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Biosynthesis of Hydrocarbons and Contact Sex Pheromone and Their Transport by Lipophorin in Females of the German Cockroach (*Blattella germanica*)

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The sites of synthesis, and transport of hydrocarbons and methyl ketone contact sex pheromone, were examined in the German cockroach. The abdominal integument of 7-day old *Blattella germanica* females showed linear incorporation of [1-^{14}C]propionate into hydrocarbon and pheromone for at least 4 h *in vitro*, whereas other body parts including the head, wings, legs, thorax, fat body, digestive tract, and vitellogenic ovaries produced negligible amounts of hydrocarbon and pheromone. The sternites synthesized significantly more hydrocarbon and methyl ketones than the tergites, and in both, the incorporation of propionate into hydrocarbon correlated well with its incorporation into the methyl ketone pheromone, suggesting that the abdominal oenocytes or epidermal cells synthesize both hydrocarbon and pheromone. The abdomen contained about 55% of the total hydrocarbon and pheromone in the female, and both were located mainly in the ovaries, integumental epidermal cells (including oenocytes), fat body, and hemolymph. Hemolymph contained up to 20% of the internal hydrocarbons and their methyl ketone derivatives, suggesting that hemolymph carries and circulates hydrocarbon and pheromone in the female. A high density lipophorin (1.109 ± 0.002 g/ml) was isolated and purified from hemolymph by KBr gradient ultracentrifugation and was shown to be the only hemolymph protein that carries hydrocarbon and pheromone. This result suggests that lipophorin loads newly synthesized hydrocarbon and pheromone from the abdominal epidermis and transports them to various tissues, including the ovaries, fat body and possibly the epicuticle. After injection of radiolabeled propionate, newly synthesized radiolabeled methyl-branched hydrocarbons appeared first in the epidermal fraction (with oenocytes) and hemolymph, and later on the epicuticle. When veins entering the fore-wings were cut so that hemolymph circulation to the wings was blocked, the amount of newly synthesized hydrocarbon on the wings was significantly lower than on intact fore-wings. Significantly more radiolabeled hydrocarbon which had been topically applied on the sternites was recovered in the hemolymph than on the surface of the wings. These results show that transport of hydrocarbon is mediated by lipophorin, which shuttles newly synthesized hydrocarbon and methyl ketone sex pheromone from the abdominal oenocytes to epicuticular and internal deposition sites, including the ovaries.

Hydrocarbon Contact pheromone Lipophorin Lipid transport Oenocytes *Blattella germanica* Cockroach

INTRODUCTION

The cuticular hydrocarbons of *Blattella germanica* females consist of *n*-alkanes, 3-methylalkanes, internally branched monomethylalkanes and dimethylalkanes, with 3,11-, 3,9-, and 3,7-dimethylnonacosane as the major components (Augustynowicz *et al.*, 1987; Carlson and Brenner, 1988; Jurenka *et al.*, 1989). In females only, the 3,11-isomer is hydroxylated and oxidized at the 2-position to form 3,11-dimethylnonacosan-2-one, a component of

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the contact sex pheromone (Chase *et al.*, 1990; 1992). Two other pheromone components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one (Nishida and Fukami, 1983), are presumably also derived from 3,11-dimethylnonacosane. Schal *et al.* (1990a) found a fourth component, 3,11-dimethylheptacosan-2-one, which is undoubtedly derived from 3,11-dimethylheptacosane by the same oxidative enzymes used for the synthesis of the C_{29} methyl ketones. The endocrine regulation of pheromone production and its biosynthesis have been reported (Chase *et al.*, 1990, 1992; Juarez *et al.*, 1992; Schal *et al.*, 1990b, 1991, 1994).

Oenocytes have been implicated in hydrocarbon biosynthesis in the desert locust, *Schistocerca gregaria* (Diehl, 1973; 1975) and in *Tenebrio molitor* larvae (Romer, 1980); in both species the epidermis synthesizes less than 1% of the amount simultaneously synthesized by the oenocytes. In the house fly, both epicuticular hydrocarbons and hydrocarbon sex pheromones are synthesized in the abdominal integument (Dillwith *et al.*, 1981), and both share common biosynthetic pathways and enzymes (Blomquist *et al.*, 1987). The location of oenocytes varies considerably among insects. The oenocytes of *Calpodes ethlius* larvae are found in the hemocoel (Jackson and Locke, 1989). In fifth instar females of *S. gregaria*, the oenocytes are found only in the abdominal peripheral fat body situated beneath the epidermis (Diehl, 1975). In these insects, hydrocarbon must be transported through the hemolymph to sites of deposition on the cuticle. For example, in the honey bee, *Apis mellifera*, identical alkenes and alkadienes in the hemolymph and on the epicuticle suggest transport of hydrocarbons from the site of synthesis to sites of usage through the hemolymph (Francis *et al.*, 1989).

The oenocytes of the American cockroach, *Periplaneta americana*, are distributed below the epidermis of the abdominal tergites and sternites (Kramer and Wigglesworth, 1950), suggesting that hemolymph-mediated transport of hydrocarbons to the epicuticle may not be necessary. Yet, the hemolymph hydrocarbon composition of *P. americana* is identical to the hydrocarbon profile of the cuticle (Chino and Downer, 1982). Lipophorin was shown to serve as a carrier for the transport of hydrocarbon from the site of synthesis (presumably oenocytes) to the cuticle (Katase and Chino, 1982, 1984).

Using *B. germanica* integument, Juarez *et al.* (1992) isolated a novel microsomal fatty acid synthetase which synthesizes the methyl-branched fatty acids that are then converted to branched hydrocarbons and contact sex pheromone. This suggests that in *B. germanica* also, epidermal cells and/or associated oenocytes synthesize hydrocarbons and pheromones. In previous experiments, we found that the hemolymph hydrocarbons of the German cockroach were qualitatively identical to epicuticular hydrocarbons (Schal *et al.*, 1994), although internal titers were generally higher. In this paper, we employ *in vitro* techniques to determine which tissues in the adult *B. germanica* female synthesize hydrocarbons and pheromone. We also examine the patterns of distribution of pheromone and hydrocarbons in various tissues and the appearance of newly synthesized hydrocarbons on the wings after [1-^{14}C]propionate injection to test the prevailing hypothesis that newly synthesized integumental lipids pass directly through the cuticle via wax canals in an outward direction. We propose that the transport of newly synthesized hydrocarbons and contact pheromone is mediated by hemolymph lipophorin and that this allows for multiple sites of deposition, in addition to the epicuticle.

