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Differences in Black Pigmentation in Lepidopteran Cuticles as Revealed by Light and Electron Microscopy*

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Summary. Black cuticles of larvae and pupae from various Lepidoptera were studied by light and electron microscopy. There are striking differences in the representation of black pigmentation, especially at the ultrastructural level. Two types may be described: 1. With the light microscope black melanin-like grana, electron-dense electron microscopically, are found in the distal parts of the exocuticle. This type is demonstrated in larvae of *Celerio euphorbiae*, *Papilio machaon*, and *Phalera bucephala*. 2. With the light microscope a dark homogeneous layer in the distal exocuticle can be recognized, however, electron microscopically no structures correlated with this dark pigment layer. This type of pigmentation was present in pupae of *Pieris brassicae* and *Aglais urticae*; in *Pieris* larvae the dark pigmented layer appeared to be limited to the epicuticle. In *Celerio* processes of the epidermal cells are involved in transporting precursors to the exocuticle. The conclusion was reached that black pigmentation in cuticles is based on different mechanisms as proposed by structural features. The two likely mechanisms are melanization and sclerotization.

Key words: Melanin – Sclerotin – Insect cuticle – Light microscopy – Electron microscopy.

Introduction

Insect color patterns have frequently been studied both chemically and biologically. In contrast to the present knowledge on the chemical structure and physiological relevance of most pigment classes, little is known about the regula-

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tion of those metabolic processes which lead to pigmentation (cf. Vuillaume, 1969; Needham, 1974; Fuzeau-Braesch, 1972).

Black pigmentation, despite its widespread occurrence in insects, has not thus far been thoroughly investigated. Without supporting analytical data, its chemical substrate has been widely accepted as "melanin". This unfounded supposition has largely been accepted as fact. The basis for this general lack of knowledge can be attributed in part to the fact that dark pigment may be produced by at least two distinct processes, i.e., melanization and sclerotization (cf. Needham, 1974). The products of both mechanisms are quite different in chemical and functional aspects despite the common precursor, tyrosine, in both pathways. Furthermore, melanization and sclerotization have been shown to be independent of each other (Fogal and Fraenkel, 1969; Mills and Fox, 1972). Therefore, experiments using labeled tyrosine may not give unequivocal results. An additional complication in the analysis of black pigment results from their insolubility in most solvents. Histochemical tests are also inadequate.

The present study deals with light and electron microscopy as primary tools in the analysis of black insect pigmentation giving a basis for labeling experiments. Thereby, striking differences concerning electron density, site and mode of deposition could be revealed. The results may be indicative of an occurrence of true melanin and sclerotin.

Material and Methods

Insects. Several Lepidopteran species were studied, all showing black pigmentation in their cuticles. Larval as well as pupal stages were examined: larvae of *Papilio machaon* (Papilionidae), *Celerio euphorbiae* (Sphingidae), *Phalera bucephala* (Notodontidae), and *Pieris brassicae* (Pieridae); pupae of *Aglais urticae* (Nymphalidae) and *Pieris brassicae*.

Small parts of the dorsal integument (cuticle and epidermis) with black spots were excised from the second abdominal segment.

Light Microscopy. The samples were fixed in Bouin's solution, dehydrated through an ethanol-isopropanol series and embedded in paraffin (Tissuemat, Serva, Heidelberg). Some of the 5–8 μm sections were examined unstained for native pigment. To facilitate discrimination between the different layers of the cuticle, the sections were stained with Azan according to Heidenhain or with Mallory's triple stain.

Additionally, semithin sections of tissue embedded for electron microscopy were examined by light microscopy. Micrographs were taken with a Zeiss photomicroscope.

Electron Microscopy. Cuticle samples were fixed in either a fixative according to Wohlfarth-Bottermann (1957) or in 2% buffered glutaraldehyde, postfixed in 1% buffered OsO_4, dehydrated in graded acetones, stained in block in a solution of uranyl acetate and phosphotungstic acid (both 1% in 70% acetone, w/v) and embedded in Epon 812 (Serva, Heidelberg). Sections were cut with glass knifes on a LKB ultramicrotome and examined with a Zeiss EM 9 A.

