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# Inducing Melanotic Nodules within the Fat Body of the Last Instar Larvae of *Epilachna varivestis* (Coleoptera) by Azadirachtin

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Injection of minute amounts of azadirachtin causes formation of numerous small (20- to 30-μm diameter) nodules in the fat body of last instar larvae of the Mexican bean beetle, *Epilachna varivestis*. About 1 day after injection, some fat body cells start degenerating by necrosis. The first visible sign is a leakage of the membranes as detected by a tracer substance (colloidal lanthanum nitrate). The remnants of the distributed fat cells are pinocytosed and possibly phagocytosed by other fat cells. Subsequently, such fat cells develop large heterolysosomes. After the degeneration of these cells, the heterolysosomes become free within the intercellular spaces of the fat body and clot with remnants of other cells by forming large aggregations which become black during condensation. During this process, hemocytes form a multilayered coat around the aggregations, which then stop growing. For several reasons it is presumed that the formation of these melanotic nodules is correlated with the interference of azadirachtin with premetamorphic events in the larvae. 

KEY WORDS: *Epilachna varivestis;* Coleoptera; Coccinellidae; Azadirachtin; melanotic nodules; ultrastructure.

## INTRODUCTION

Tetranortriterpenoid azadirachtin (for structure, see Kraus et al., 1985) has been known as a natural insecticide for several years (Steets and Schmutterer, 1975; Steets, 1976). In insects it induces various symptoms after oral application, e.g., occurrence of certain black head and thorax areas in larvae (Schmutterer and Rembold, 1980). These black areas arise from subcuticular deposits of melanin caused by increased autolysis of epidermal regions, especially the imaginal anlagen (Schlüter, 1981, 1985a). Similar melanotic changes can be induced within the fat body by injection of pure azadirachtin into the hemolymph (Schlüter, 1986). Its distribution, development, and evaluation within $L_4$ larvae of the Mexican bean beetle, *Epilachna varivestis*, is described here in detail.

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## MATERIALS AND METHODS

*E. varivestis* belongs to the coleopteran family Coccinellidae. Up to 10 larvae were cultured in plastic containers (18 × 23 × 6 cm) the cover of which was fenestrated; these holes were closed by gauze for regulation of atmospheric humidity and better ventilation. The larvae were fed on bean (*Phaseolus vulgaris*) leaves placed in small water-filled glasses. Single larvae were kept in Petri dishes with wet paper at the bottom and with a fresh bean leaf daily.

The investigation described here was done on 0- to 3-day-old $L_4$ larvae; earlier stages did not show any melanotic nodules.

Azadirachtin was obtained from the seed of the neem tree, *Azadirachta indica*. Azadirachtin (0.05–0.2 μg) dissolved in ethanol (90%) was injected into each larva using a 1-ml Hamilton syringe. The needle penetrated the thorax from its left side; the injection area was neglected during subse-



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quent investigations while all other regions were investigated.

Tissues were fixed in 2.5% glutaraldehyde in phosphate or cacodylate buffer, *p*H 7.2–7.4, for 4–6 hr, and embedded in Araldite. For histological investigations, semithin sections were stained with toluidine blue (1% in water with 0.1% borax). For transmission electron microscopy, ultrathin sections were contrasted with uranyl acetate and lead citrate.

For scanning electron microscopy, samples were embedded in gelatine capsules filled with Araldite without accelerator and transferred into liquid nitrogen for 15 min. These deep-frozen capsules were crushed using a hammer and Araldite was dissolved again in propylene oxide. Samples were critical-point dried and sputter coated as described previously (Schlüter, 1985d).

To ascertain the distribution of melanin, semithin sections were stained using a modified Warthin–Starry technique (Van Duinen et al., 1983). Control tissues were incubated without silver nitrate and subsequently bleached in 10% $H_2O_2$ for 12–14 hr. For a phenoloxidase test, small tissue samples were fixed in glutaraldehyde in phosphate buffer, *p*H 7.2, and incubated 8–10 hr in 0.1% DOPA/phosphate buffer on a shaker. The control samples were incubated in solutions containing 0.5% phenylthiourea.

