THE FATE OF AMPHIBIAN EGG MELANOSOMES

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INTRODUCTION

Numerous studies have been directed toward cytoplasmic differentiation of the amphibian egg (Kemp, 1953, 1956; Wartenberg and Gusek, 1960; Wartenberg and Schmidt, 1961; Balinsky and Devis, 1963; Hope et al., 1964 *a*, 1964 *b*; Wischnitzer, 1966). One of the egg's components is a pigment granule, presumably containing melanin, which is synthesized and deposited within an organelle[1] (melanosome; see Fitzpatrick et al., 1966 for terminology) during oocyte development.

[1] In this discussion the term "egg melanosome" will be used to denote a membrane-bounded, opaque pigment granule originating in oocyte development.

Balinsky (1970) states that egg pigment may not be important for the development of the embryo; however, Stebbins (1951) suggests that it may protect the egg from deleterious radiation.

Depending upon the species, egg color ranges from a light tan to black (Stebbins, 1951). Presumably this is due, in part, to the amount of egg melanosomes present. The majority are found in the cortical layer of the animal hemisphere, whereas the white vegetal hemisphere is practically devoid of them. During cleavage some are displaced from the periphery of the embryo to its interior by the formation of cleavage furrows (Selman and Waddington, 1955; Zotin, 1964; Selman and Perry, 1970) and later by morphogenetic movements (for review, see Balinsky, 1970). Up to the time of hatching, melanosomes are abundant in cells making up the epidermis; but during early larval stages they become scarce. Eventually, cytocrine melanosomes originating from newly differentiated epidermal melanocytes repigment the epidermis. In the present study, egg melanosomes which remain in the periphery of the embryo were examined during development in order to more clearly determine their distribution, fate, and possible functional role.

MATERIALS AND METHODS

Observations utilized in this study were made on embryos and larvae of *Rana pipiens*. Embryos were obtained by natural or induced spawnings (Rugh, 1962). Embryonic stages were determined by the table of Shumway (1940). Larvae used were in the interval stages between stage 25 (Shumway, 1940) and stage I (Taylor and Kollros, 1946).

Tissues were fixed for 2 hr with 4% glutaraldehyde in 0.1 M *s*-collidine buffer at pH 7.4 followed by postfixation in 2% osmium tetroxide in 0.1 M *s*-collidine buffer at the same pH. After fixation, tissues were dehydrated in an ethanol series and infiltrated in Epon 812 under vacuum and then polymerized. Tissue blocks were sectioned with a diamond knife. Sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken on an RCA EMU-4A. Thick sections (1–2 μ) were stained with toluidine blue and examined by light microscopy.

In order to clearly demonstrate melanosome caps resulting from cytocrine activity of epidermal melanocytes, larvae were sacrificed and whole-mount preparations were made of fixed (formaldehyde, acetic acid, and 70% ethanol, 2:1:17), dehydrated, and unstained pieces of epidermis. It was found that in prepations in which nuclei were stained, the visible presence of nuclei under melanosome caps obscured the definition of the cap in black and white photographs. Nuclei and caps are not in the same plane of focus; as a result, nuclei appear as dark diffuse areas under caps which in turn are black. In all cases, the verification of nuclei under melanosome caps was made by through-focusing with phase-contrast microscopy.

RESULTS

The animal hemisphere of a fertilized egg is heavily laden with egg melanosomes, whereas they are absent or few in number in the vegetal hemisphere. A grey transitional zone separates the two. The majority of egg melanosomes are localized in the cortical region and are intermingled with yolk platelets (Fig. 1). Below the cortex, their density diminishes; however, some are found deep within the egg. From the beginning of cleavage to the 32-cell stage (stage 7), there is an apparent shift in some of the larger yolk platelets from the vegetal to the animal hemisphere (Fig. 2). Excluding those moving inward with cleavage furrows, the majority of egg melanosomes further condense at the cortex's periphery and a few are found below it. At the neural groove stage (stage 15), cells making up the prospective epidermis still possess yolk platelets and, in addition, now contain large vacuoles (Fig. 3). Egg melanosomes are homogeneously distributed throughout the cytoplasm, the highest number being found in prospective apical epidermal cells. At the tailbud stage (stage 19), the boundary between the epidermis and the yet to be developed dermis is clearly established (Fig. 4). A further segregation of egg melanosomes to apical epidermal cells is seen. Within these cells, an uneven distribution of egg melanosomes is now found for the first time. The majority of egg melanosomes are localized between the nuclei and the apical portions of the cells. In tail epidermal cells of stage-25 embryos, egg melanosomes are greatly reduced in number. In addition, they are now aggregated above the nuclei of most epidermal cells (Fig. 5). The "capping" of apical and basal cell nuclei by egg melanosomes is not homogeneous throughout the tail. Cells in the anterior portion of the tail have fewer caps and more individual egg melanosomes than do those in the posterior portion. Tail epidermis is essentially two layers thick; apical cells are conical while basal cells are triangular in appearance. The basement membrane complex which separates epidermis from underlying dermis is apparent as well as differentiating fibroblasts and dermal melanophores.