MATERIALS AND METHODS

Insects

German cockroach nymphs were reared in 2-liter glass jars and fed Purina rat chow #5012 and water *ad libitum*. Females were separated from males on the day of adult emergence (day 0) and reared in groups in 1-liter plastic cages with food and water as above. Both nymphs and adults were kept at 27 ± 0.3°C under a 12:12 light:dark cycle. All insects used in experiments were 7-day old grouped virgin females (basal oocyte length about 1.8 mm), except where indicated otherwise. Such females exhibit the highest rates of propionate incorporation into pheromone, while the lowest rates are found in previtellogenic and gravid females (Schal *et al.*, 1991).

Chemicals

Sodium [1-^{14}C]propionic acid (55 mCi/mmol) and [carboxyl-^{14}C]inulin (1.7 mCi/g) were purchased from NEN Research Products, DuPont Co., Boston, MA. [11,12-^{3}H]3,11-Dimethylnonacosane and [11,12-^{3}H]3,11-dimethylnonacosan-2-one were gifts from Dr G. Prestwich (Stony Brook, NY) and were prepared as described in Chase *et al.* (1992). All other chemicals were from Sigma (St Louis, MO) or Bio-Rad (Richmond, CA).

Hemolymph volume

Chilled females were injected with [carboxyl-^{14}C]inulin (12,500 dpm) in 1 μl saline near the hind coxa and returned to their normal rearing conditions. After predetermined time intervals 2 μl of hemolymph was collected from the severed cercus of each female and radioactivity was measured with a Beckman 3801 liquid scintillation spectrometer. Chilling does not change hemolymph volume in cockroaches (Wharton *et al.*, 1965).

Lipid extraction and quantification

Cuticular lipids were extracted by immersing insects in *n*-hexane for two 5 min washes and a third rapid rinse. After removal of cuticular lipids, various tissues were homogenized (Polytron, Brinkmann, NY) and extracted with chloroform : methanol : water (Bligh and Dyer, 1959). *n*-Hexacosane and heptacosan-14-one were

included as internal standards during extraction for quantification of the hydrocarbons and the methyl ketones, respectively, by gas–liquid chromatography (GLC). Lipids were loaded in hexane and separated on Biosil-A (Bio-Rad) mini-columns: Hydrocarbons were eluted first with 7 ml hexane, and more polar lipids, including methyl ketones, were eluted with 7 ml freshly prepared 3% ethyl ether in hexane. Lipid samples were analyzed on a HP 5890 GLC equipped with a flame-ionization detector and a HP 3390A integrator. Splitless injection was made into a 30 m x 0.53 mm i.d. DB-1 (J & W Scientific, San Jose, CA) or a 15 m x 0.53 mm i.d. SPB-5 (Supelco, Bellefonte, PA) column, programmed from 150 to 270°C at 15°C/min and then to 310°C at 2°C/min. The injector and detector were maintained at 320°C. For radiochemical experiments, polar lipids containing methyl ketones were further separated on 20 × 20 cm pre-coated silica gel 60 F_{254} (layer thickness 0.2 mm) thin-layer chromatography (TLC) plastic-backed plates (EM Science, Cherry Hill, NJ). [11,12-^{3}H]3,11-Dimethylnonacosan-2-one was used as a standard to mark the methyl ketone spot. The plates were developed twice in 93 : 7 hexane : ethyl ether, the methyl ketone spots were cut, and radioactivity was measured by LSC.

In vivo studies on hydrocarbon synthesis

Insects were injected with [1-^{14}C]propionate in 0.6 μl saline. The labeled propionate used in these experiments will only label methyl-branched hydrocarbons and their derivatives (Chase *et al.*, 1990). After the times indicated, the external hydrocarbons were extracted for 1 min with hexane, followed by two hexane rinses. A hemolymph sample was then collected, and the abdominal integument (including epidermis) was isolated by stripping and removing other internal tissues, such as fat body. The integument was washed three times with saline. The dried epidermal tissue and hemolymph were extracted separately with chloroform overnight. Hydrocarbons were eluted with hexane from BioSil-A columns and radioactivity was assayed by LSC.

In vitro synthesis of hydrocarbon and pheromone

Abdominal tergites and sternites were removed with forceps under a microscope and washed twice with saline. Extreme caution was taken to separate the fat body from the epidermal layer. Fat body, ovaries, digestive tract, and the left colleterial gland were dissected and washed twice in fresh saline to remove hemolymph before incubations or lipid extractions. Antennae, head, wings, legs, thorax and abdomen were separated from adjacent tissues and these body parts were extracted with the hemolymph they normally contained.

Tissues were incubated in 150 μl *B. germanica* saline solution (BG-SSA, pH adjusted to 7.2, osmotic pressure 410 mOsm) (Kurtti and Brooks, 1976) at 28–30°C with either sodium [1-^{14}C]propionic acid or [^{3}H]3,11-dimethylnonacosane. An orbital waving shaker (The Waver, VWR) was used to oxygenate the tissues (Katase and Chino, 1982). After 4 h, or as indicated, the tissues were homogenized, lipids and incubation medium were extracted and hydrocarbons and methyl ketones were separated chromatographically.

Hemolymph collection and lipophorin isolation

Hemolymph was collected from 30–60 ice-chilled females. One cercus was severed and a 50 μl micro-capillary pipette which contained 10 μl saline–EDTA solution (0.15 M NaCl, 10 mM ethylenediamine–tetra-acetic acid (EDTA), 0.05 M sodium phosphate buffer, pH 7.0, and glutathione added just before use to a final concentration of 5 mM) was used to collect the hemolymph. The hemolymph–saline mixtures were pooled into a cooled tube and centrifuged at 10,000 ***g*** for 2 min to pellet the hemocytes. Phenylmethyl sulfonyl fluoride (PMSF) was added to the supernatant to a final concentration of 2 mM to inhibit hemolymph proteases.

Lipophorin was isolated by a modification of the KBr density-gradient ultracentrifugation method described by Shapiro *et al.* (1984). The hemolymph supernatant and 2.57 g KBr in saline were mixed and adjusted to a final volume of 5.8 ml. The KBr–hemolymph mixture (density = 1.311 g/ml) was placed into a Beckman 13.5 ml QuickSeal tube and overlaid with 7.7 ml of freshly-prepared 0.9% NaCl (density = 1.006 g/ml). Centrifugation was with slow acceleration and deceleration at 285,000 ***g*** for 18 h at 4°C in a Beckman L8-70M ultracentrifuge using a fixed-angle rotor (70.1Ti). Fractions (≈ 420 μl per fraction) were collected from the top of the tube with a slow peristaltic injection of a saturated KBr solution into the bottom of the tube. The densities of KBr fractions were determined gravimetrically at room temperature.