Results

Light microscopically, black pigments revealed two modes of deposition in cuticles, either as granular material or as a homogeneous layer.

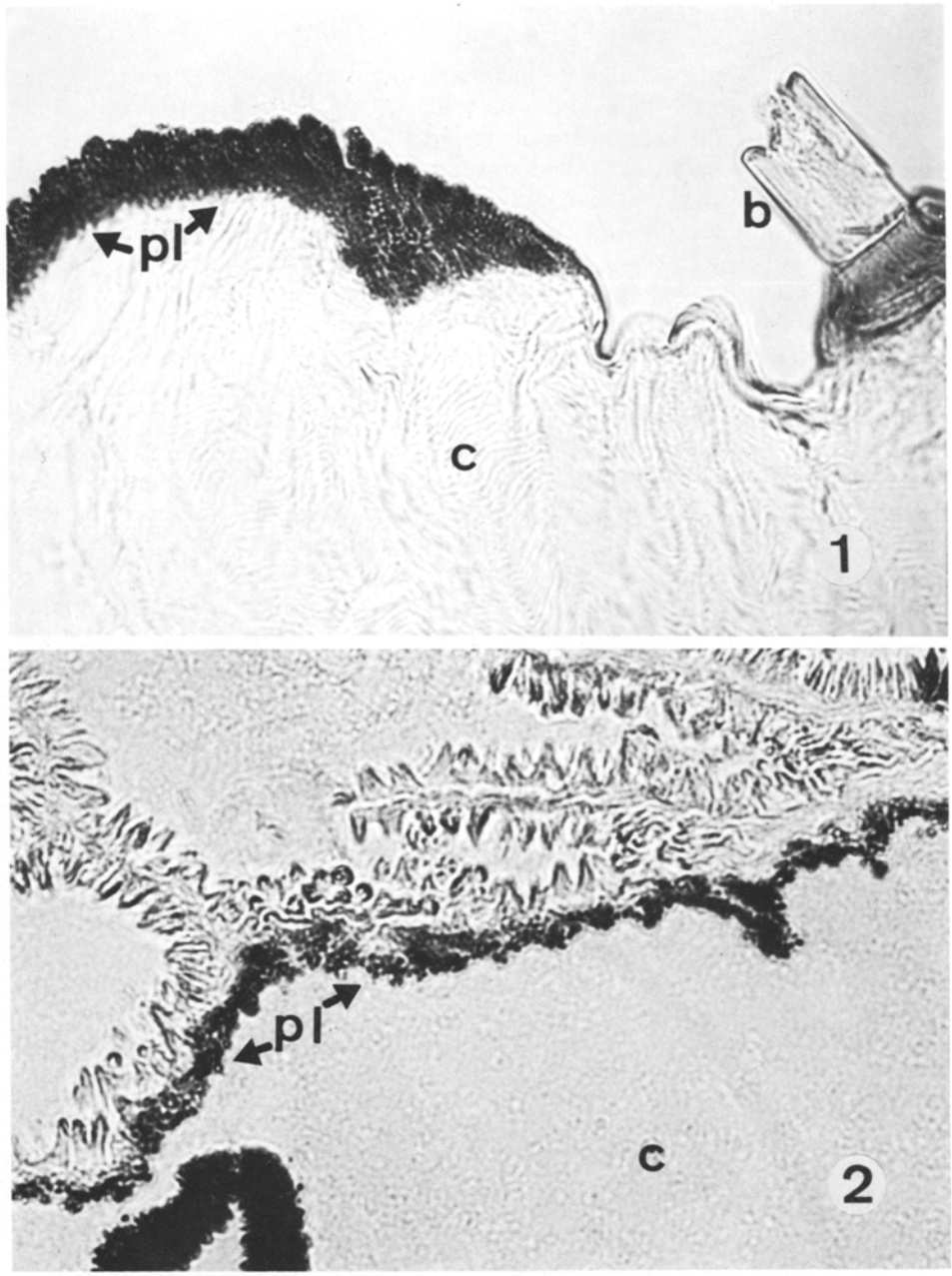


Fig. 1. Light micrograph of the larval cuticle (*c*) of *Phalera bucephala* showing the pigment layer (*pl*) consisting of numerous pigment grana. The bristle (*b*) is without pigment. ×400