Electron microscopy of the aforementioned

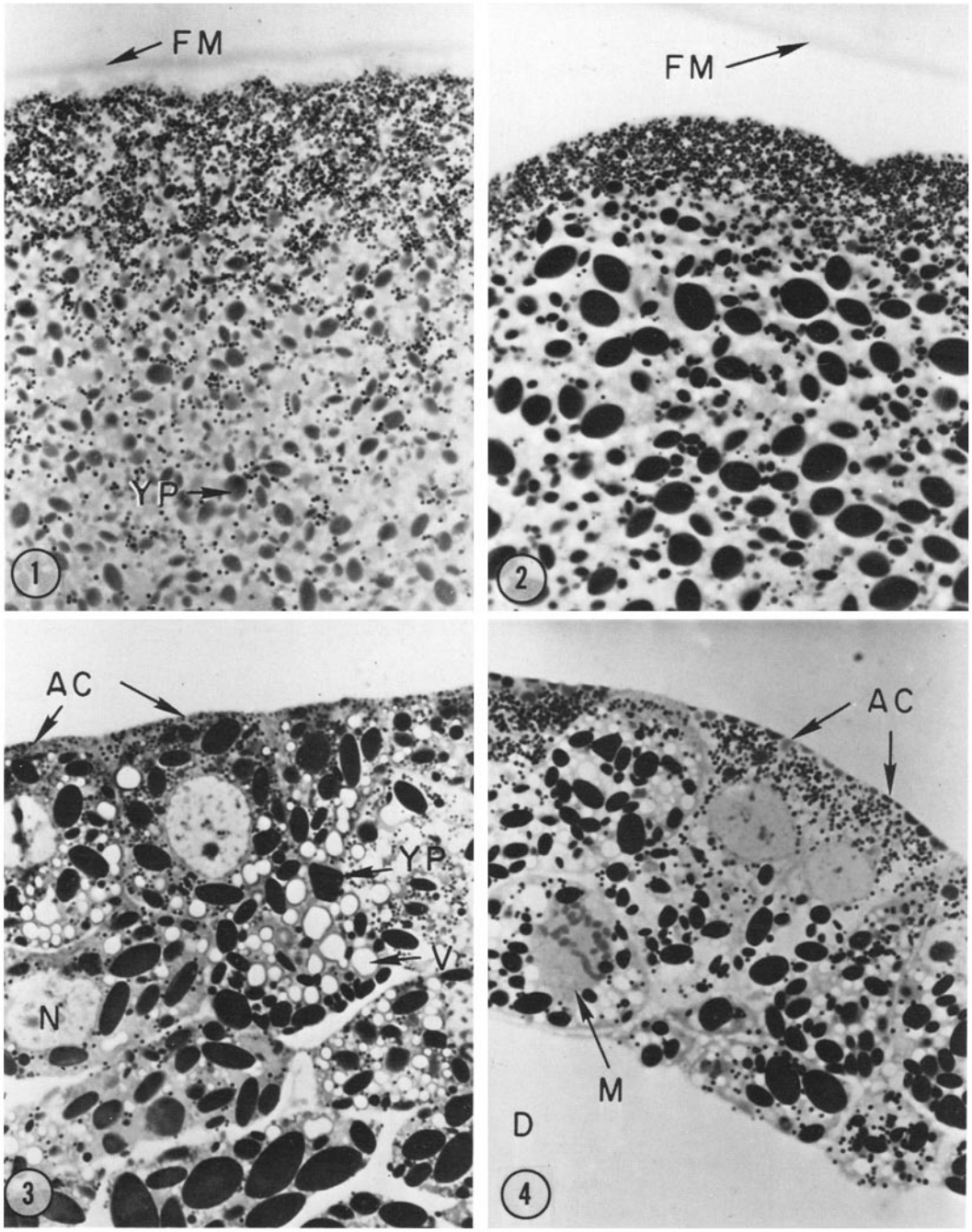


FIGURE 1 Light micrograph of a portion of the animal hemisphere of a fertilized egg (stage 2). Egg melanosomes appear as small black granules intermingled with yolk platelets (*YP*). Their density is greatest in the cortex; however, a few are scattered below the cortex. *FM*, fertilization membrane. × 950.

FIGURE 2 Portion of blastomere (stage 7), approximately the same area as Fig. 1, demonstrating the condensation of egg melanosomes in the cortex. In addition, larger yolk platelets from the vegetal hemisphere are now intermingled with those from the animal hemisphere. *FM*, fertilization membrane. × 950.

FIGURE 3 Cells making up the prospective dorsal epidermis of a neural groove stage (stage 15). Egg melanosomes are homogeneously distributed, the greatest numbers being found in apical cells (*AC*). In addition to yolk platelets (*YP*), vacuoles (*V*) are now seen. *N*, nucleus. × 950.

FIGURE 4 In stage 19, prospective epidermis is clearly separated from the yet to be developed dermis (*D*). Egg melanosomes are further segregated into apical cells (*AC*). In addition, a gradient is established within these cells; the majority of egg melanosomes lie between nuclei and outer apical cell membranes. *M*, mitotic figure. × 950.

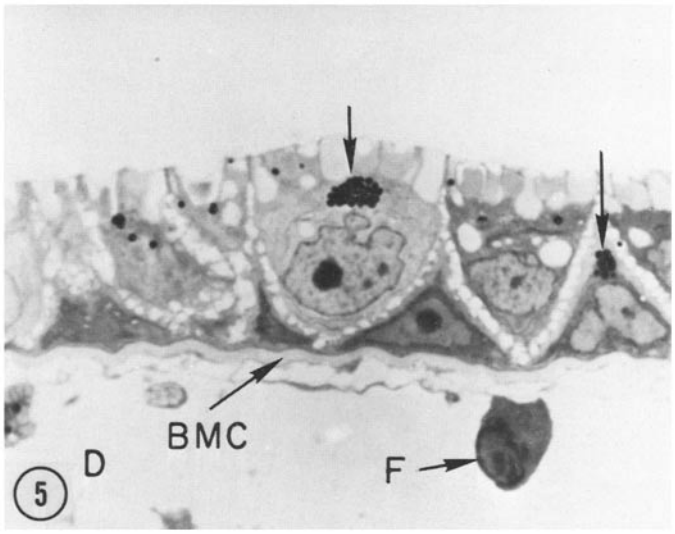


FIGURE 5 Tail epidermis and developing dermis of stage 25 embryo. Egg melanosomes further condense, forming caps over both apical and basal cell nuclei (arrows). Individual melanosomes not participating in cap formation are also found in apical regions of each cell. *BMC*, basement membrane complex; *D*, dermis; *F*, fibroblast. × 1250.

