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Immunohistochemical Contribution to the Study of Morphine Metabolism in Calliphoridae Larvae and Implications in Forensic Entomotoxicology

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ABSTRACT: Morphine was detected by immunohistochemistry on sections of third stage larvae of *Calliphora vomitoria* (Diptera, Calliphoridae) reared on minced beef meat previously treated with morphine hydrochloride. The detection was performed with an avidin-biotin-peroxidase-complex method. Positive specimens showed specific staining of the haemolymph and a more intense immunoreaction in an area located at the limit between exocuticle and endocuticle. These results constitute an evidence of morphine accumulation inside the cuticle of Diptera larvae during their development. During the pupariation, the larval cuticle is transformed into the sclerotized puparium. This study consequently points out the possibilities of analyzing empty puparia when suitable tissues or living necrophagous insects are absent.

KEYWORDS: forensic science, immunohistochemistry, morphine, *Calliphora vomitoria,* forensic entomology

It has been well established that insects, particularly Diptera larvae, can be used as alternate specimens for toxicologic analyses when conventional postmortem samples are not available (1–3). The metabolism of the drugs by insects in interpretation of results of such analysis has certainly a great significance, but this metabolism is largely unknown and there have been especially few studies in which larvae reared on artificial media containing different concentrations of common drugs were analyzed (4,5). In a new approach to the study of larval metabolism of drugs, we investigated the potential for detection of morphine in larval tissues of *Calliphora vomitoria* L. (Diptera, Calliphoridae) using immunohistochemical techniques.

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Materials and Methods

Approximately 500 eggs of *C. vomitoria* were placed on 1 kg of minced beef meat that had been combined with a morphine hydrochloride solution to give a concentration of 10 mg/kg of morphine. Another colony of approximately 500 eggs was established on 1 kg of untreated minced beef. Both colonies were placed into a thermoregulated cabinet (Aqualitic, BIOBLOCK SCIENTIFIC) at 25°C. On Day 5, when larvae had reached the active feeding 3rd instar, 50 larvae were removed from each colony. After a thorough washing in PBS-buffer, the larvae were fixed in 4% buffered paraformaldehyde. Ten of the fifty larvae were then embedded in paraffin. Samples were sectioned at 4 μm and treated immunohistochemically using an avidine-biotin-complex (AB-complex) technique for morphine.

First, endogenous peroxidase activity was blocked with 5% H_2O_2. To reduce nonspecific binding to the primary antibody, specimens were treated with buffered goat serum (dilution 1:100 in PBS-buffer). Morphine detection was accomplished using a polyclonal morphine-antibody from a rabbit (SIGMA M 9023) as the primary antibody (dilution 1:1000 in PBS-buffer) which has 100% cross reactivity with codeine. The secondary antibody was a biotinylated antirabbit IgG antibody (DAKO E 0432) (Dilution 1:300 in PBS-buffer). Detection of the specific antigen binding was accomplished using the avidin-biotin-peroxidase-complex method (BIOSYS VECTOR PK 4000) with 3-amino-9-ethyl-carbazole as chromogen (BIOGENEX HK121-5K). Finally, specimens were counter-stained with hematein for 2 to 3 sec. At the same time and to avoid possible cross reactivity of the morphine with the assay, control tests were performed on each larvae in which primary and secondary antibodies were successively omitted. This method was done for larvae from both control and test colonies.

Results

All immunochemical procedures and histological stainings were done on sections taken from 3rd instar *C. vomitoria* larvae. Each positive specimen showed specific staining of the haemolymph just under the integument (Fig. 1), while controls and test larvae in which the primary or the secondary antibody were omitted showed no staining reaction. The immunoreaction was observed to be more intense at the boundary between exocuticle and endocuticle (Figs. 2, 3).

Discussion

The integument of *C. vomitoria* larvae (Fig. 4) consists of a single layer of epidermal cells and an outer cuticle. There are three