Polyacrylamide gel electrophoresis

The purity of lipophorin was checked by polyacrylamide gel electrophoresis (PAGE). KBr gradient fractions were dialyzed against 1 liter of 50 mM sodium phosphate buffer with 5 mM EDTA for 4 h (with one change of buffer). SDS-PAGE was performed according to the procedure of Laemmli (1970). Gel concentrations for separating gel and stacking gel were 7 and 2.5%, respectively. Native gels were performed in 3.75% polyacrylamide gel according to the system of Davis (1964). Gels were stained with Coomassie brilliant blue G-250 according to Blakesley and Boezi (1977). The molecular weight of lipophorin was determined by the method of Lambin *et al.* (1976) with standards from Bio-Rad.

Transport of hydrocarbon to the wings

Since the wings accumulate, but do not synthesize, appreciable amounts of hydrocarbon (below), we performed *in vivo* experiments to examine hydrocarbon transport to the wings. The costa, subcosta, radius, and media of the fore-wings of some insects were severed at their proximal end in order to block hemolymph circulation into the wings; such wings folded normally over the tergites. Operated and control females were either

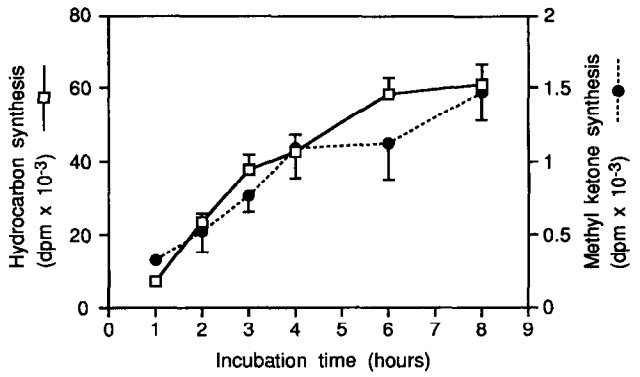

FIGURE 1. Time course of hydrocarbon and methyl ketone pheromone synthesis by abdominal integument incubated *in vitro* with 0.13 μCi [1-^{14}C]propionate. Abdominal integument was dissected from 7-day old grouped females (basal oocyte length 1.8–2.0 mm; mated on day 6). Data are means $\pm$ SEM (n = 9–10 per mean).

injected with [1-^{14}C]propionate in 0.6 μl saline or received [11,12-^{3}H]3,11-dimethylnonacosane in 0.2 μl hexane as a topical application on their sternites. After the indicated time intervals, wings and hemolymph were extracted. External lipids of the wings were extracted in hexane for 1 min followed by 2 rapid rinses; internal lipids were extracted as described above. Hydrocarbon was eluted with hexane from BioSil-A columns and radioactivity was assayed by LSC.

All data are presented as mean $\pm$ SEM.

RESULTS

Site of hydrocarbon and pheromone synthesis

Previous work (Juarez *et al.*, 1992; Gu *et al.*, 1993) and preliminary results indicated that the integument was the site of hydrocarbon and pheromone synthesis in *B. germanica* females. A time-course study of the *in vitro* incorporation of radiolabeled propionate into hydrocarbon and methyl ketone fractions showed linear incorporation for at least 4 h and continued synthesis for up to 8 h (Fig. 1). After 8 h, 20.8 $\pm$ 1.8% (n = 10) of the propionate was incorporated into methyl-branched hydrocarbons and 0.50 $\pm$ 0.07% into methyl ketone pheromone components. We chose 4 h for further *in vitro* tissue incubations.

Incubations of various tissues for 4 h clearly showed that the abdominal integument most efficiently (13.9 $\pm$ 1.5%, n = 10) incorporated radiolabeled propionate into methyl-branched hydrocarbons (Fig. 2). Pheromone synthesis was also maximal in the abdominal integument (0.36 $\pm$ 0.07% propionate incorporation), suggesting that oenocytes or epidermal cells are responsible for the synthesis of both hydrocarbon and sex pheromone. In contrast, synthesis in each of the other tissues, including head, wings, legs, thorax, abdominal fat body, digestive tract, and vitellogenic ovaries, was less than 2% of that in the abdominal integument. We also incubated individual tergites or sternites with sodium [1-^{14}C]propionate for 4 h. The sternites incorporated propionate much more efficiently and produced about 60–65% of the total abdominal integumentary hydrocarbons and methyl ketones, while the tergites produced the remaining 35–40% (Fig. 3). The second through seventh tergites produced similar amounts of lipids, while all sternites produced larger amounts. However, the third and fourth sternites incorporated radiolabel into hydrocarbons (2.4 $\pm$ 0.5%, n = 10) and pheromone (0.11 $\pm$ 0.04%) most efficiently. The incorporation of radiolabeled propionate into hydrocarbons and methyl ketones by tergites and sternites was approximately correlated with the two dimensional surface area of the incubated tissues (unpublished). This suggests that oenocytes or epidermal cells which synthesize hydrocarbon and methyl ketones may be evenly distributed underneath the tergites and sternites. Moreover, in most incubations the incorporation of propionate into hydrocarbon correlated well with its incorporation into methyl ketones, suggesting that the same cells associated with the abdominal cuticle synthesize both hydrocarbon and the contact sex pheromone.

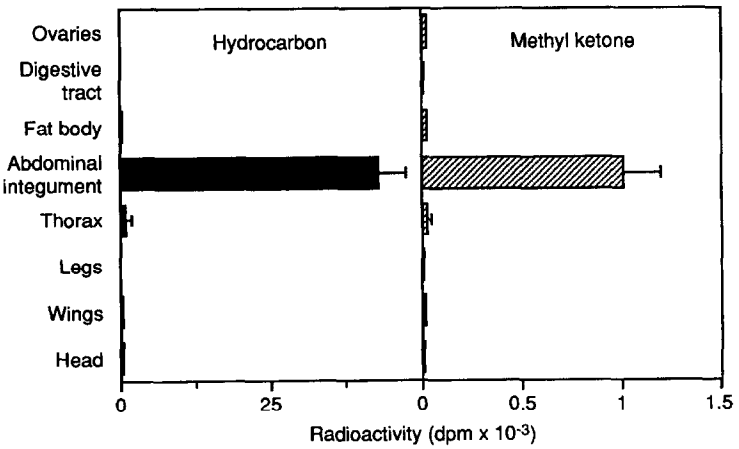

FIGURE 2. Hydrocarbon and methyl ketone pheromone synthesis by various tissues incubated *in vitro* with 0.14 μCi [1-^{14}C]propionate for 4 h. Tissues were dissected from 7-day old grouped females (basal oocyte length 1.8–2.0 mm; mated on day 6). Data are means + SEM (n = 10 per mean).