confirms the light microscope observations. Structures that might explain a mechanism by which egg melanosomes aggregate over nuclei were not observed. Aggregations of egg melanosomes in apical epidermal cells are situated above nuclei (Fig. 6). A discrete layer of mitochondria separates egg melanosomes from mucinogenic free borders which are composed of capped mucous vesicles. These goblet-shaped vesicles appear to be encircled by filaments running parallel to one another and to the apical surface. In a few cells, the apical borders are ciliated and lack mucous vesicles. Other components of the cytoplasm include at least several Golgi complexes, rough endoplasmic reticulum concentrically distributed around nucleus, smooth endoplasmic reticulum randomly distributed, and large membrane-bounded vesicles which are similar in appearance to the capped mucous vesicles. Some apical cells possess bundles of filaments, whereas others are completely devoid of them. All basal cells examined had these filaments. Except lacking mucous vesicles, basal cells seem to be similar to apical cells in organelle composition. Apical cells are attached to one another and to basal cells by desmosomes. Rarely do they come into contact with the basal lamina. Basal cells are attached to the basal lamina by hemidesmosomes. Many basal cells possess caps or individual egg melanosomes which are usually found in their apical regions (Fig. 7). Egg melanosomes are also found in cells of the dermis. No extracellular egg melanosomes have been found in either the epidermis or dermis. Occasionally, membrane-bounded bodies lie in or near the melanosome cap. They are quite prevalent in cells in which caps are reduced to a few melanosomes. Here, they are usually associated with a Golgi complex. Their internal structure reveals a homogeneous, partially dense, or crystalline matrix. One to several melanosomes have been found to occupy these bodies (Fig. 8).

