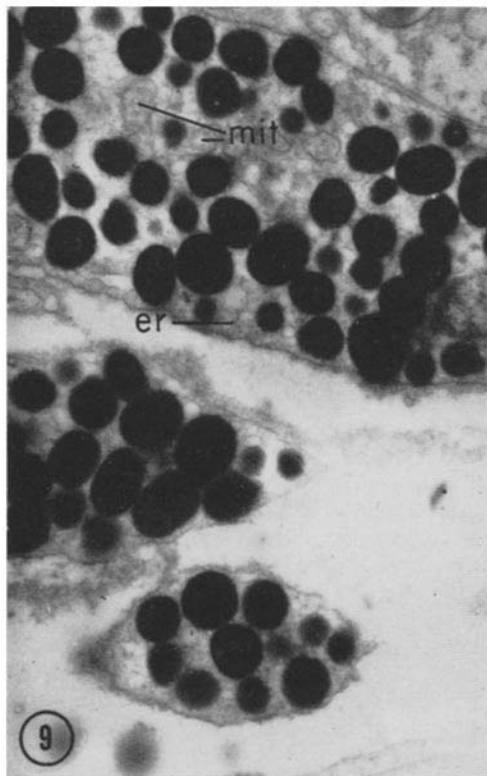
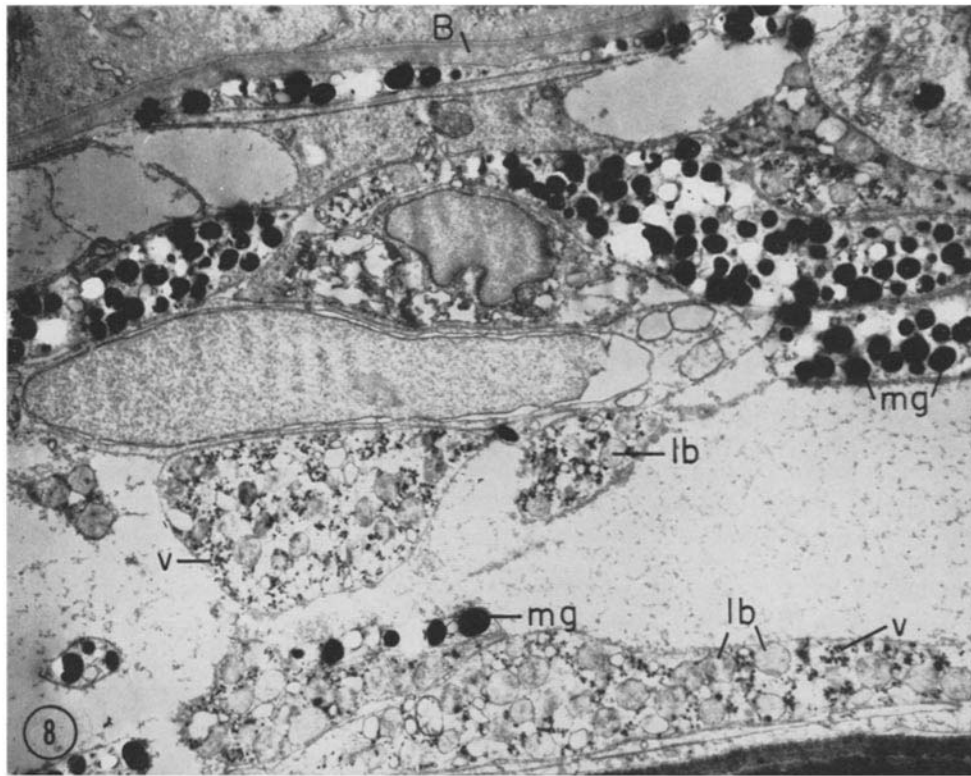
#### FIGURE 9

Higher magnification of melanophore dendrite cross-sections in black moor scale. Only very little endoplasmic reticulum (*er*) and few mitochondria (*mit*) are seen among the dense structureless melanin granules.  $\times 16,500$ .

#### FIGURE 10

Cross-section of lipophore dendrites in a xanthic scale. As in the melanophores, only sparse endoplasmic reticulum (*er*) is seen and few mitochondria (*mit*) with small tubular cristae. The large bodies (*lb*) have a distinct membrane and generally contain amorphous material. Interspersed among these are the small vesicles (*v*) with dense membranes, often seen in great abundance (*v*<sub>1</sub>).  $\times 16,500$ .





melanocytes into multi-dendritic forms. It is reasonable to extend the same concept to the formation of lipophores from lipocytes. The evidence in the present studies supports the idea that under appropriate stimuli the yellow lipocytes are converted to melanocytes. That an analogous transformation of lipophores to melanophores may occur is suggested by the demonstration of cells from black moor scales in which mature melanin granules were found together with unmelanized large bodies (Fig. 16). Such cells probably correspond to the melanolipophores observed by Biederman (22). Furthermore, in salamander embryos Niu (23) has observed a slow transformation of yellow pigment cells into melanophores following melanophoral disintegration.

