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SHORT COMMUNICATION

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Hydrocarbon synthesis by enzymatically dissociated oenocytes of the abdominal integument of the German Cockroach, *Blattella germanica*

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Abstract In insects, hydrocarbons waterproof the cuticle, protect the insect from the external environment, and serve as semiochemicals or their metabolic precursors. In the German cockroach, *Blattella germanica*, hydrocarbons are synthesized by the abdominal integument, but the precise site of biosynthesis is not known. We developed a method for separation of oenocytes from other cells in the abdominal integument using enzymatic dissociation followed by Percoll gradient centrifugation. Radiolabeled propionate was then used to monitor de novo synthesis of hydrocarbons by dissociated cells. Oenocyte-enriched cell suspensions of abdominal sternites synthesized hydrocarbons, whereas suspensions enriched with epidermal cells did not. Our results show conclusively that hydrocarbons are produced by oenocytes not only in insects whose oenocytes are localized within the hemocoel, but also in those insects whose oenocytes are within the abdominal integument. Furthermore, these data support a hemolymph pathway for transport and delivery of hydrocarbons to both external and internal tissues, including the epicuticle, fat body, and ovaries.

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Introduction

All insects depend on cuticular lipids for water balance, protection from pathogens and environmental stresses, and as mediators of intra- and interspecific communication. In most insects, hydrocarbons are the predominant cuticular lipids (Nelson and Blomquist 1995).

Oenocytes, so called because of their pale amber color, are characteristically very large – among the largest somatic cells in insects – and are rich in mitochondria and smooth endoplasmic reticulum, suggesting participation in lipid synthesis (Rinterknecht and Matz 1983; Rinterknecht 1985). Indeed, in vitro incorporation of Na-^{14}C-acetate into hydrocarbons by oenocytes in several insect species has implicated these cells as the site of hydrocarbon biosynthesis (Diehl 1973, 1975; Romer 1980).

Although of ectodermal origin, the anatomical location of oenocytes varies considerably amongst insect species and even across different developmental stages. Nevertheless, oenocytes are generally localized in the abdomen, as for example, in cell clusters within the abdominal hemocoel in larvae of the Brazilian skipper *Calpodes ethlius*, and the beetle *Tenebrio molitor* (Jackson and Locke 1989). Because oenocytes that are arranged in discrete clusters within the hemocoel are readily accessible for experimentation, most investigations have concentrated on species with this arrangement.

In many insects, however, including the German cockroach (*Blattella germanica*), the American cockroach (*Periplaneta americana*), and the fruit fly (*Drosophila melanogaster*), oenocytes are found within the abdominal integument, separated from the hemocoel by a basal lamina (Kramer and Wigglesworth 1950; Liang and Schal 1993; Ferveur et al. 1997). In *B. germanica*, radiotracer studies demonstrated that only the abdominal sternites and tergites synthesize hydrocarbons (Gu et al. 1995). Hydrocarbons are then loaded into a high density hemolymph lipoprotein, lipophorin, and transported to the cuticle, fat body, and oocytes (Schal et al. 1998; Fan et al. 2002). The oenocytes of *Drosophila* are also

localized within the abdominal integument (Johnson and Butterworth 1985), and using an elegant genetic approach, Ferveur et al. (1997) reported that targeted expression of the *transformer* gene in oenocytes of male fruit flies resulted in feminization of the hydrocarbon pheromone mixture. These results most convincingly demonstrated that the oenocytes produce hydrocarbons, including hydrocarbon sex pheromones, in a species such as *B. germanica*, in which oenocytes are within the abdominal integument.

Nonetheless, definitive biochemical localization of hydrocarbon biosynthesis within the epidermis has been hampered by an inability to separate the integument into its component cell types. Herein we report procedures for separating oenocytes from epidermal cells, and we explicitly demonstrate, using radioisotope tracers, that only oenocytes synthesize hydrocarbons.

Materials and methods

Animals

The *B. germanica* colony was maintained at 27± .5°C and ~50% relative humidity under a 12:12 (light:dark) photocycle, and given Purina 5012 rat chow (Purina Mills, St. Louis, Mo.) and water ad libitum. Newly emerged adult females were separated from the colony within 12 h of eclosion, and maintained as virgins in groups. Five-day-old females, which produce large amounts of hydrocarbons, were used in all experiments.