With subsequent development, epidermal melanin units (Hadley and Quevedo, 1966) form as a result of epidermal melanocyte differentiation. The extent of an epidermal melanin unit is dependent upon the synthetic activity of the epidermal melanocyte. Aggregations of cytocrine melanosomes cap epidermal nuclei (Fig. 9). Larvae whose epidermal melanocytes lack cytocrine activity also lack cytocrine melanosome caps.

DISCUSSION

Amphibian egg pigment color ranges from tan to black (Stebbins, 1951). This color is due to membrane-bounded granules, some of which are homogeneously opaque and appear similar to melanosomes reported in melanophores of several amphibian species (Bagnara et al., 1968; Taylor,

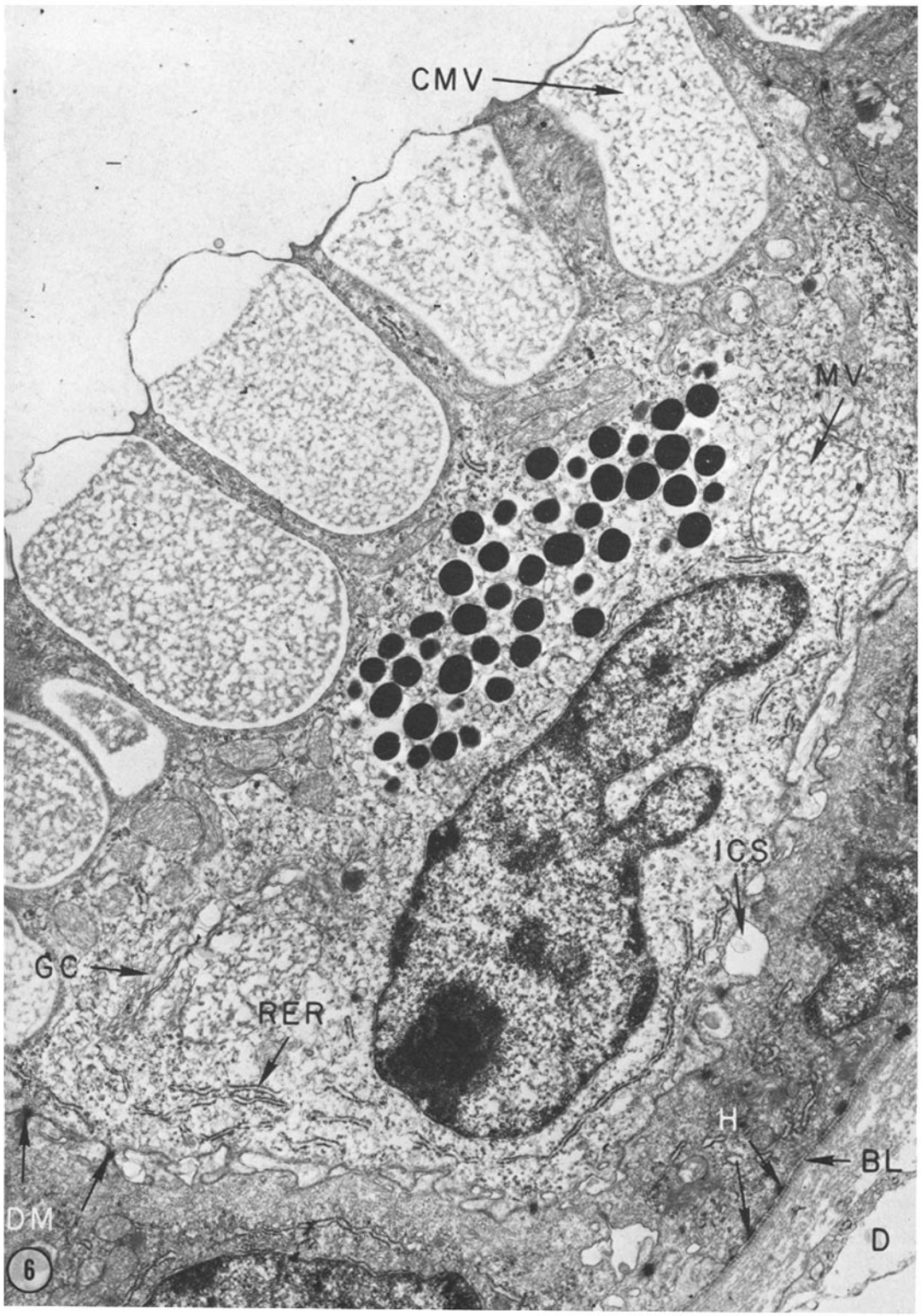


FIGURE 6 Electron micrograph of an apical cell and portions of basal cells in tail epidermis of stage 25 embryo. Egg melanosomes aggregate over the nucleus and form a cap. The apical border of the cell is composed of capped mucous vesicles (*CMV*). Separating the egg melanosome cap from the apical border is a layer of mitochondria. Other cytoplasmic components include the Golgi complex (*GC*), rough endoplasmic reticulum (*RER*), and mucous vesicles (*MV*). *DM*, desmosome; *H*, hemidesmosome; *BL*, basal lamina; *D*, dermis; *ICS*, intercellular space. × 8750.

1969; Bagnara and Taylor, 1970; Taylor, 1971). Others appear as particulate granules (Karasaki, 1959, 1963; Hope et al., 1964 *b*; Wischnitzer, 1966; Eppig, 1970). As yet, this type has not been reported in amphibian melanophores. It has been assumed that egg pigment is melanin (Wartenberg and Schmidt, 1961; Balinsky and Devis, 1963), but chemical verification of this point is lacking. Circumstantial