The formation of melanin granules has been described in detail in mammalian cells (1, 3, 4, 24, 25). Melanin accumulates in tyrosinase-active vesicles, melanosomes, arising from the Golgi zone in melanocytes. The melanin first appears in

a distinct pattern which becomes obliterated as the granule is completed. The melanophore in the goldfish contains no melanosomes but only the mature melanin granules in which no internal structures have been seen. The developmental stages of these granules, if they are to be seen, would be found in the sparse melanocytes. However, in experiments on induced melanogenesis *in vivo* (9), melanophores were seen to appear very shortly after the first melanocytes were observed, suggesting that the duration of the latter phase is rather brief.

The lipophore, like the melanophore, contains uniform characteristic granules, the large bodies (premelanin granules), but no evidence of their stages of growth. The small dense vesicular component in the cytoplasm of lipophores seems to be structurally unrelated to the large bodies and possibly has something to do with the yellow pigment substance. If the inference from the present observations is correct, namely, that the large

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FIGURE 11

Portion of a melanophore in a black moor scale incubated 46 hours in tissue culture without ACTH. The membranes surrounding several of the melanin granules have become evident by swelling.  $\times 18,000$ .

FIGURE 12

Lipophore dendrite in a control xanthic scale incubated in tissue culture without ACTH. The large bodies appear swollen and mostly empty. The small dense vesicular component of the cytoplasm is often diminished or aggregated as seen in this micrograph.  $\times 9000$ .

FIGURE 13

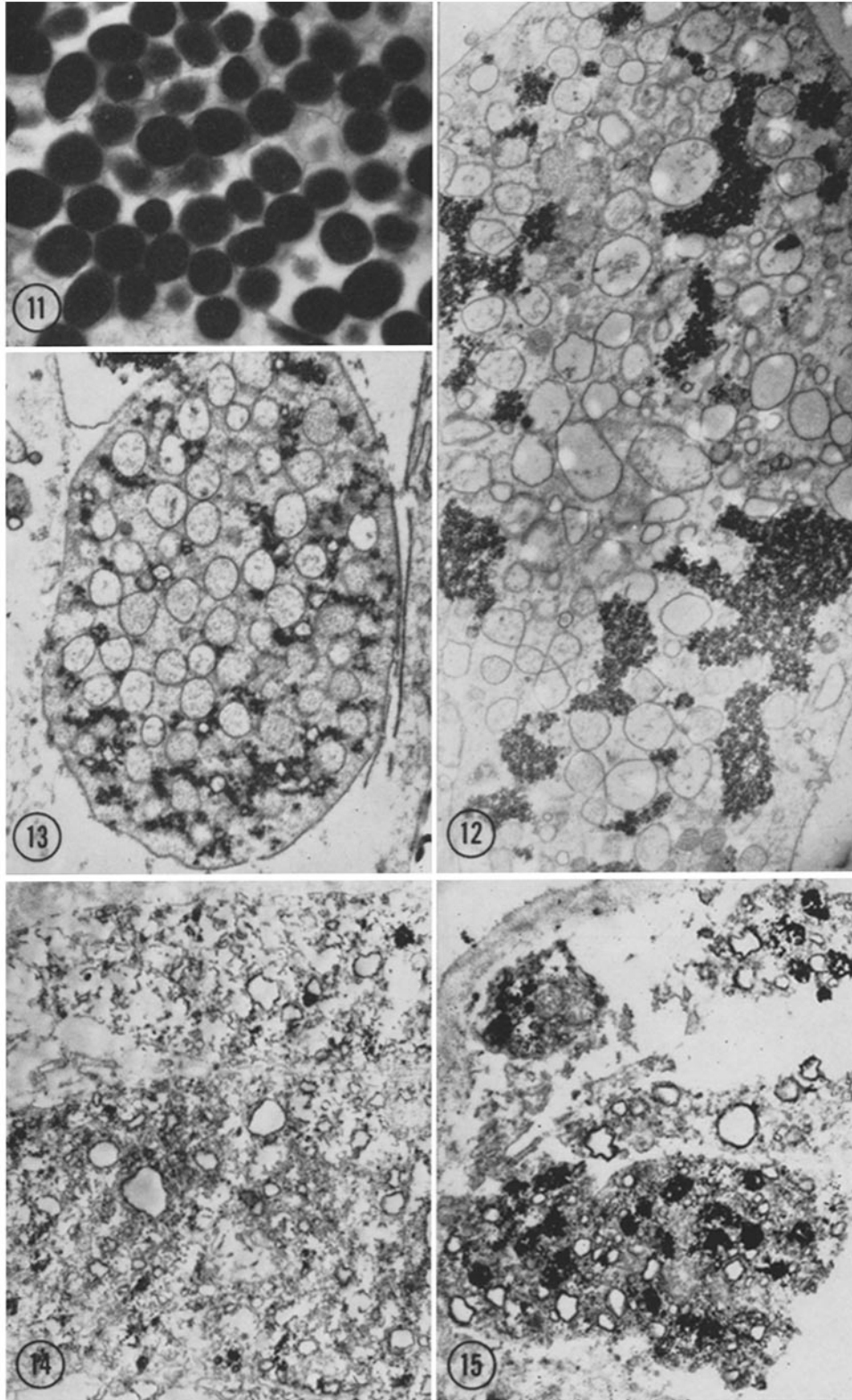
Lipophore dendrite in a xanthic scale incubated in tissue culture with ACTH present in the medium. The cells appear less distorted and swollen and the large bodies are normal in size and are generally filled with typical amorphous material. The small dense vesicles show some aggregation.  $\times 9000$ .