## RESULTS

### *Induction, Distribution, and Morphology of Nodules*

The rate of development in insects depends on temperature. The $L_4$ stage of *E. varivestis* takes 5 days at 25° ± 2°C until fastening (i.e., beginning of prepupa phase). After an additional 1.5–2 days, the prepupa molts into the pupa stage.

Pupation is prevented by injection of azadirachtin into 40-hr-old $L_4$s; this means before the critical stage of ecdysis. Initially, 0.05–0.2 μg per specimen seems not to impair development; e.g., body weight increased as in the controls. The prepupa stage, however, is never reached and later disorder of motility occurs; many of the larvae are no longer able to hold fast to leaves. Application of 2.5 μg prevents weight increase and larvae die earlier than at lower doses. Application of 0.05–0.2 μg azadirachtin to early $L_4$s prolongs this larval stage. Therefore, tissue reactions caused by azadirachtin that occur after several days can easily be observed.

In nearly all treated specimens the fat body of thorax and abdomen is dotted by homogeneously distributed black nodules 6–12 days after injection (Fig. 1). The nodules may occur singularly or arranged in groups. They are spherical and usually 20–30 μm in diameter. They reach their final size 8–12 days after injection.

Such nodules cannot be initiated by azadirachtin in earlier larval stages. They are, moreover, not formed in late $L_4$s after the critical stage of ecdysis, in pupae, and in adult animals.

The center of each nodule consists of nonhomogeneous condensed material that causes artifacts like lipid droplets ("shatters") when sectioned on an ultramicrotome (Fig. 2). Broken fragments indicate a low penetration rate by Araldite. This center is surrounded by a multilayer of flattened hemocytes interconnected by fibrous structures (Figs. 8, 9). Additional hemocytes are located in close vicinity to each nodule.

FIG. 1. Photograph of an isolated fat body lobe dotted with melanotic nodules (arrows). Bar = 300 μm.

FIG. 2. Electron micrograph from a section of a young nodule within fat body (FB). The nonhomogeneous center is surrounded by mostly flattened hemocytes (H). N, nuclei of these; NF, nucleus of fat body cell. Bar = 5 μm.