Fig. 2. Light micrograph of the larval cuticle (*c*) of *Papilio machaon*. The pigment layer (*pl*) is composed of black grana. ×160

A. Black Pigment in Grana Form

The granular storage of black pigment was found in larvae of *Papilio, Phalera* and *Celerio*. With the light microscope black grana of different shapes could be distinguished in the distal parts of exocuticles (Figs. 1, 2). At the ultrastructural level these grana were electron dense and inhomogeneous in structure (Fig. 3). They were surrounded by a matrix and fibrillar components (Fig. 4). In all cases the epidermal cells were free from similar electron dense granules.

In the cuticle of *Celerio*, which had been fixed immediately after the last larval ecdysis, the epidermal cells exhibited processes which passed through the cuticle into the distal part of the exocuticle (Figs. 3, 5). Within these processes, grana were observed surrounded by a membrane and composed of material with different electron densities (Fig. 6). The distribution of these grana was such that the most electron dense grana were found in the distal parts of the processes, whereas the less dense ones were confined to the proximal parts. Supposedly, these processes are responsible for the transport of precursors of the black pigment (cf. Klaus, 1969). During transport, the synthesis of pigment or, at least, a metabolic alteration of the precursor may occur as indicated by the electron density of the distal grana.

In cuticles of *Papilio* and *Phalera*, which were examined in the middle of the last larval instar, no epidermal processes were found in the cuticle. Thus, no definite statement can be given concerning their appearance during ecdysis.

B. Black Pigment as a Homogeneous Layer

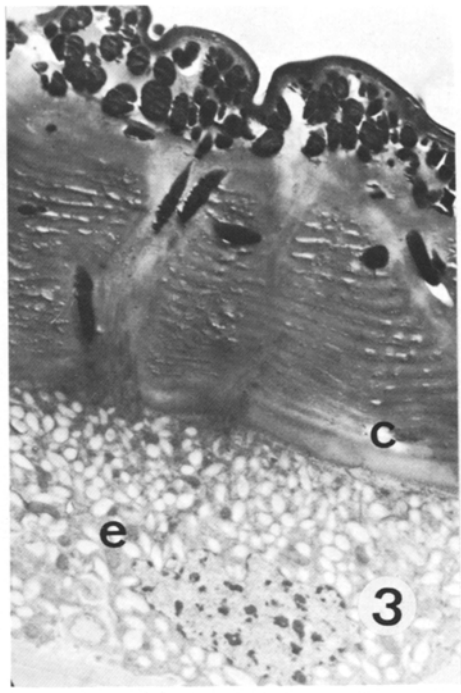
The black pigment was found to be homogeneously deposited in a distinct part of the cuticle in pupae of *Pieris* (Fig. 7) and *Aglais*. Examination of sections with the light microscope revealed areas of brown appearance, homogeneously pigmented in the distal parts of the exocuticles. These areas were not sharply outlined, but rather passed in a continuous pattern into the otherwise colorless regions of the cuticles. Similar brown areas were also found at the base of the bristles. In this case the pigmented layer was

Fig. 3. Electron micrograph of cuticle (*c*) and epidermis (*e*) of a larva of *Celerio euphorbiae*. The pigment grana are located in processes of epidermal cells. × 5,000

Fig. 4. Electron micrograph of the distal part of the larval cuticle (*c*) of *Papilio machaon* showing pigment grana (*pg*); *ep* epicuticle. × 12,000