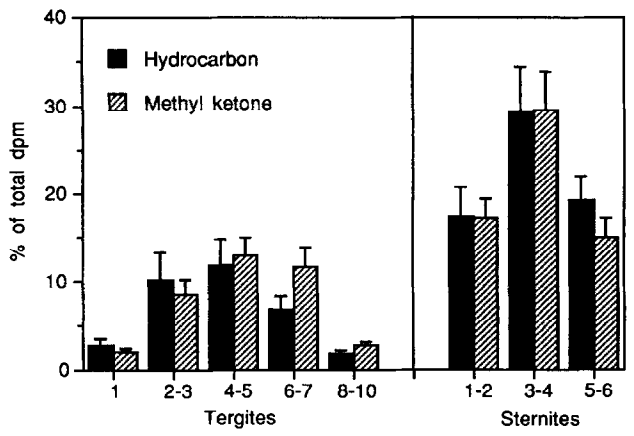

FIGURE 3. Incorporation of [1-^{14}C]propionate into hydrocarbons and methyl ketone pheromone by tergites or sternites. Each 7-day old grouped female (basal oocyte length 1.8–2.0 mm; mated on day 6) was dissected and each tissue was incubated *in vitro* with 0.14 μCi [1-^{14}C]propionate for 4 h. Data represent the percentage of newly synthesized lipid of the total synthesized by all tergites and sternites of each respective female. Data are means + SEM ($n = 10$ per mean).

Distribution of hydrocarbon and pheromone

We previously described the patterns of accumulation of C_{27} and C_{29} methyl ketone pheromone components both internally and on the cuticular surface through the first two gonadotrophic cycles of *B. germanica* (Schal *et al.*, 1994). In this study, we were specifically interested in the tissue distribution patterns of hydrocarbon and pheromone in 7-day old females in order to determine whether sites of synthesis are adjacent to sites of lipid deposition.

The epicuticular lipids were localized primarily on the abdomen, thorax and wings (Fig. 4). Because only the abdominal integument synthesizes appreciable amounts of hydrocarbon and methyl ketones (Fig. 2), these data suggest that these lipids are either transported internally from sites of synthesis to specific target sites, or that they are translocated mechanically on the epicuticle, possibly through grooming activities. Of particular interest are the wings, which exhibit only minor lipid synthesis *in vitro* (Fig. 2) and minor internal stores (Fig. 4), but accumulate large amounts of hydrocarbons and their derivative pheromone components on the surface.

Because it was impossible to separate hemolymph entirely from each body part, the internal lipids of some body parts included lipids also contained in their hemolymph fraction. Insect hemolymph volume was determined with radiolabeled inulin injections. The titer of inulin in the hemolymph did not change significantly from 2 h (1013 $\pm$ 25 dpm per 2 μl hemolymph) to 5 h after injection (1018 $\pm$ 32 dpm per 2 μl hemolymph) ($n = 46$, $F = 1.206$, $P = 0.319$). Hemolymph volume was determined 2 h after injection because we wanted to avoid errors due to its subsequent metabolism, absorption, or excretion (see Wharton *et al.*, 1965). Each 7-day old grouped virgin female has 24.7 $\pm$ 0.6 μl of hemolymph ($n = 13$), which is nearly 25% of its wet body mass. The hemolymph of females contained 19.3% of the internal hydrocarbon, 11.4% of the internal C_{29} methyl ketone, and 7.2% of the total internal C_{27} methyl ketone. These results indicate that, in the female, hemolymph carries and circulates hydrocarbons and methyl ketone pheromone components (Fig. 4).

Not surprisingly, about 55% of the total and 75% of the internal hydrocarbons and methyl ketones in females were contained in internal abdominal tissues (Fig. 4), primarily in the ovaries, integumental epidermal cells (including oenocytes), fat body, and hemolymph (Fig. 5). The digestive tract and left colleterial gland contained only minor amounts of these lipids. We were unable to determine the lipid content of the tracheal system, the epicuticle of which most likely was not extracted with the external hexane washes. Since the ovaries, fat body and hemolymph do not synthesize appreciable amounts of hydrocarbon and pheromone, large stores of lipids in them indicate that these lipids were transported through the hemolymph and deposited over time.

Lipophorin carries hydrocarbon and pheromone

Hemolymph was fractionated by KBr gradient ultracentrifugation. Absorbance of all KBr fractions at

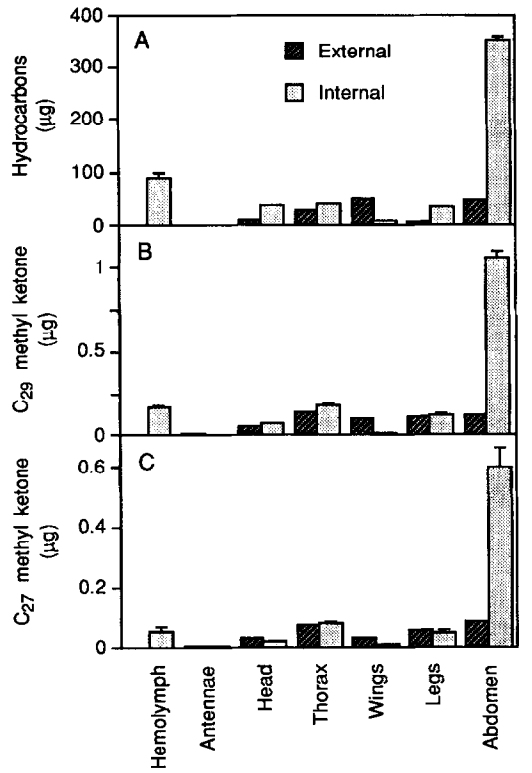

FIGURE 4. Distribution patterns of (A) total hydrocarbons (total external = 141 μg; internal = 473 μg), (B) 3,11-dimethylnonacosan-2-one (total external = 0.54 μg; internal = 1.44 μg), and (C) 3,11-dimethylheptacosan-2-one (total external = 0.28 μg; internal = 0.76 μg) in various body parts internally and on the epicuticle in 7-day old grouped virgin females, with basal oocyte length 1.8-2.0 mm. Data are means + SEM ($n = 5$ per mean).