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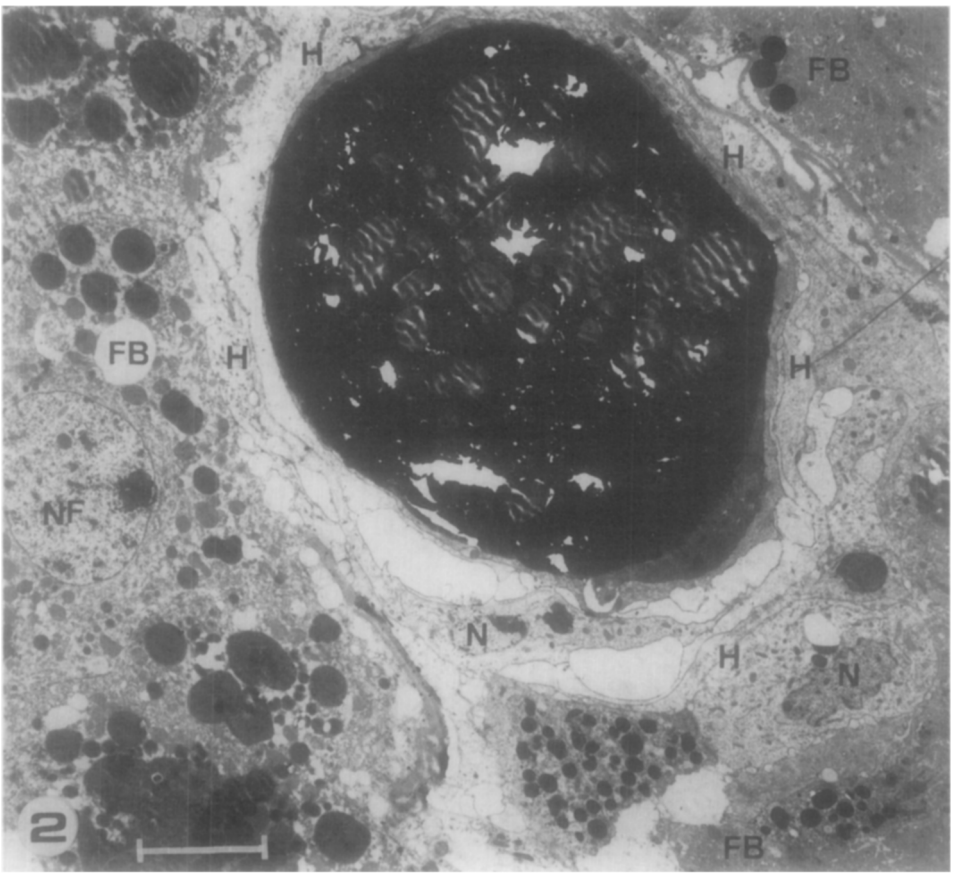
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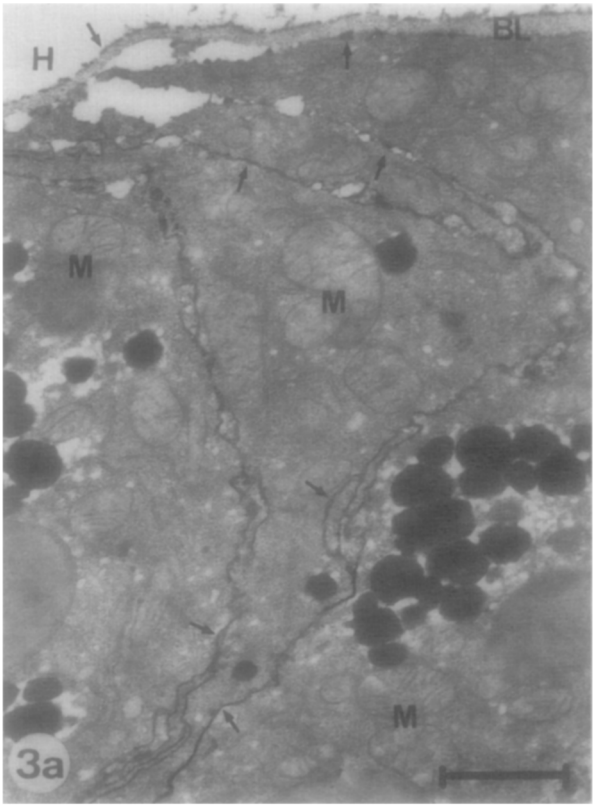
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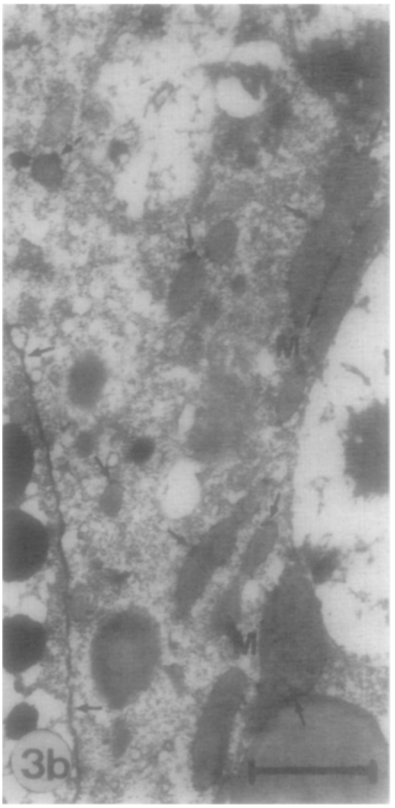
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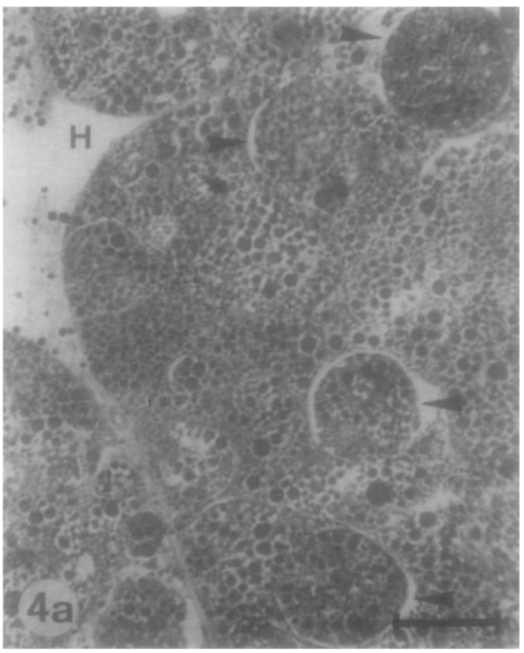
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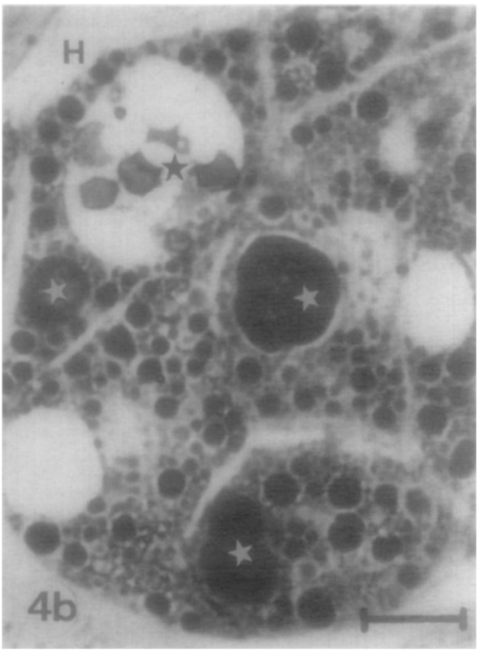
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*Development of Melanotic Nodules*