Enzymatic dissociation of the integument

Sternites 3 and 4 were removed from ten CO_2-immobilized 5-day-old females and incubated at 27°C for 30 min with shaking in 100 μl of enzymatic dissociation solution [10 mM EDTA, 0.3% trypsin, and 0.1% collagenase in *B. germanica* saline A (BG-SSA, Kurtti and Brooks 1976)] in a 96-well microtiter plate. Sternites were then washed three times with BG-SSA, each for 2 min with shaking. Because oenocytes are not extensively coupled (Caveney and Berdan 1982), most oenocytes were readily separated from the integument into the medium during this washing step. This oenocyte sample is denoted "oenocyte-enriched". Epidermal cells, on the other hand, are tightly coupled with gap junctions, and following the mild enzyme treatment these cells remained associated with the intact integument. The remaining cellular layer, together with the basal lamina, was peeled from the cuticle in *B. germanica* saline B (BG-SSB, containing 20% fetal bovine serum, pH 7.4, osmotic pressure 410 mOs) (Kurtti and Brooks 1976) to stop enzyme activity, transferred into 1% fetal bovine serum in L15B medium (Specialty Media, Lavallette, N.J.) (Munderloh and Kurtti 1989), and pipetted up and down ten times to separate the epidermal cells from each other; we refer to this sample as "epidermal cell-enriched".

Each cell suspension was centrifuged at 400 *g* for 4 min to pellet the cells, and washed twice with 1 ml L15B containing 1% fetal bovine serum. The final volume of each cell suspension was adjusted to 100 µl. A 20 μl aliquot was stained with 1% eosin to facilitate cell counts and size measurements. Because most cells in suspension were oval or elliptical, cell diameter was measured in a hemocytometer as the mean of the maximum length and width of each individual cell. Viability of cells was checked with 0.1% trypan blue.

Separation of cells in Percoll density gradients

Percoll gradients were formed by mixing 150 µl of 10× L-15B medium and 1.35 ml Percoll reagent (Sigma, St. Louis, Mo.) in a 1.7 ml microcentrifuge tube. The mixture was centrifuged at 16,000 *g* for 120 min at room temperature (Pertoff et al. 1978).

The "oenocyte-enriched" or "epidermal cell-enriched" samples were carefully layered on top of the Percoll gradient and centrifuged at 200 *g* for 8 min. Fractions of 100 µl were then carefully removed from the top, representing a linear density gradient ranging from 0.968 to 1.147 g/ml. As before, a 20 μl aliquot of each fraction was used for cell counts and cell size measurements. The rest of the fraction was used to quantify hydrocarbon biosynthesis. Percoll was not removed because it did not appear to interfere with hydrocarbon biosynthesis.

Hydrocarbon biosynthesis

Each 80 µl fraction was added to 120 µl L-15B medium containing 0.074 MBq sodium-(1-^{14}C) propionate (specific activity: 2.04 GBq/mmol; NEN Research Products, Boston, Mass.) and incubated for 3 h at 27°C. To separate neutral lipids, cells were first homogenized for 30 s (Kontes micro ultrasonic cell disruptor, Vineland, N.J.), extracted in hexane:methanol:water (2:1:1), vortexed vigorously, and finally centrifuged at 2,000 *g* for 10 min. An aliquot of the hexane phase was loaded on a ~500 mg silica gel (Selecto, Fisher) minicolumn in a glass wool-stoppered Pasteur pipette and hydrocarbons were eluted with 7 ml hexane. The solvent was reduced with a gentle stream of N_2, and radioactive hydrocarbons were assayed by liquid scintillation spectrometry (LS5801, Beckman, Fullerton, Pa.).

Transmission electron microscopy

Cell suspensions were pelleted by centrifugation for 2 min at 400 *g*, washed twice with phosphate-buffered saline (PBS), and fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 M PBS (McDowell and Trump 1976). The cell suspensions were centrifuged at ~250 *g* for 30 s, rinsed twice in 0.1 M PBS (pH 7.2–7.4), and embedded in 3–4% water agar (Dykstra 1993). Freshly dissected sternites 3 and 4 were also rinsed with PBS and fixed in the same manner. Agar-embedded cells and sternites were postfixed in 2% aqueous osmium tetroxide, dehydrated in an ethanolic series, passed through 100% acetone, and embedded in Spurr's epoxide resin. Ultrathin sections (80–90 nm) were mounted on 200-mesh copper grids, and stained with saturated methanolic uranyl acetate and lead citrate (Dykstra 1993).