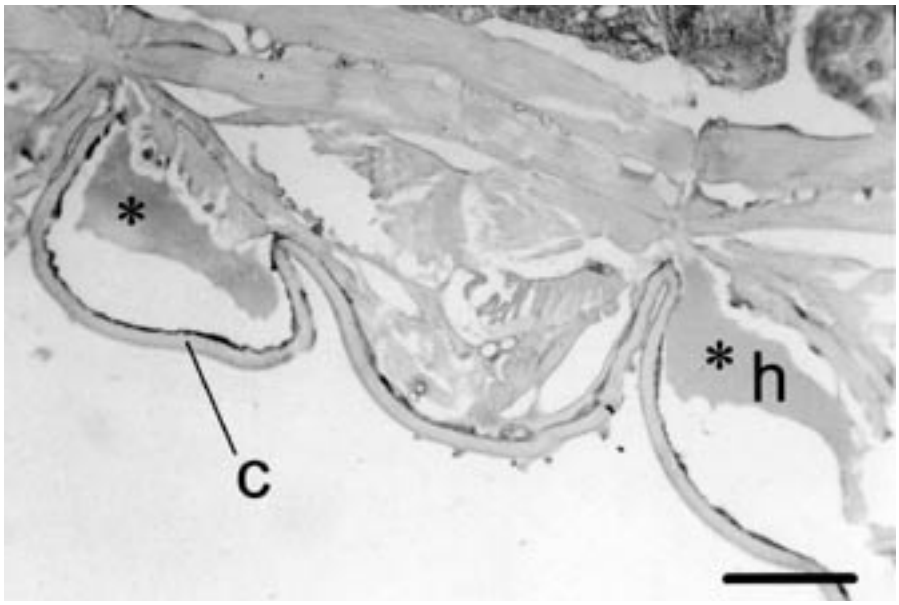

FIG. 1—*Positive morphine staining (*) of the haemolymph of* Calliphora vomitoria *larva. Abreviations denote the following: c, cuticle; h, haemolymph. Internal scale: 150 μm.*

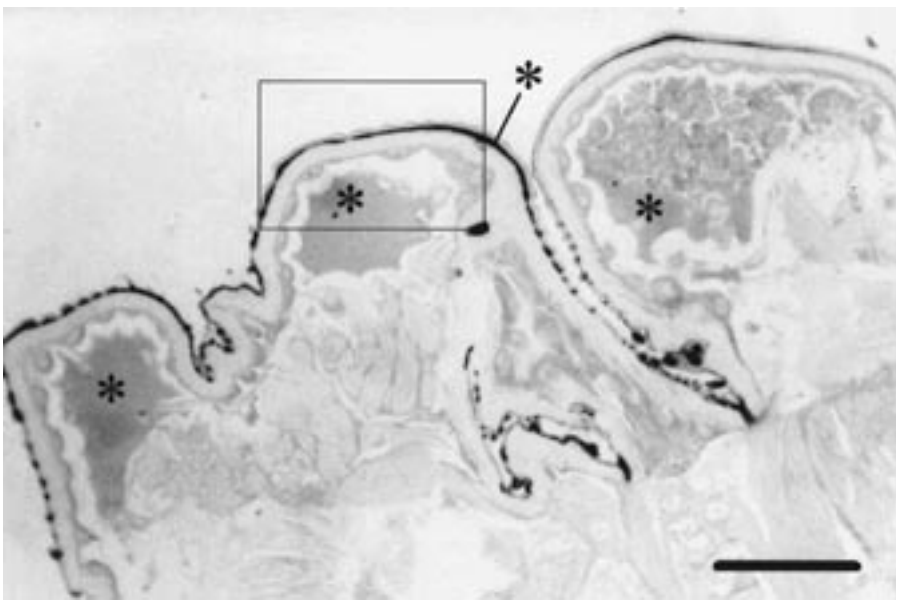

FIG. 2—*Positive stainings (*) of haemolymph and cuticle of* Calliphora vomitoria. *The surrounded area is magnified on Fig. 3. Internal scale: 150 μm.*

distinct layers to the cuticle: endocuticle, exocuticle, and epicuticle. In maggots, both the endocuticle and exocuticle are flexible. The outermost layer is the thin epicuticle which is impermeable and responsible for water conservation (6). In the higher Diptera, there are modifications of the cuticle during the process of pupariation. During this process, the soft endocuticle becomes sclerotized and is transformed into the hard dark shell of the puparium, which is identicle in biochemical nature to the exocuticle of other insects (7).

Immunohistochemical detections of drugs such as phenobarbital (8) or methamphetamine (9) have been used to study the metabolism and distribution of drugs in various organisms. Balkon et al. (10) developed immunofluorescence techniques for the detection of morphine in postmortem tissues. Additionally, immunohistochemical detection of substances at injection site has been used to confirm subcutaneous route of administration (11). Within the same context, Wehner et al. (12) observed morphine-specific immunoreaction in skin sections from areas of suspected injection. The protocol was the same as in the present study, but the authors used 3,3′-diaminobenzidine (DAB) as chromogen.

Positive morphine-specific staining were observed in haemolymph and integument of the larvae, but the digestive tracts were negative. Haemolymph or insect blood is the medium for all biochemical exchanges between organs, transport of hormones, distribution of blood materials, and removal of waste products to the excretory organs. Presence of morphine in the haemolymph re-