The development of nodules starts about 1 day after injection. At this early stage, cell membranes of some fat body cells are altered in their properties: after incubation of fat body from treated specimens in lanthan nitrate, this substance occurs within certain cells, especially aggregated with mitochondria (Fig. 3b). In untreated control specimens, lanthan nitrate, however, is only found within the intercellular spaces close to the cell membranes (Fig. 3a).

Cells with their obviously damaged membranes then become segregated from neighboring cells and are spherical. Their cytoplasm occurs condensed initially but becomes loosened at the end (Fig. 4a). Finally, the membrane disrupts and the cytoplasm is found in the intercellular space (Fig. 4b). Neighboring cells which remain unchanged start to pinocytose and, perhaps, to phagocytose the components of the lysed cells as revealed by injection with horseradish peroxidase. This tracer occurs not only within the intercellular spaces but also in vesicles of the surrounding cells (Fig. 5). Within these cells large heterolysosomes develop (Fig. 4b). Finally these cells also degenerate. Their fragments aggregate containing initially intact organelles (e.g., cisternae of endoplasmic reticulum), lipid droplets, and material from protein vacuoles (Fig. 6). At this stage the occurrence of melanin can be shown (inset).

The aggregations become more compact and simultaneously phenoloxidase-positive hemocytes occur within the fat body areas (Fig. 7). Most of these are granulocytes; cystocytes occur in lower numbers. They finally surround as a multilayer the melanotic aggregates. During this process they are flattened and build up their characteristic interconnections mentioned above (Figs. 8, 9). Their inner layers degenerate and become involved within the melanotic center of the nodule that has reached its final stage and definitive size.