Results

An enzymatic dissociation of the integument underlying sternites 3 and 4, followed by mechanical disruption of cell connections, effectively dissociated the integument into a suspension of ovoid cells (Fig. 1D). Cell sizes of the dissociated cells were bimodally distributed: The majority of the cells (65%) were <20 µm in diameter, but 35% of the cells were >30 µm and up to 85 µm in diameter (distribution not shown). Because the two cell categories were separated by a clear gap between 20 and 30 µm, they appeared to represent different cell types. A milder enzymatic digestion, as described in the Materials and methods section, was then used to specifically separate the oenocytes from the integument. In this "oenocyte-enriched" sample, 60% of the cells were >30 µm in diameter, averaging 48.65±2.77 μm (n=84),

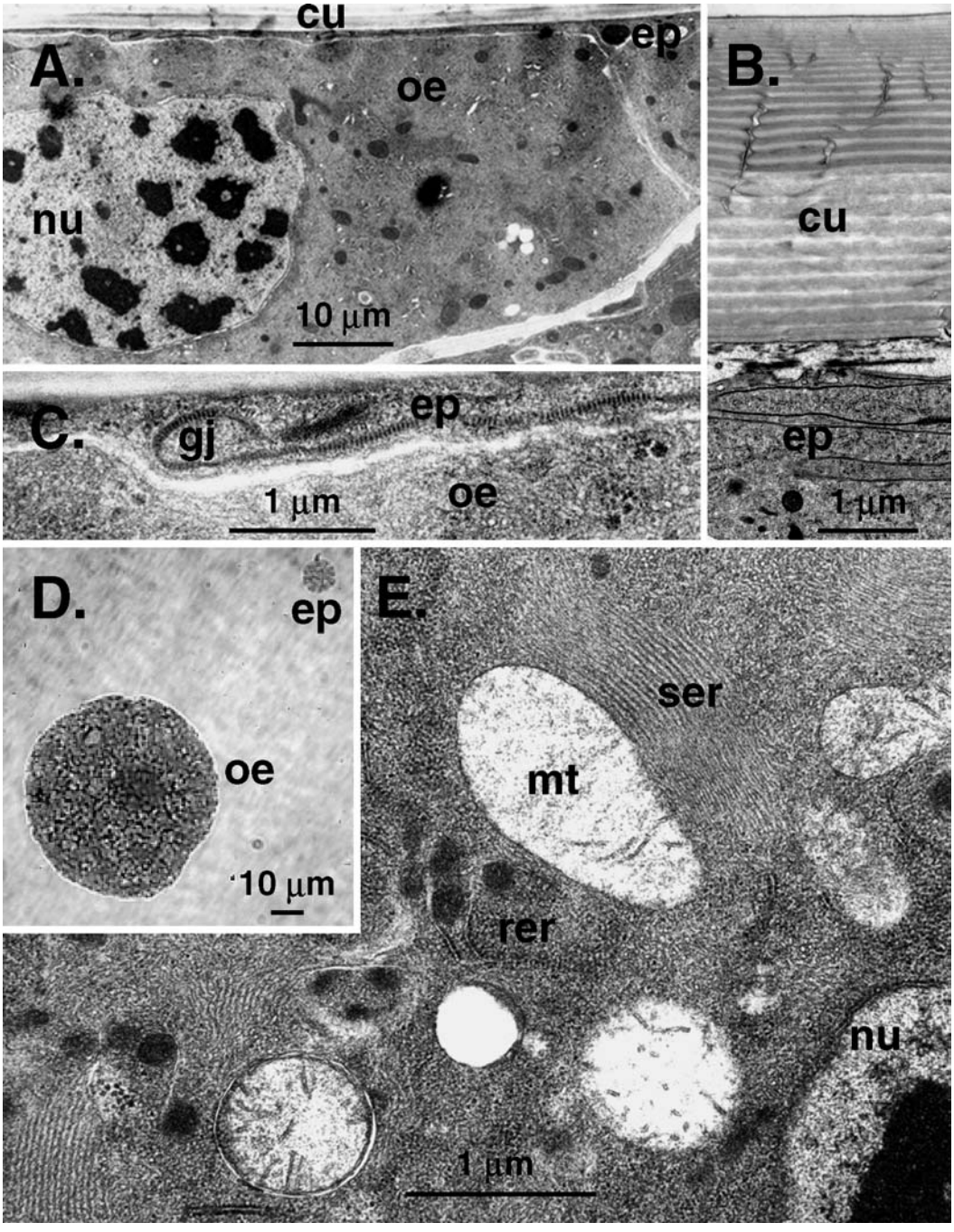

Fig. 1 Light and transmission electron microscopy of the cuticle, oenocytes, and epidermal cells of *B. germanica*. **A** Section through an intact sternite, showing the cuticle (*cu*), epidermal cells (*ep*), and a large oenocyte (*oe*); *nu* nucleus. **B**, **C** Higher magnification of epidermal cells, showing extensive coupling with gap junctions (*gj*). **D** Light micrograph of a dissociated oenocyte and epidermal cell, showing their round shape, and striking differences in volume. **E** Ultrastructure of a dissociated oenocyte following Percoll density gradient centrifugation, showing extensive smooth endoplasmic reticulum (*ser*), less rough endoplasmic reticulum (*rer*), abundant mitochondria (*mt*), and large nucleus (*nu*) with dense chromatin