Fig. 5. Electron micrograph of cuticle (*c*) and epidermis (*e*) of *Celerio* larva with processes of epidermal cells (*p*); *pg* pigment grana. × 10,000

Fig. 6. Electron micrograph of an oblique section of the cuticle (*c*) of *Celerio* larva. Processes of epidermal cells (*p*) passing through the cuticle show in their proximal part premelanosome-like material. × 20,000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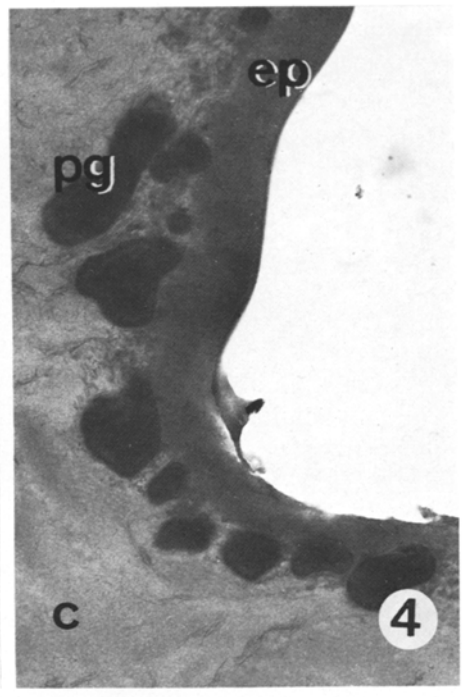
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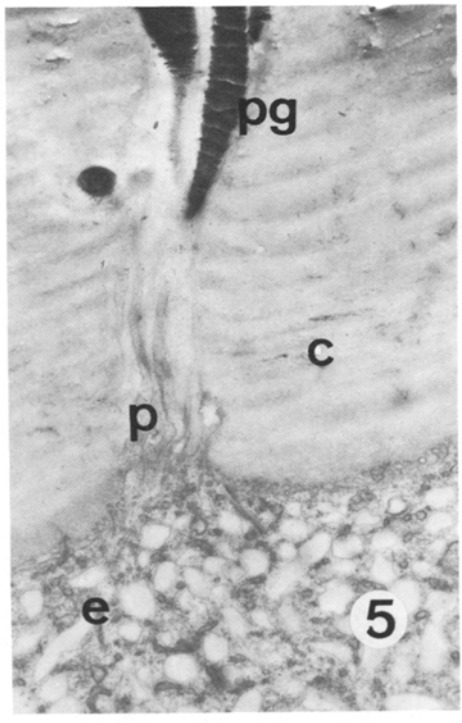
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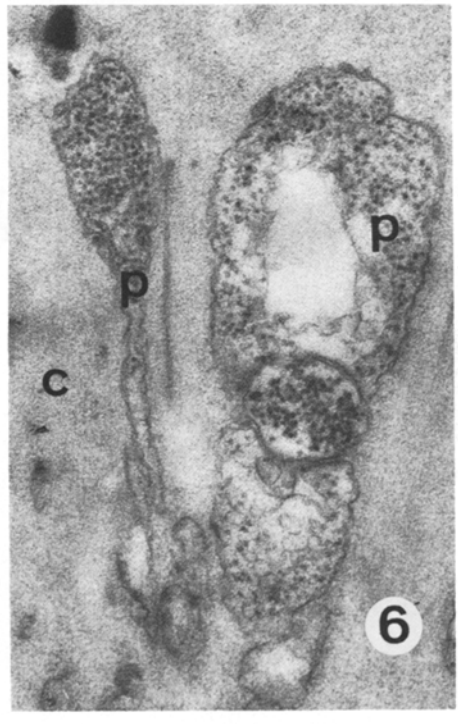
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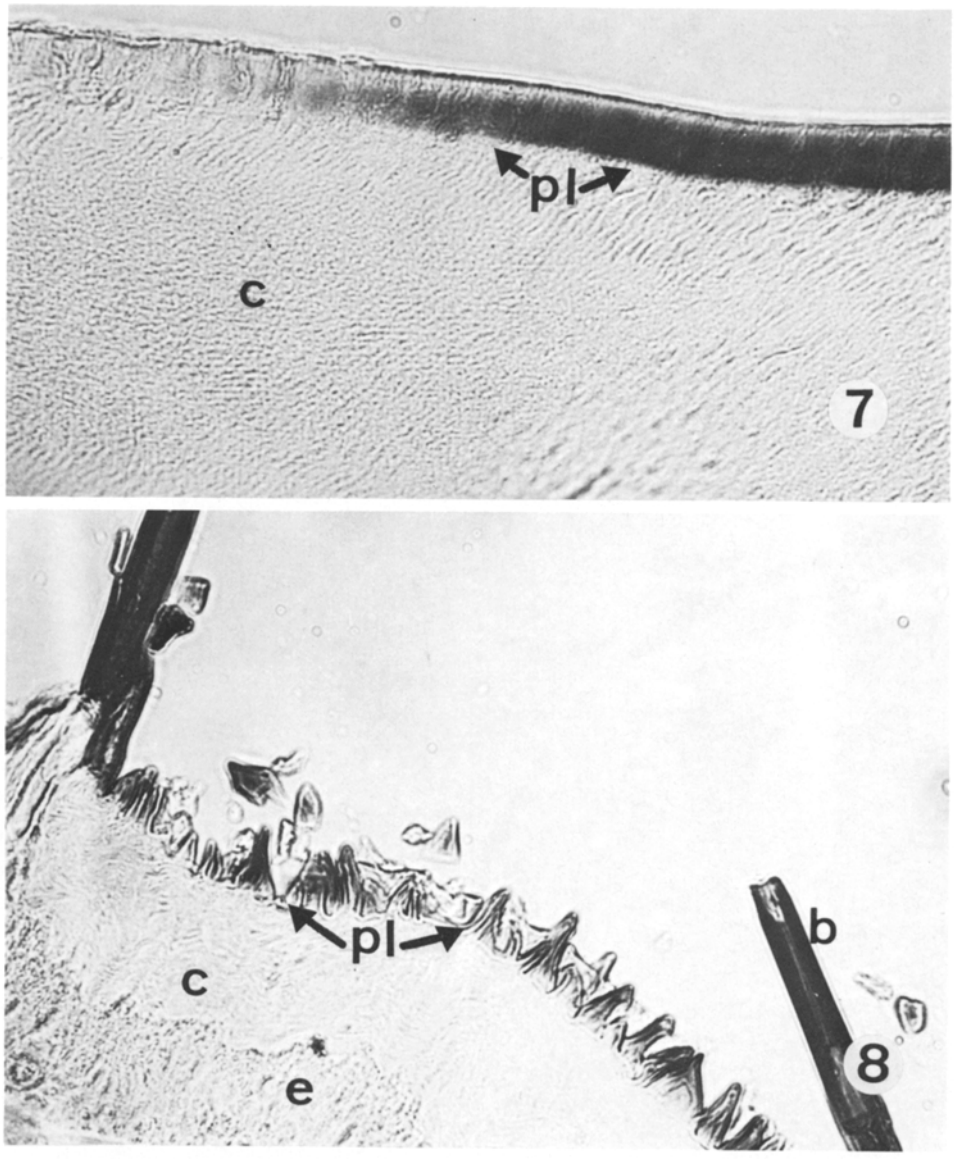