evidence indicates that it is melanin, however. For example, eggs of albino frogs and salamanders lack pigment (Taylor, personal observations). In addition, Eppig (1970) has shown that egg pigment granules have the ability to further melanize if, during embryogenesis, they come to rest in differentiating retinal melanoblasts but not in neighboring cells. On the basis of this and their ultrastructural appearance, we believe that the term "egg melanosome" is consistent with pigment cell terminology (Fitzpatrick et al., 1966) and should be used until chemical analysis indicates otherwise.

At least four egg melanosome distribution-gradients are present during embryogenesis. The first two are established in the ovary: a peripheral-central and an animal-vegetal gradient. In other words, the highest concentration of egg melanosomes is in the cortex of the animal hemisphere. Apparently in *Rana temporaria* a size gradient is also present; egg melanosomes located at the animal pole are approximately twice as large as those elsewhere (Wartenberg and Schmidt, 1961). Melanosome gradients remain intact until gastrulation; however, the peripheral-central gradient is somewhat disturbed when cleavage furrows move some egg melanosomes to the interior. With the onset of gastrulation, the animal-vegetal gradient is reduced drastically as a result of morphogenetic movements, whereas the peripheral-central gradient remains intact. Those melanosomes that move to the interior have been reported to be in phagocytes or free within larval cerebrospinal fluid (Kordylewski, 1969) and in retinal-pigmented epithelium, mesenchymal cells, blood cells, and extracellular spaces of the eye (Eppig, 1970).