but ~40% were <20 μm (9.27±0.25 μm; n=71) (Fig. 2A). Ultrastructural details of sectioned sternites (Fig. 1A–C) indicated that the smaller cells are epidermal cells, while cells >30 μm are oenocytes.

A second sample was generated by further mechanical disruption of the integument after most oenocytes had been removed. This "epidermal cell-enriched" sample contained ~85% epidermal cells and only 15% oenocytes (Fig. 2B). Ultrastructural details of both samples were consistent with these designations (Fig. 1A, C, E). Percoll gradient centrifugation of each of these enzymatically dissociated cell suspensions further enriched certain

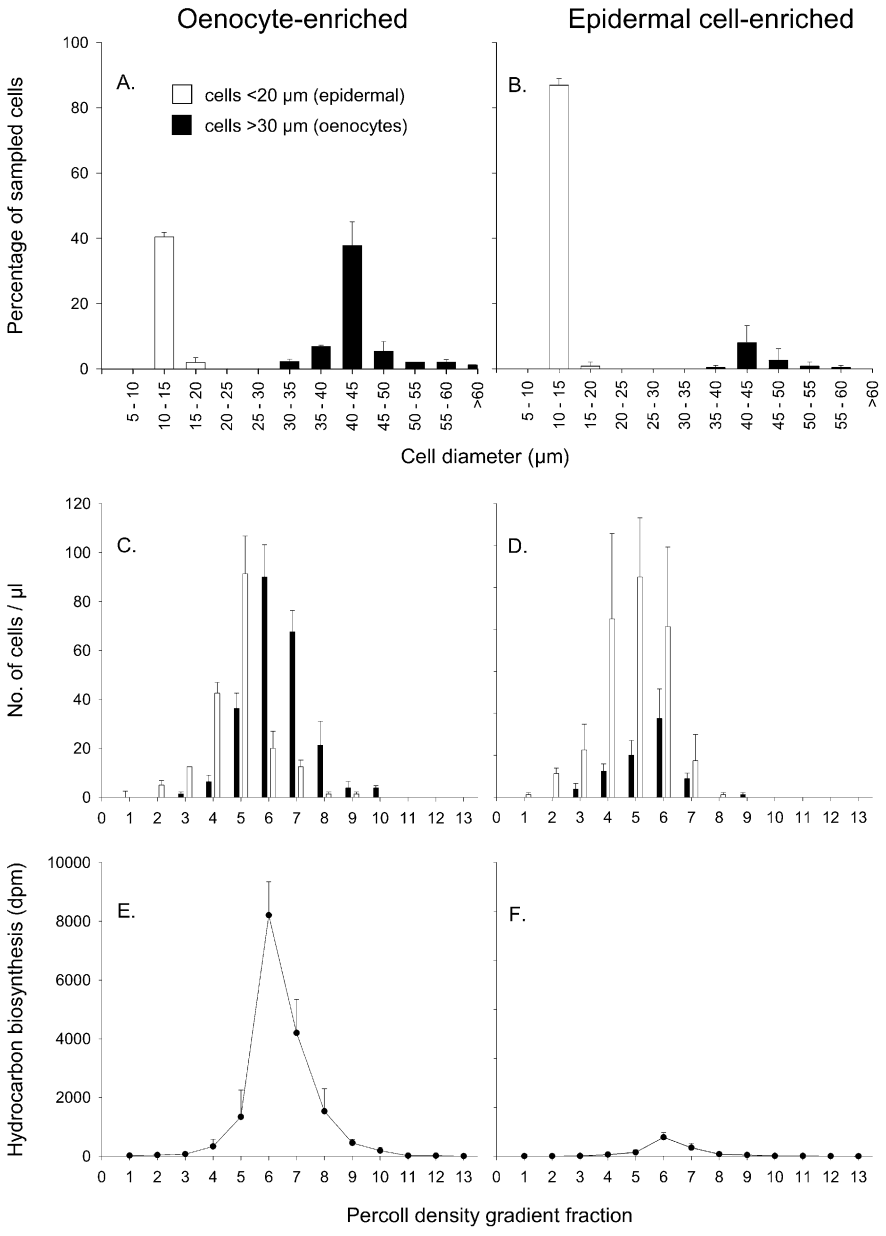

Fig. 2 Enzymatic dissociation of abdominal integument, followed by Percoll gradient separation of cell types, and assays for hydrocarbon synthesis in *B. germanica*. Sternites 3 and 4 from ten 5-day-old virgin females were separated enzymatically into oenocyte-enriched (**A**) and epidermal cell-enriched (**B**) samples. Each cell suspension was then fractionated on a Percoll density gradient (**C**, **D**) and hydrocarbon synthesis by each fraction was measured with a radiochemical assay (**E**, **F**). The procedure was replicated ten times

fractions based on their densities. In both gradients, epidermal cells predominated in more buoyant fractions, whereas most oenocytes were found just below the epidermal cells (Fig. 2C, D).

Hydrocarbon synthesis was measured in each fraction of the Percoll gradient. Because the majority of *B. germanica* hydrocarbons are methyl-branched, and propionate labels methyl-branched hydrocarbons, incorporation rates of up to 20% can be achieved with ^{14}C propionate (Gu et al. 1995; see Young and Schal 1997 for validation of the parameters of the incubation, extraction, fractionation, and analysis of radiobiosynthesized hydrocarbons). The data demonstrate a relationship between presence of oenocytes and de novo production of hydrocarbons by dissociated cells (Fig. 2E, F). In density gradient fractions from both samples, maximal rates of hydrocarbon biosynthesis were found in fraction 6, corresponding to the largest number of oenocytes. We therefore conclude that hydrocarbons are produced by oenocytes and not by other cell types within the integument.

Discussion

The insect integument is a complex, multifunctional tissue, composed of epidermal cells that serve as both transepithelial barriers and in transepithelial transport. In addition to epidermal cells, a multitude of specialized cells reside within the integument, including sensillum cells, glandular and duct cells, hairs and scales, and oenocytes. The oenocytes of many insects are located

beneath the epidermal cells, in close proximity to the basal lamina, and with no direct contact with the cuticle.