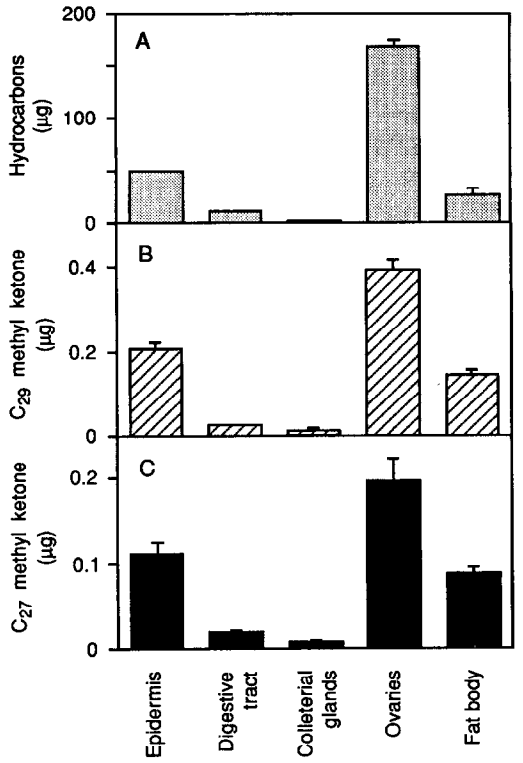

FIGURE 5. Distribution patterns of (A) total hydrocarbons, (B) 3,11-dimethylnonacosan-2-one, and (C) 3,11-dimethylheptacosan-2-one in various abdominal tissues. Data are means+SEM ($n=5$ per mean).

280 nm indicated that there were two major hemolymph proteins: lipophorin between fraction 6 and 13, and vitellogenin between fraction 26 and 30 [Fig. 6(A)]. The absorbance peak at 455 nm represents carotenoids which are carried by lipophorin (Shapiro *et al.*, 1984). The purity of some of the KBr gradient fractions was confirmed by SDS-PAGE (Fig. 7) and native-PAGE (not shown). Fractions 7–13 represent lipophorin which was composed of two apoproteins, apoLp-I and apoLp-II, with relative molecular weights of 212,000 and 71,000, respectively. The density of lipophorin is 1.109 ± 0.002 g/ml.

Each KBr fraction of the hemolymph was extracted and hydrocarbons and methyl ketones were quantified by GLC [Figs 6(B) and (C)]. Hemolymph hydrocarbon and pheromone were associated only with lipophorin fractions, and none with vitellogenin fractions. The comparative ratios among total hydrocarbons, C_{29} methyl alkane (3,7-, 3,9- and 3,11-dimethylnonacosane), C_{27} methyl ketone, and C_{29} methyl ketone were similar for each fraction. These results clearly show that, in the hemolymph of *B. germanica*, only lipophorin carries significant amounts of hydrocarbon and pheromone. Together with other results they also suggest that lipophorin loads newly synthesized non-polar lipids from the epidermis and transports them to various tissues, including the ovaries, fat body and possibly the epicuticle.

Transport of newly synthesized hydrocarbon and pheromone

The involvement of lipophorin is also supported by findings that injected radiolabeled hydrocarbon and newly synthesized methyl ketones were associated only with lipophorin fractions after KBr gradient fractionation (Fig. 8). These results, and the abundance of epicuticular hydrocarbons on non-synthesizing body parts (e.g. wings, legs), suggested that newly synthesized hydrocarbon was transported and redistributed by lipophorin to epicuticular deposition sites. We examined this with a time-course study of the distribution of labeled hydrocarbon after propionate injection into 3-day old females; such females synthesize hydrocarbon at a high rate and deposit newly synthesized hydrocarbon on the cuticular surface (Schal *et al.*, 1994). The radiolabeled propionate which was injected into the hemolymph would be expected to enter the oenocytes or epidermal cells, where it would be incorporated into hydrocarbon, which in turn would be transported either directly to the epicuticle, or indirectly through the hemolymph (internal hydrocarbons). Figure 9 shows that within 10 min after propionate injection, newly synthesized hydrocarbons appeared in the epidermis, as expected. Nearly twice as much radiolabeled hydrocarbon appeared in the

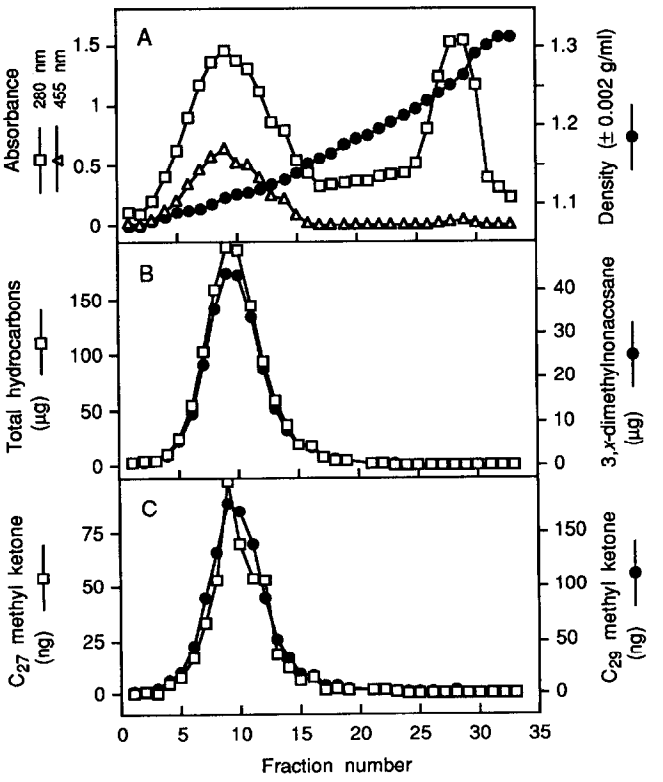

FIGURE 6. KBr gradient fractionation of about 0.4 ml hemolymph from 50 *B. germanica* 7-day old females with basal oocyte length 1.8–2.0 mm. (A) Absorbance at 280 and 455 nm and densities of KBr fractions. (B) Amount of total hydrocarbons and 3,7-, 3,9- and 3,11-dimethylnonacosane, the major hemolymph hydrocarbons, associated with each KBr fraction. (C) Amounts of C_{27} and C_{29} methyl ketone pheromone components associated with each KBr fraction.