Fig. 7. Light micrograph of the pupal cuticle (*c*) of *Pieris brassicae*. The pigment layer (*pl*) homogeneously spread out in the exocuticle, does not consist of pigment grana. ×320

Fig. 8. Light micrograph of the cuticle (*c*) and epidermis (*e*) of a larva of *Pieris brassicae*. The pigment layer (*pl*) is limited to the epicuticle. Bristles (*b*) are also pigmented. ×130

thicker than in the other dark spots of the cuticle. In larvae of *Pieris* the dark pigmentation seemed to be limited to the epicuticle (Fig. 8).

In contrast to light microscopic studies, however, using electron microscopic methods no electron dense material could be demonstrated, neither as distinct grana nor in a homogeneous layer in those parts of the cuticle where the dark pigment was expected to be found as revealed by light microscopy.

Cuticles of *Pieris*, which were examined at two hour intervals during the larval to pupal transformation (Kayser-Wegmann, in preparation), did not show processes of epidermal cells extending into the cuticle at any time. Only microvilli were present.

Discussion

This study presents evidence that black cuticle pigmentation in insects is heterogeneous in nature. In different species, black pigmentation, despite a similar macroscopic appearance, may exhibit striking differences at the microscopic level. The different microscopic features are possibly related to two different chemical substrates causing black coloration, i.e. melanin and sclerotin.

Melanins, because of their chemical heterogeneity, are classified as eumelanins, phaeomelanins and allomelanins (cf. Nicolaus, 1968). Eumelanins are derived from tyrosine and are formed by a complex process involving quinones and free radicals. Phaeomelanins are synthesized from both tyrosine and cysteine. Allomelanins are found only in plants and are formed from nitrogen-free precursors. More recent experiments have shown that tryptophane and other aromatic compounds can also be utilized as substrates by the enzyme tyrosinase to form black pigment (de Antoni et al., 1974).

In vertebrates melanin is synthesized within melanocytes and is translocated by dendrites to keratocytes for final deposition (Klaus, 1969). As presented here, quite similar results are obtained with insects. In *Celerio* melanin-like grana are located in the outer parts of the exocuticle, thus providing the basis for black coloration. Synthesis of the pigment probably occurs within those grana which are present in epidermal cell processes passing through the cuticle. This granular material may be compared with premelanosomes, containing enzymes and substrates as is known from vertebrates (cf. Nicolaus, 1968). In both cases the content of the grana is transformed from granular to homogeneously electron dense material.

In studies with insects, several reports have dealt with the granular storage of black pigment in exocuticles. Hackmann (1967) concluded that the black pigment of the puparium in *Lucilia* must be a melanin as judged by its solubility in alkali and its chemical degradation to indol derivatives. Fuzeau-Braesch (1970) using labeled tyrosine identified the black pigment in the larvae of *Tyria* as a melanin. Barbier (1972), studying the same insect with the light and electron microscopes, described melanin-like grana in the distal parts of the exocuticle. From histochemical results he concluded that the grana are composed of melanin and not sclerotin. However, Barbier assumed an extracellular transport of precursors via cuticle pore canals. Furthermore, pigment grana have also been

found in the exocuticle of *Mallacosoma*. Due to overlying filaments in the epicuticle, producing a Tyndall effect, the cuticle appears blue (Byers, 1975).

Sclerotization is a special ability of arthropods to harden and darken their exosceleton. During this process N-acetyldopamine is linked to proteins either in a quinoid form, thus producing dark sclerotins, or via its side chain to form colorless tanned cuticles (Andersen, 1971). The precursor is supplied by hemocytes to the epidermal cells and secreted there into the procuticle for tanning (Post, 1972).

Returning to the pupal cuticles of *Pieris* and *Aglais*, the black spots and areas may be the result of a quinoid sclerotization, whereas the otherwise colorless parts of the cuticles may be tanned by the side chain process. The actual existence of this latter mechanism in the *Pieris* cuticle is supported by Andersen and Barrett (1971). Additionally, the absence of electron dense granules (supposed to contain precursors in the case of the melanin-like pigment) either in cellular processes or in the epidermal cells itself is thought to be an argument in favor of sclerotization.

In contrast to the pupa, in the larva of *Pieris* black material was observed in the epicuticle, not in the exocuticle. As summarized by Richards (1967), black pigment may be present in the epicuticle as well as in the exocuticle of insects, but more commonly in the exocuticle. Whether these two sites of color production are related to different modes of sclerotization is presently unknown.

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