Cuticular hydrocarbons are produced by the abdominal integument of *B. germanica* (Gu et al. 1995), and we now demonstrate that in this cockroach only the oenocytes within the integument synthesize hydrocarbons. Although previous studies with other insects also concluded that oenocytes produce hydrocarbons, because of technical constraints of separating the oenocytes from epidermal cells, none has addressed this question with biochemical assays of the integument.

Critical to our investigations was the enzymatic dissociation of the abdominal cellular integument. We took advantage of the prevalence of cellular junctions between epidermal cells, and the apparent infrequency of gap and septate junctions between oenocytes and epidermal cells. Consequently, a mild enzymatic treatment that digested the basal lamina also liberated most of the oenocytes, while at the same time the much more numerous epidermal cells remained attached to each other and to the overlying cuticle. Thus, a cell suspension was obtained that was highly oenocyte-enriched compared with the integument from which it was derived. Separation of these cells by Percoll density gradient centrifugation further enriched specific fractions with oenocytes and effectively demonstrated that oenocytes produce hydrocarbons.

Oenocytes are among the largest somatic cells in insects, reaching a diameter of up to 170 μm (Romer 1991). Their large volume is usually achieved by polyploidization, especially in hemimetabolous insects, and their other main characteristics, extensive smooth endoplasmic reticulum and absence of glycogen, suggest an overall similarity to steroidogenic cells and participation in lipid metabolism. Consistent with this, in vitro assays with oenocytes conclusively showed that radiolabeled substrates are incorporated into ecdysone and hydrocarbons (Diehl 1973, 1975; Romer 1980, 1991).

Because oenocytes in the German cockroach are localized within the abdominal integument, transfer of hydrocarbons to tissues that do not produce hydrocarbons, such as the head, thorax, and internal tissues of the abdomen (e.g., ovaries) requires transport through the hemolymph. A high-density carrier protein, lipophorin, appears to serve this function in all insects examined thus far (Chino 1985; Schal et al. 1998). Hydrocarbons synthesized by oenocytes are transferred to lipophorin, probably through a plasma membrane reticular system (Jackson and Locke 1989). Lipophorin, in turn, shuttles hydrocarbons to the fat body, ovaries, and to the cuticle, presumably through epidermal cells. Lipophorin-mediated transport, therefore, appears to play a major, if not exclusive, role in hydrocarbon delivery to the cuticle. Testing this hypothesis is a major thrust of our current research and the ability to isolate oenocytes from the integument should facilitate these studies.

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