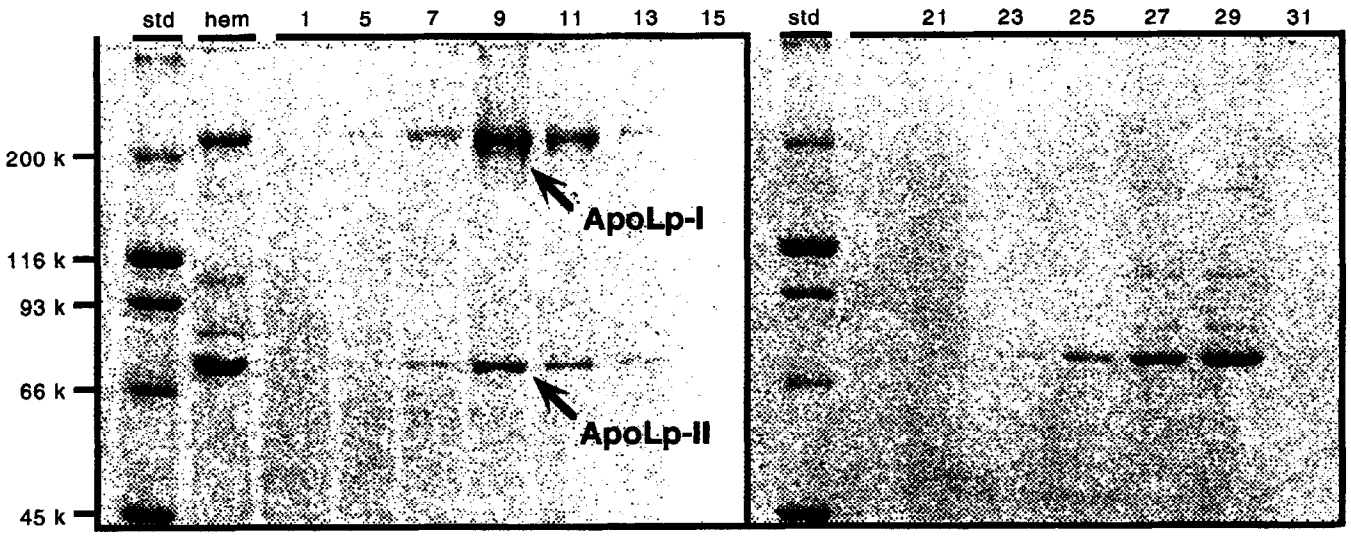

FIGURE 7. Electrophoretic analysis of KBr gradient fractions by SDS-PAGE. 325 μl hemolymph was collected from 27 4–9-day old females and separated in a KBr gradient by ultracentrifugation. The KBr fractions were dialyzed and 15 μl of each fraction and 2 μl of hemolymph (Hem) were loaded on each gel. Molecular weight markers (std) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

hemolymph, and only a slight amount was evident on the epicuticule. After 1 h, the amount of radiolabeled hydrocarbon increased on the cuticular surface while hemolymph hydrocarbon declined, probably as they were taken up by fat body and ovaries. These results are consistent with our previous data showing that there was an almost linear increase in the percent incorporation of propionate into the cuticular hydrocarbons for up to 8 h, whereas there was little increase in the percent incorporation of radioactivity into the internal hydrocarbon after 2 h (Chase *et al.*, 1990). These data indicate that in 3-day old grouped virgin females newly synthesized hydrocarbons are taken up by lipophorin at a very fast rate. They also suggest that the newly synthesized epidermal hydrocarbons are either transported to the hemolymph first and then to the cuticular surface or, less likely, that this pathway gives way after the first hour to a direct transport of newly formed hydrocarbon from the epidermis through cuticular pores. It is also possible that while newly synthesized hydrocarbon is transported directly to the cuticular surface, only transport to internal deposition sites, such as the ovaries, is lipophorin mediated.

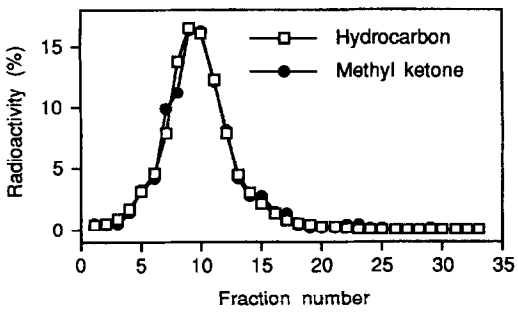

FIGURE 8. Percentage of radiolabeled hydrocarbon and newly synthesized methyl ketone pheromone carried by each KBr fraction in relation to the total radiolabeled hydrocarbon and pheromone in all KBr fractions. [11,12-^{3}H]3,11-Dimethylnonacosane (1 μCi in 0.2 μl hexane) was injected into each of 20 7-day old grouped virgin females. After 4 h, 120 μl of hemolymph collected from the insects was subjected to KBr fractionation. Lipids were extracted from each KBr fraction and the hydrocarbons and methyl ketones were assayed for radioactivity by LSC.

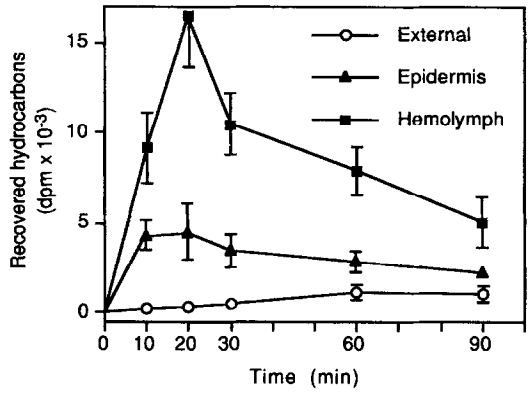

FIGURE 9. Time-course of recovery of newly synthesized hydrocarbons from the external surface, abdominal cuticular epidermis, and hemolymph after injection of [1-^{14}C]propionate (0.26 μCi) into each 3-day old grouped virgin female. The insects were extracted with hexane for 1 min. Then, hemolymph samples were collected, the abdominal cuticular epidermis dissected, washed three times with saline solution, extracted, and the hydrocarbons were fractionated and assayed for radioactivity by LSC. Data are means $\pm$ SEM (n = 6 per mean). Hemolymph volume of a 3-day old grouped virgin female is 21.8 μl (CS, unpublished data).

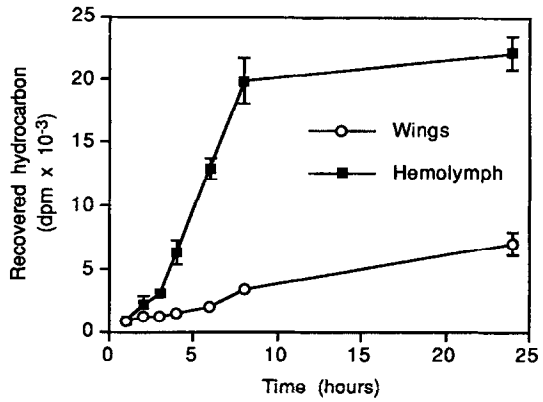

FIGURE 10. Time-course of recovery of hydrocarbon from hemolymph and wings after topical application of [11,12-^{3}H]3,11-dimethylnonacosane (0.14 μCi) onto the sternites of 5-day old grouped virgin females. Hemolymph was collected, wings were extracted, and the hydrocarbons were fractionated and assayed for radioactivity by LSC. Data are means $\pm$ SEM ($n = 10$ per mean).