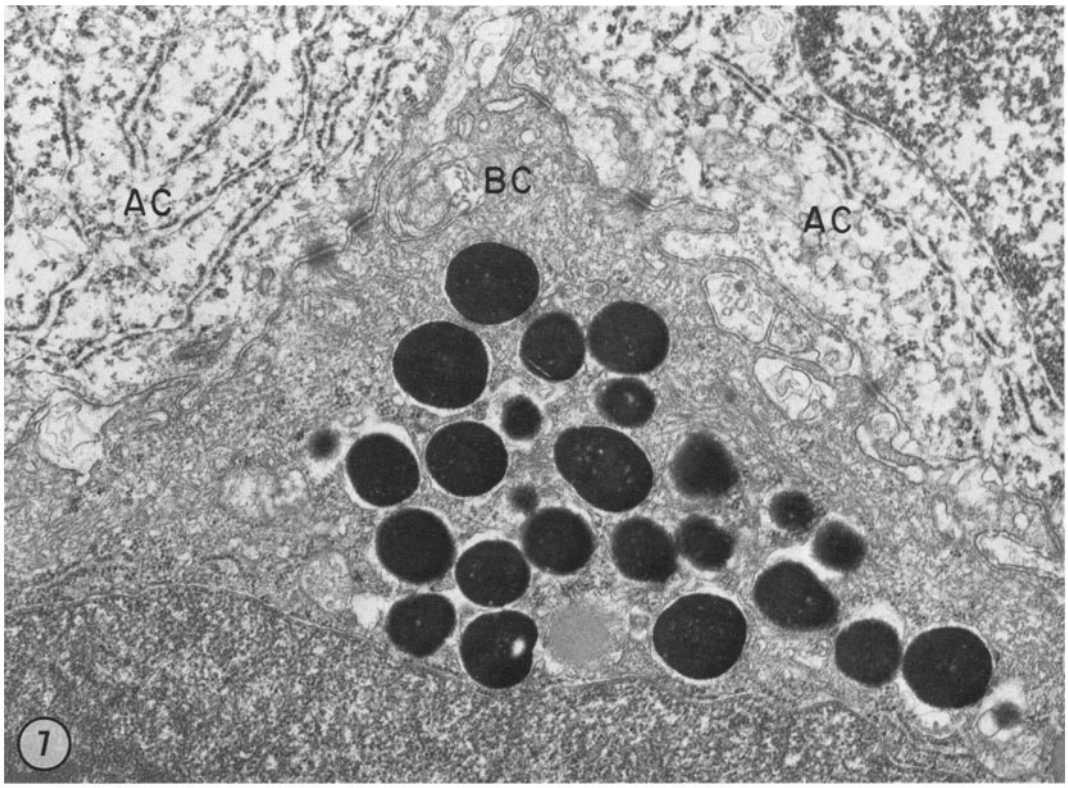
A third gradient is established in cells which participate in the formation of the presumptive epidermis. Here, apical cells have more egg melanosomes than do those cells below them. Later, this gradient is further amplified with the establishment of a fourth gradient, intracellularly within apical and basal epidermal cells themselves. The majority of egg melanosomes are localized between the nuclei and the apical membranes. The climax of the fourth gradient is the formation of egg melanosome caps over nuclei.

Newly differentiated epidermal melanocytes produce cytocrine melanosomes which cap epidermal cell nuclei. The mechanism appears to be similar to that reported for adult frogs and is the functional basis for the epidermal melanin unit (Hadley and Quevedo, 1966). Cytocrine melanosomes are believed to screen nuclei against deleterious radiation (for review, see Quevedo, 1969). Because of the constancy of their peripheral positions during embryogenesis, it would seem reasonable to assume that a possible role for egg melanosomes is also one of protection against deleterious radiation. Since the timing of the epidermal melanocyte's differentiation coincides with the loss of egg melanosome caps, it seems reasonable to suggest that cytocrine melanosome caps are now assuming the function of the egg melanosomes.

The results of this investigation demonstrate the morphological means by which egg melanosomes may protect embryos and young larvae during development. Differentiating epidermal melanocytes provide cytocrine melanosomes which assume this protective role at a later developmental stage. Simple as the egg melanosome's role appears, there remain questions yet to be answered. Among these are the mechanisms by which distribution gradients are established during vitellogenesis and later in the presumptive epidermis. In addition, events leading to cap formation are still to be determined. Finally, the timing of epidermal melanocyte differentiation and the loss of egg melanosome caps suggests a control mechanism

FIGURE 7 Junction between two apical cells (*AC*) and one basal (*BC*) cell. Note egg melanosome aggregation over the basal cell nucleus. × 20,500.

FIGURE 8 Egg melanosomes incorporated into a membrane-bounded body as a melanosome complex. The egg melanosomes' limiting membranes are missing. × 25,050. *Insert:* Enlarged portion of the complex demonstrating its organized crystalline matrix. × 66,550.