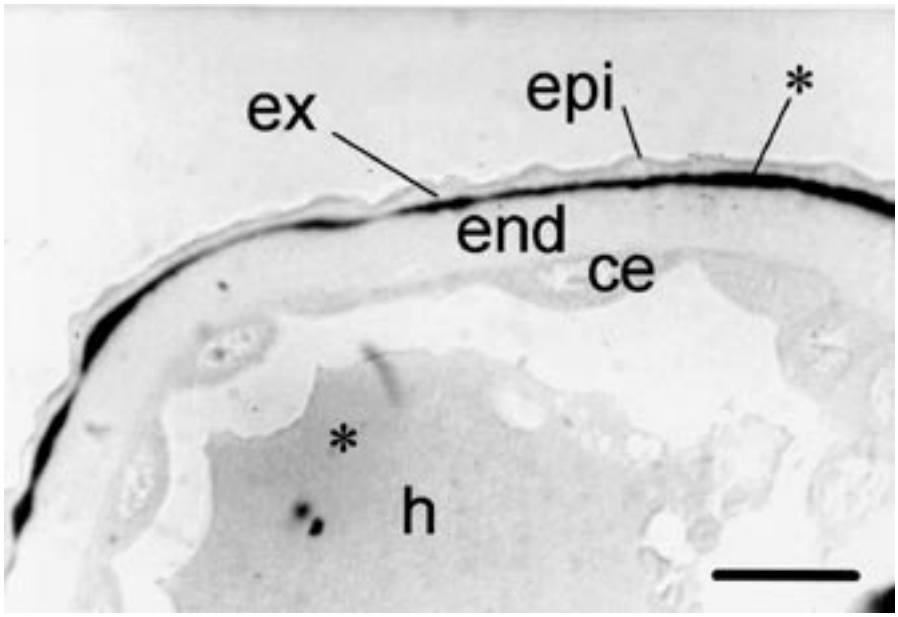

FIG. 3—*Positive stainings of cuticle and haemolymph at high magnification. Note the intense immunoreaction at the limit between exocuticle and endocuticle. Abbreviations denote the following: ce, epidermal cell; end, endocuticle; epi, epicuticle; ex, exocuticle. Internal scale: 40 μm.*

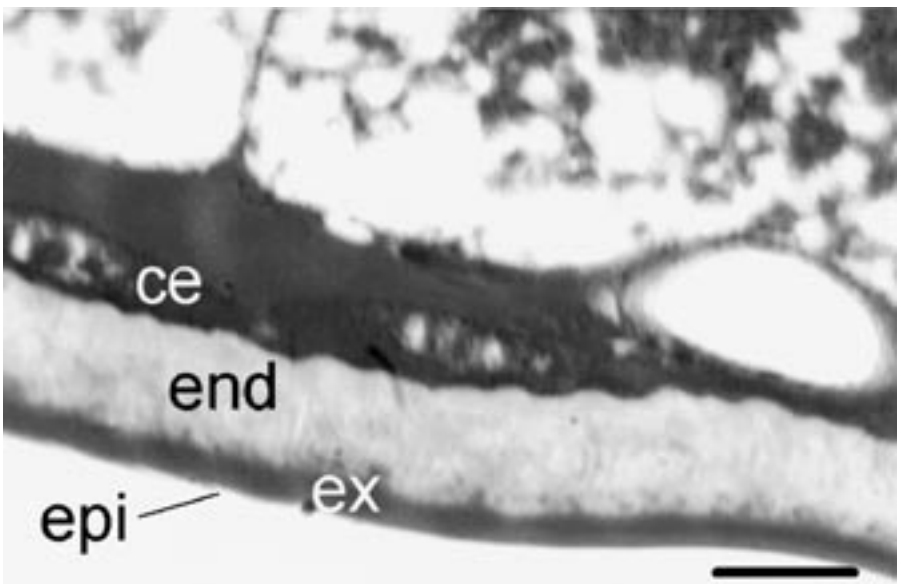

FIG. 4—*Histological section (4 μm) of the integument of* Calliphora vomitoria *larva (trichrome staining). Abbreviations denote the following: ce, epidermal cell; end, endocuticle; epi, epicuticle; ex, exocuticle. Internal scale: 15 μm.*

sults from the rapid absorption of digested materials through the intestinal epithelium into the haemolymph (7). In fly larvae, simple water-soluble molecules are generally excreted from the haemolymph by Malphigian tubules. Malphigian tubules are four blindly ending tubules arising near the junction of the midgut with the hindgut. The blind ends of these tubules float free in the haemolymph and absorb materials. More complex molecules are initially degraded by large cells, called pericardial nephrocytes located at each side of the circulatory structures. Products of this degradation are returned to the haemolymph for excretion via the Malphigian tubules.

Morphine specific staining in the cuticle was derived from morphine in internal larval tissues being retained due to the waterproof nature of the epicuticle. There are numerous transcuticular tubes, termed pore canals, in the endocuticle and exocuticle. In larvae of *Sarcophaga,* these pore canals are relatively coarse structures, approximately 1 μm in diameter. The pore canals contain filamentous cytoplasmic processes from epidermal cells, with 50 to 70 arising from each cell, giving 15 000 canals per sq mm. From these processes arises the noncellular cuticle (13). In the larvae of *C. vomitoria,* morphine in the haemolymph is excreted by epidermal cells and

deposited in close proximity to pore canals in the cuticular matrix. In this respect, the cuticle acts as a storage, similar to adipocytes and pericardial nephrocytes (7). When the 3rd instar larval cuticle is sclerozited to form puparial case, morphine deposited in close proximity to the pore canals is incorporated into the puparial case during the sclerotization of the integument.

This study is a further contribution to the study of metabolism of drugs in fly larvae using immunochemical techniques. We confirm the previous studies which have detected drugs in empty puparia using techniques employed in hair extractions (14–16). This further serves to focus attention on the significance of empty puparia in cases where normal toxicological specimens or living necrophagous insects are not present.

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