Hydrocarbon transport to the wings

The wings do not synthesize hydrocarbon (Fig. 2), but their surface contains a large amount of hydrocarbon [Fig. 4(A)]. Hydrocarbons on wings may come from two sources: from mechanical translocation of lipids from the cuticular surface of other body parts, presumably from tergites and sternites which are sites of synthesis, or from lipophorin. After topical application of [11,12-^{3}H]3,11-dimethylnonacosane onto the sternites, up to 6-fold more labeled hydrocarbon was recovered from the hemolymph than from the external surface of the wings (Fig. 10). This suggested that translocation of hydrocarbon from the abdominal epicuticle to the wings was less significant than its uptake by lipophorin, and that the external hydrocarbons of the wings may come from a hemolymph transport pathway. Therefore, we severed the bases of the costa, subcosta, radius, and media of the fore-wings in order to block hemolymph transport, while the wings remained folded normally over the abdomen (Fig. 11). Twenty four hours after [1-^{14}C]propionate injection, the severed fore-wings contained only 30% of the newly synthesized hydrocarbons found on normal control wings. The hemolymph in both the fore- and hind-wings together contains a small amount of hydrocarbon; therefore it does not contribute significantly to the difference between intact and cut wings. Moreover, in both groups, the intact hind-wings had the same amount of hydrocarbon despite their larger surface area and their physical proximity to the abdominal epicuticle. To control for differences in grooming between operated and non-operated insects, we cut the veins of one fore-wing and left the contralateral wing as control. The results (data not shown) were similar to those presented in Fig. 11. These data show that most of the newly deposited hydrocarbons on the surface of the wings are transported through the hemolymph, while less than 30% may come from external lipid migration or grooming activity.

DISCUSSION

Hydrocarbon and pheromone are synthesized by integumentary tissue

Our *in vitro* experiments clearly demonstrate that methyl-branched hydrocarbons and methyl ketone contact sex pheromone components of *B. germanica* females are synthesized in the integument, especially by tissues associated with the abdominal tergites and sternites. Although the head and legs were not dissected completely to expose their epidermis to the incubation medium, the epidermal layer of the thorax was well exposed, and the thorax produced only negligible amounts of hydrocarbons and methyl ketones. Therefore, it is unlikely that low rates of production of lipids by the head, thorax and legs were due to low availability of substrate. In the *in vivo* experiments, propionate was efficiently incorporated into hydrocarbons and methyl ketones within 30 min, indicating that injected propionate circulated freely in the hemocoel and became quickly accessible for lipid biosynthesis. The amount of newly synthesized lipids correlated well with the surface area of the incubated tergites and sternites, lending further support to the conclusion that the thorax and legs, which have relatively large surface areas, synthesize significantly less lipids than the abdominal integument.

The ratio of newly synthesized hydrocarbons and methyl ketones was similar for each tissue, suggesting that the same cells, oenocytes and/or epidermal cells, produce both. The epidermis usually forms a continuous sheet of single-layered cells beneath the cuticle, covering skeletal invaginations such as apodemes, fore- and hind-gut and

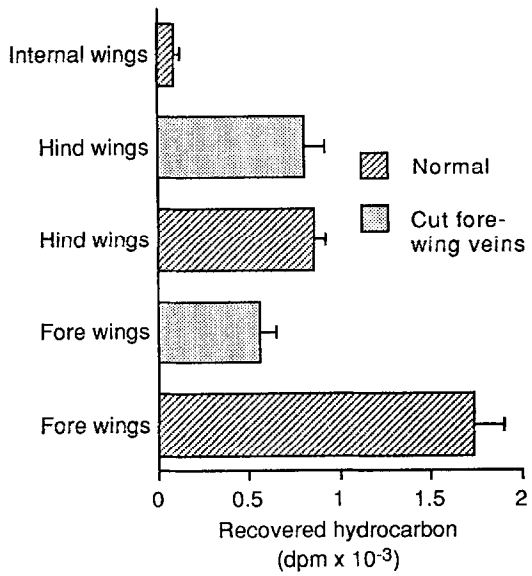

FIGURE 11. Newly synthesized hydrocarbons recovered from pairs of fore-wings with cut veins, pairs of normal fore-wings or hind wings, and internal wings (including hemolymph in both fore- and hind-wings) after injection of [1-^{14}C]propionate (0.24 μCi) into each 5-day old grouped virgin female. After a 24 h incubation, wings were extracted, and the hydrocarbons were fractionated and assayed for radioactivity by LSC. Data are means + SEM ($n = 9$–10 per mean).

the inner surfaces of evaginated structures such as wings, styli, and spurs (Hepburn, 1985). Since our results showed negligible lipid synthesis in the alimentary canal, wings, and integumentary tissues other than the abdominal integument, it appears that oenocytes are involved in the synthesis of hydrocarbons and contact sex pheromone. Preliminary electron microscopy results suggest that oenocytes are common underneath the abdominal cuticle and relatively less common under the cuticle of other body parts in *B. germanica* females. Oenocytes in *B. germanica*, as in other insects, have extensive smooth endoplasmic reticulum (SER) suggesting that these cells synthesize lipids (Liang and Schal, 1993), whereas epidermal cells normally lack enlarged SER and are much smaller than oenocytes (D. Liang, personal communication).

In immatures and adults, one function of the oenocytes is presumably to replenish cuticular lipids which are lost due to abrasion (Diehl, 1975). In the German cockroach female, however, the production of hydrocarbons and contact sex pheromone is regulated by stage-specific feeding and corpus allatum activity during the gonotrophic cycle (Schal *et al.*, 1994). Large amounts of hydrocarbons are deposited in the ovaries prior to ovulation (Fig. 5) and are found in the ootheca upon oviposition (Schal *et al.*, 1994). Therefore, the adult female experiences cycles of synthesis of hydrocarbons and methyl ketones. The oenocytes are expected to undergo the same cyclical activation prior to ovulation and rest during pregnancy.

Lipophorin shuttles pheromone and hydrocarbon

The specific capacity of lipophorin to accept hydrocarbons from epidermal tissue was demonstrated *in vitro* in *P. americana* (Katase and Chino, 1982, 1984; Chino and Downer, 1982). To our knowledge, our experiments provide the first evidence that lipophorin is also directly involved in pheromone transport in insects. Lipophorin loads sex pheromone from the oenocytes and delivers it to various internal tissues and the epicuticle, whereas other hemolymph proteins, such as vitellogenin, carry only trivial amounts of hydrocarbons and pheromone. Injected radiolabeled 3,11-dimethylnonacosane, a precursor to C_{29} methyl ketone pheromone components (Chase *et al.*, 1990, 1992; Juarez *et al.*, 1992), is thus loaded by lipophorin (Fig. 8), delivered to the epidermis, where it is oxidized to pheromone, which is then loaded onto lipophorin for delivery to the epicuticle.