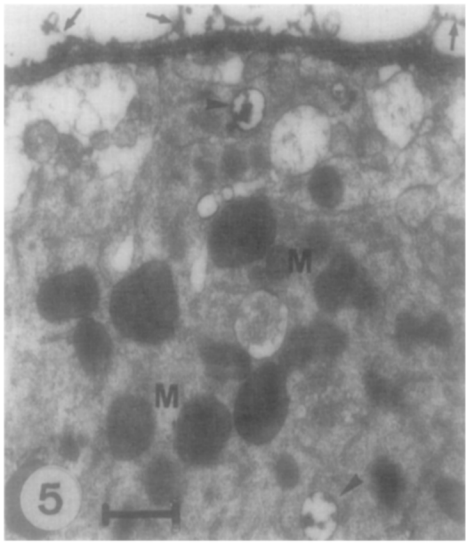
## DISCUSSION

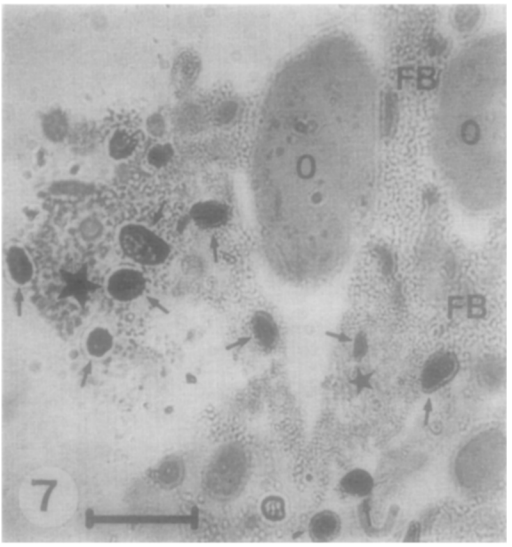
Melanotic nodules are widespread among insects. They usually are initiated as defense reactions against pathogens like fungi (cf. Vey and Fargues, 1977) or bacteria (cf. Ratcliffe and Gagen, 1977; Walters and Ratcliffe, 1983). They develop while coagulating hemocytes secrete substances after contact with the pathogen. At this area the pathogens aggregate and become melanized. Subsequently, hemocytes often build a multilayered sheath around the foreign body (Ratcliffe, 1982). Larger biotic foreign bodies (e.g., eggs of parasitic insects or nematodes) or abiotic implants are immediately encapsulated by hemocytes (cf. Schmit and Ratcliffe, 1978). Cellular changes and hemocyte reactions during melanotic nodule formation have been described by Salt (1970) and Nappi (1984).

Similar mechanisms occur as in the formation of the nodules described above. These, however, are not initiated by foreign bodies but from humoral substances obviously activated by azadirachtin. In this context it should be mentioned that melanotic nodules could also been induced by application of juvenile hormone or juvenile hormone-like substances (Bryant and Sang,

FIG. 3. Electron micrograph of sections through fat body cells incubated with lanthan nitrate of an untreated (a) and of a treated (b) specimen. In (a) the membrane tracer (arrows) is found close to the basement lamina (BL) and cell membranes outside of cytoplasm. Azadirachtin application causes membrane penetration of some cells, in which lanthan nitrate can be seen within cytoplasm, particularly aggregated with mitochondria (M). H, hemocoel. Bars = 1 μm.

FIG. 4. Sections through segregated spherical cells (a, arrow heads) and at the stage of advanced damage (b, black star). H, hemocoel; white stars mark growing heterolysosomes in neighboring cells. Bars (a) = 40 μm, (b) = 15 μm.