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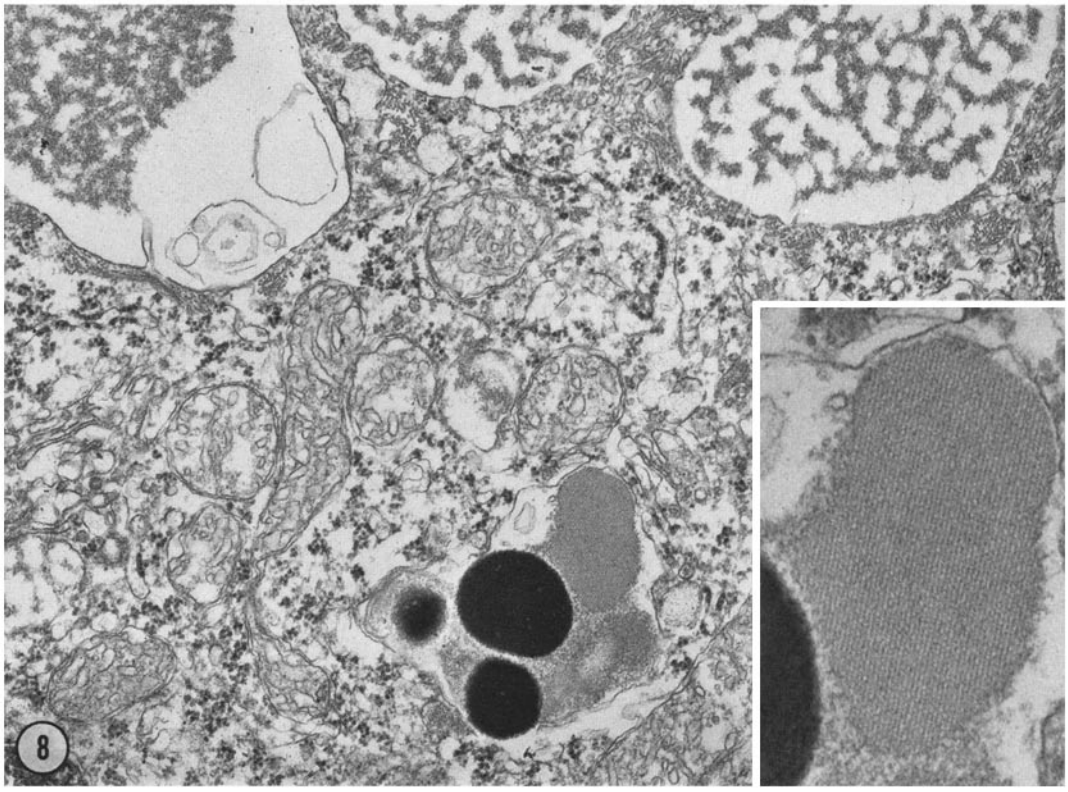

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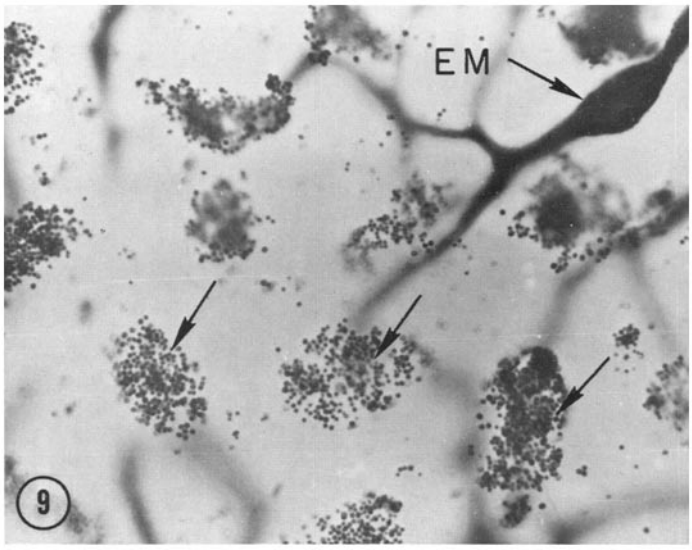

FIGURE 9 Light micrograph of unstained, whole-mount preparation of larval epidermis. Cytocrine melanosome caps (arrows) cover underlying nuclei. The presence of the nuclei was verified by phase-contrast microscopy. Epidermal melanocyte (*EM*) is responsible for cytocrine melanosome production. × 1100.

involving communication between these melanocytes and/or a control mechanism involving an external stimulus such as deleterious radiation. The answers to these and other questions may provide clues relative to the embryo and its environment and to the nature of cell organization and communication during embryonic development.

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