FIGURE 14

Lipophores in a xanthic scale incubated in tissue culture without ACTH and stained with ammoniated silver nitrate. This micrograph and those which follow show not only the tissue distortion resulting from incubation in tissue culture but also the effects of the staining procedure before fixation. Corresponding to the light micrograph, Fig. 7, only small scattered metallic deposits of stain are found.  $\times 9000$ .

FIGURE 15

Lipophore dendrites in a xanthic scale incubated in tissue culture with ACTH and stained with ammoniated silver nitrate. In agreement with Fig. 6, the staining intensity is increased as a result of the presence of ACTH in the culture medium; compare with Fig. 14. The deposits of stain are composed of large numbers of fine metallic granules, the characteristic pattern seen in the large bodies (11).  $\times 9000$ .



bodies of lipophores are structures analogous to mature melanin granules, then they are also essentially similar to the unmelanized "ghost" granules found in human albino melanocytes (5). These latter granules also show the staining characteristics of premelanin (26) and are distinguished from melanosomes by having reached mature size without melanization.

The final step leading to the melanization of lipocytes is still not clear. The finding of both soluble and particle-bound tyrosinase in xanthic goldfish skin (27) shows that the process of pigmentation has not been interrupted by the absence of this enzyme. Tyrosinase is synthesized in the ribosome fraction of the cytoplasm (28). It is probable that the localization of tyrosinase, in particular its being bound to characteristic organelles in the cytoplasm of yellow pigment cells, is a determinative step in initiating the melanization of these bodies. In support of this concept Kim *et al.* (29), comparing homogenates of skin from xanthic and black goldfish, found particle-bound tyrosinase activity to be three times larger in the black goldfish, whereas the enzyme in the soluble fraction was twice as high in the xanthic goldfish. This particulate fraction would contain (a) mature melanin granules, in which little tyrosinase activity is found (30), and (b) the characteristic large

bodies of lipophores, which appear to be the site of melanin formation. Thus, the available evidence suggests that the mechanism for the blocking of melanization in the xanthic goldfish is a disturbance of the association of tyrosinase with characteristic organelles in the cytoplasm. The nature of this association between an enzyme and a cytoplasmic structure and the mechanism by which ACTH promotes the association remain subjects for further study.