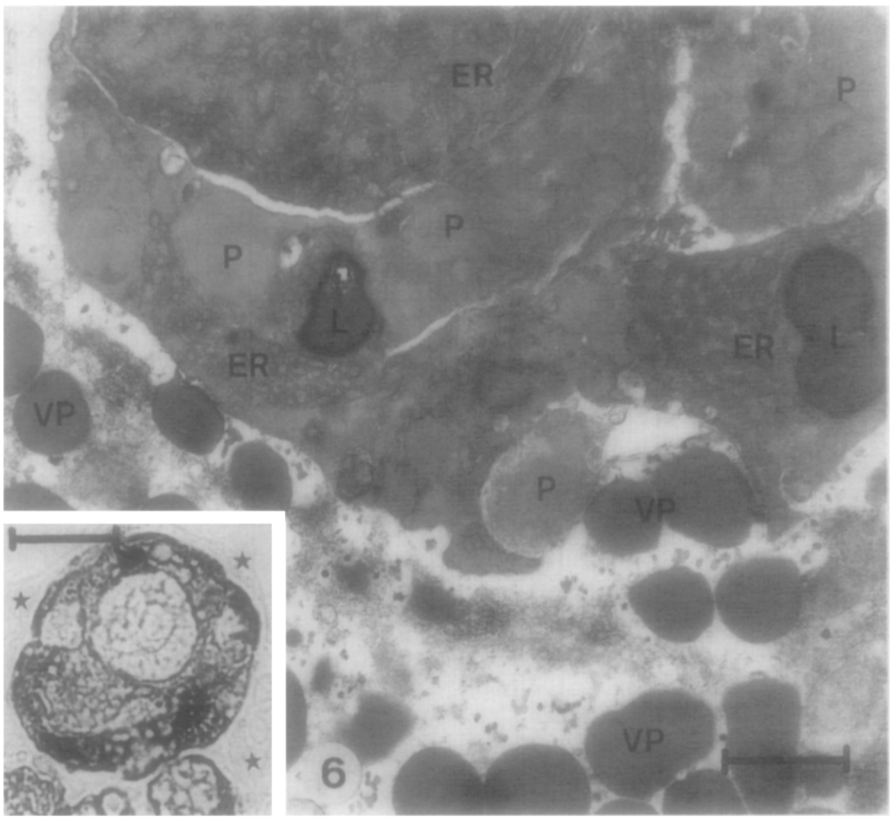
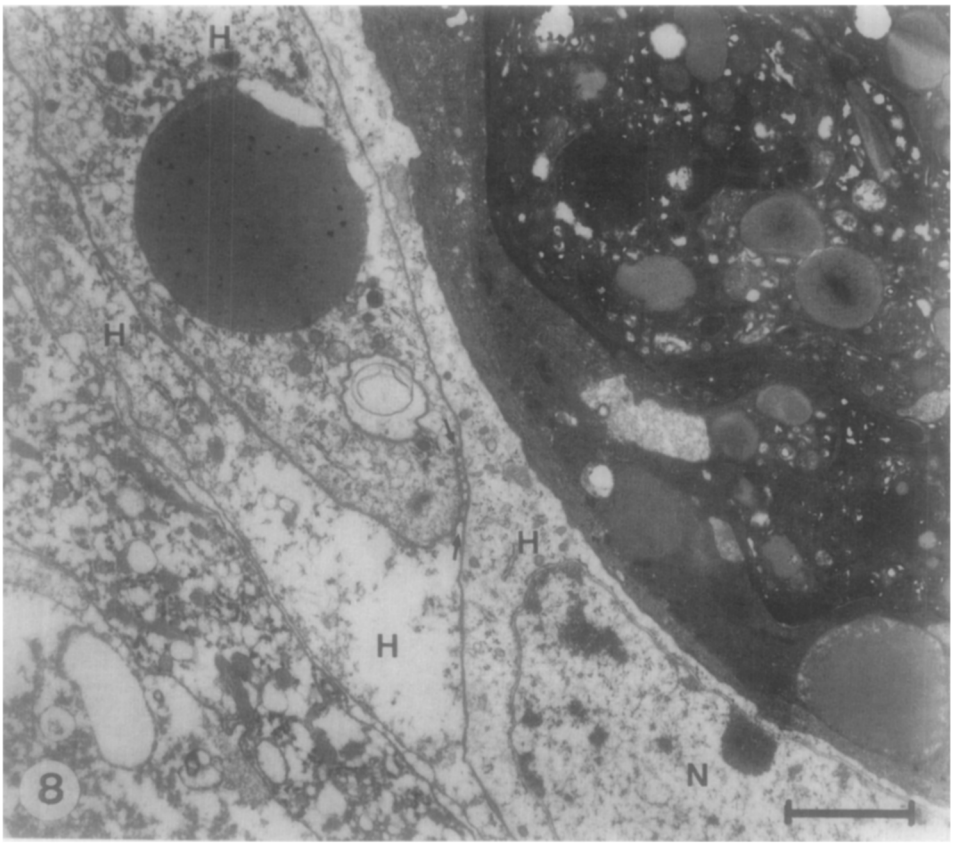




FIG. 5. Section of a so far intact fat body cell after injection of horseradish peroxidase. The tracer is found at the membranes along intercellular spaces (arrows) but also within vesicles (arrowheads). M, mitochondria. Bar = 0.5 μm.

FIG. 6. Electron micrograph from the region of a developing heterolysosome within a degenerating fat body cell. ER, incorporated cisterna of endoplasmic reticulum; L, lipid droplets; P, proteinaceous material; VP, protein vacuoles. The inset is a light micrograph of a section through melanized cells within unstained (not melanized) fat body tissue (stars). Bars = 1 μm, inset = 15 μm.

FIG. 7. Light micrograph of a small tissue sample providing the presence of phenoloxidase-containing hemocytes (arrows) in the vicinity of a damaged fat body area (star). FB intact regions; O, oenocytes. Bar = 35 μm.



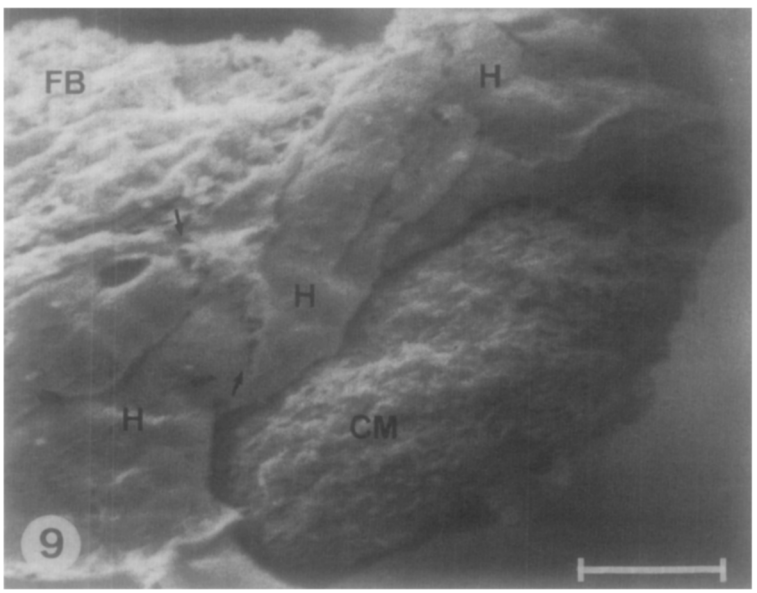




FIG. 8. Electron micrograph of a section through a nodule. Upper right half shows a sector of the melanotic center. H, encapsulating hemocytes with connecting structures (arrows); N, nucleus. Bar = 1 μm.

FIG. 9. Scanning electron micrograph of a freeze-broken fat body lobe containing a nodule. CM, melanotic center, partly laid open; FB, parts of the fat body; H, enveloping hemocytes with connecting structures (arrows). Bar = 12 μm.

1969; Madhaven, 1972) and after exstirpation of stomodeal ganglia (Dorn, 1978).

Barigozzi (1958, 1969) denoted melanotic nodules as "pseudotumors." This term would appear to be no longer correct. Neoplastic growth typical of true tumors (Gateff, 1978) is lacking; melanotic nodules show a limited growth. Even pseudotumors, which by definition imply swelling, are inflammatory structures. Probably they do not originate from a specific reaction to azadirachtin because (1) after injection they require a longer period to develop, and (2) melanotic nodules within the region of the wing anlagen may also be induced by the antibiotic chartreusin (Holst and Schlüter, 1984). It is remarkable, however, in the case reported here that a high specificity of developmental stage in which pseudotumor can be induced and of reactive tissue exists. Neem substances or pure azadirachtin do not cause such melanotic nodules if fed to or injected into different larval stages, the pupa, or imago, although damage of other tissues may occur. This indicates that their development is closely related to premetamorphic changes within the fat body at that time.

Actually, large amounts of stored protein coagulate with other cellular elements within the $L_4$'s fat body during genesis of nodules. Fat cells of later $L_4$s also contain larger amounts of future cuticular proteins and tyrosine. The latter is stored in specific vacuoles (Locke, 1984) and is one of the ground substances for sclerotization of the cuticle. Azadirachtin inhibits all metabolic processes involved in molting as necessary materials remain within the fat body cells (Schlüter, 1985c). From the degenerated cells they finally reach the big cytolysosomes by autophagy. There melanin is synthesized via tyrosine metabolism by polymerization of indolchinone as its early reaction shows. Hemocytes are not necessary at this stage; they react later due to the azadirachtin reaction. How this reaction is induced remains unsolved. Probably a breakdown of cells is necessary that sets free the specific metabolites within the hemolymph.

Another related phenomenon should be noted: melanotic reactions described here cannot be induced in young $L_4$ larvae (Schlüter, 1985b). This fact can also be explained by the retaining of future cuticular substances within the fat body cells for only in the case of mixture of numerous substances in the hemolymph, autolyzed tissues (epidermis, imaginal anlagen), and cuticular material does reaction occur leading to the typical melanization.

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