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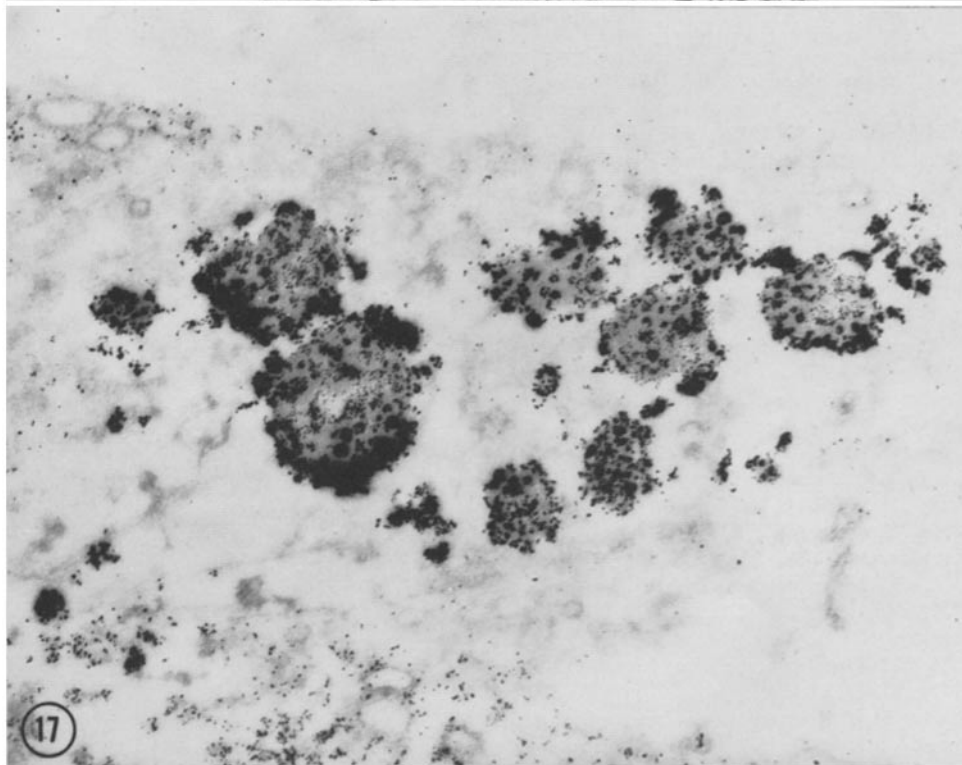
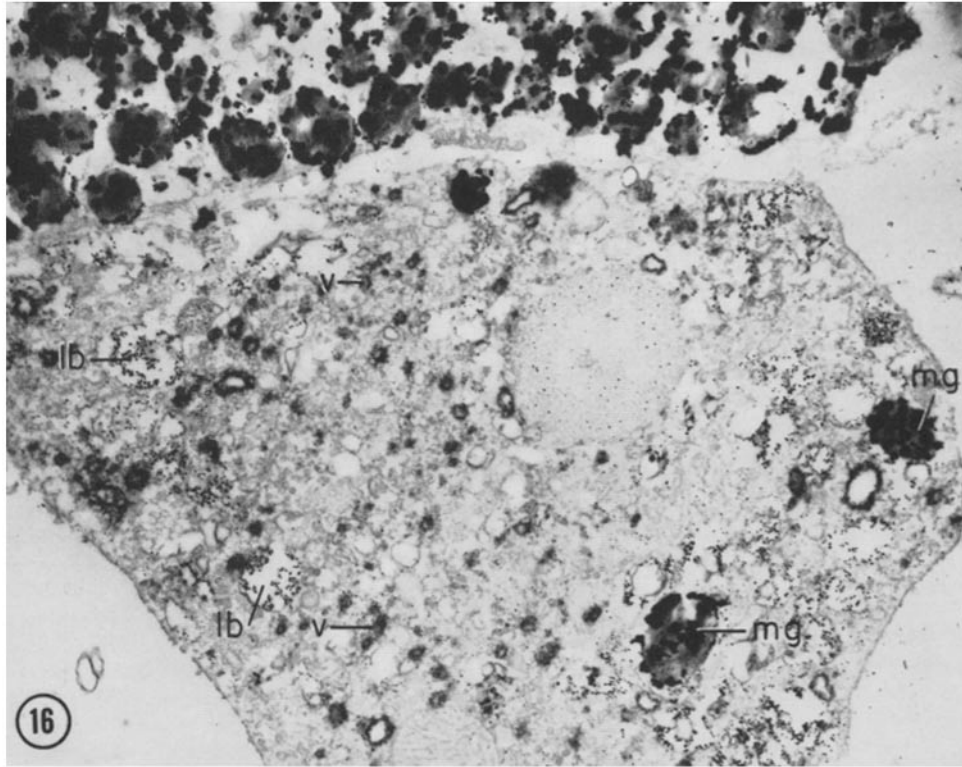
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FIGURE 16

A melanolipophore in a black moor scale incubated in tissue culture with ACTH and stained with ammoniated silver nitrate. At the top of the micrograph are seen typically stained mature melanin granules in a melanophore. The large spherical crystallites of stain are deposited in the smooth dense background of melanin. Below, in the cytoplasm of the melanolipophore, similar melanin granules (*mg*) are seen as well as lipophore large bodies (*lb*). The latter are stained characteristically by fine metallic particles. Small aggregates of dense vesicles (*v*) are seen; these show no particular affinity for the ammoniated silver nitrate stain.  $\times 15,500$ .

FIGURE 17

Melanin granules in a melanocyte induced in a xanthic goldfish scale by ACTH in tissue culture and stained with ammoniated silver nitrate. These structures are known to be melanin granules by their characteristic homogeneous dense background. The formation of metallic aggregates in the staining of these melanin granules is intermediate between that of mature melanin and the unmelanized large bodies of yellow pigment cells.  $\times 33,000$ .



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