Although the fat body did not synthesize hydrocarbon and pheromone (Fig. 2), it contained large amounts of both (Fig. 5). These lipids may be associated with lipophorin that is "trapped" in the fat body. Alternatively, hydrocarbon- and pheromone-loaded lipophorin might unload some of its neutral lipids at the fat body as it accepts diacylglycerol. Newly formed lipophorin may thus contain its constituent hydrocarbons when it is assembled and released from the fat body. The hydrocarbon profile of the fat body is qualitatively identical to that of lipophorin and the ovaries, suggesting that, in addition to triacylglycerol, phospholipids, and other more polar lipids, the fat body may also be a site for hydrocarbon and pheromone storage and exchange.

The ovary is a major deposition site for hydrocarbons and methyl ketones

In *Manduca sexta*, less than 1% of the total egg lipid is derived from *de novo* synthesis by the follicles (Kawooya and Law, 1988). The remaining egg lipid originates in the fat body and is transported to the ovary by lipoproteins; vitellogenin accounts for only 5% of the total egg lipids, and the remaining 95% is attributed to lipophorin. We also found that *B. germanica* ovaries did not synthesize hydrocarbon and pheromone (Fig. 2). The hydrocarbon composition of the ovaries is similar to that of the epidermis, fat body, and hemolymph, suggesting that lipophorin may transfer lipids from the oenocytes and fat body to the ovaries.

Although the ovaries and fat body are major internal deposition sites for pheromone, the function of pheromone in the oocytes and fat body is unknown. Because lipophorins of locusts and the American cockroach display no species-specificity in their ability to accept hydrocarbons from oenocytes (Katase and Chino, 1984), it is likely that lipophorins display no specificity in the deposition of hydrocarbon derivatives as well. The lipophorin of *B. germanica* may nonspecifically unload its pheromone and hydrocarbons to oocytes and fat body. Alternatively, while lipophorin acts as a reusable shuttle for lipid transport (Kanost *et al.*, 1990), in some insects, lipophorin can be endocytosed and internalized by the follicles into the oocytes and therefore, like vitellogenin, it may contribute its protein subunits and loaded lipids to the oocytes (Kulakosky and Telfer, 1990; King and Tobe, 1993). HDLp-A in *M. sexta* is not recycled back into the hemolymph and does not function as a reusable lipid shuttle between the fat body and the ovary (Kawooya *et al.*, 1988). *Blattella* lipophorins may also undergo a similar endocytosis, explaining the appearance of hydrocarbons and methyl ketones in oocytes. Whether the lipophorins are selectively taken-up into the oocytes and undergo transformations in the embryo remains unknown.

Hydrocarbons that are supplied to the oocytes may be utilized as cuticular lipids and energy sources by the embryo or by the first instars. Catabolism of hydrocarbons may also provide metabolic water to the embryo. Presently, it is unknown whether the pheromone components and hydrocarbons in the ootheca are metabolized into other substrates or remain unchanged through embryogenesis. However, we do know that late in embryogenesis the ootheca contains a similar amount of hydrocarbons ($\approx$ 270 μg) as that lost by the female at oviposition (Schal *et al.*, 1994). This suggests that egg hydrocarbons are not catabolized by the embryos.

Lipid transport pathways to the cuticular surface

When lipophorin containing labeled hydrocarbons was injected into locust hemocoel, the labeled hydrocarbons soon appeared on the cuticular surface (Katase and Chino, 1984). Our *in vivo* experiment also showed that newly synthesized labeled hydrocarbons appeared on the epicuticle. While internal hydrocarbons and methyl ketones were clearly transported by lipophorin through the hemolymph to target sites, epicuticular pheromone and hydrocarbons from the oenocytes appear to have two possible transport pathways: (a) a direct pathway in which newly synthesized hydrocarbons and methyl ketones are delivered directly from oenocytes to the surface, possibly through epidermal cells first and then through cuticular pore canals; (b) an indirect lipophorin-mediated pathway, in which newly synthesized lipids are shuttled from the oenocytes to the epidermal cells by hemolymph lipophorin, and then to the epicuticle through pore canals.

The direct pathway predicts that newly synthesized lipids should be excreted to the surface before they appear in the hemolymph and that oenocytes should be either directly connected to cuticular pore canals, or to epidermal cells which in turn are connected to pore canals. However, in *B. germanica* oenocytes are located beneath the epidermal cells. Electron micrographs of epidermal cells show that they have microvilli which connect them directly to cuticular pore canals through microtubules between the epidermal cells and the cuticle (D. Liang, personal communication). In the mature American cockroach, the epidermal cells are much attenuated and their nuclei displaced by the enlarged oenocytes which come into close contact with the cuticle (Kramer and Wigglesworth, 1950). However, the thin epidermal cells separate the oenocytes from the cuticle. Therefore, lipids would have to traverse the epidermal cells to reach the pore canals. Hydrocarbons do not seem to be stored to any appreciable extent inside the locust oenocytes (Diehl, 1975). Yet, in *B. germanica* the epidermal fraction, which includes oenocytes, had considerable amounts of hydrocarbons and pheromone (Fig. 5), suggesting that the epidermal cells may contain large amounts of lipids. Our finding that newly synthesized integumentary hydrocarbons are first internalized into the hemocoel and then appear on the epicuticle (Fig. 9) suggests that hydrocarbons carried by lipophorin are delivered to epidermal cells and then to the epicuticle.

For integument that does not synthesize hydrocarbons, such as the wings, the epicuticular lipids may come from surface lipid translocation from the tergites and sternites, and/or from a lipophorin-mediated hemolymph pathway. Our results (Fig. 11) indicate that the lipophorin-mediated pathway plays a major role in lipid deposition on the surface of the wings. Together, these results support the hypothesis that in *B. germanica* females an indirect lipophorin-mediated pathway plays an important role in the transport of non-polar lipids to the epicuticle. The unloading of hydrocarbons at epidermal cells, and their further transport through pore canals to the cuticular surface remain poorly understood mechanisms.

For hydrocarbon-synthesizing integument, such as tergites and sternites, a direct pathway for lipid deposition to the epicuticle may also be involved because oenocytes are connected directly to epidermal cells. It is likely that lipids may be transferred directly from oenocytes to epidermal cells and through cuticular pores without the involvement of lipophorin. Whether this pathway is indeed employed and the relative importance of these two pathways for external lipid deposition on tergites and sternites, remains unclear.

In contrast to our results with *B. germanica*, after [1-^{14}C]acetate was injected into *Triatoma infestans* females, epicuticular hydrocarbon labeling was shown to precede the appearance of label in hemolymph hydrocarbons (Juarez and Brenner, 1989). It thus appears that, in relation to the different locations and coupling of oenocytes to epidermal cells or cuticle, different insects may employ different pathways for hydrocarbon delivery to the cuticular surface. For example, in *Culicoides nubeculosus* females, the abdominal dorsal and ventral integument differ considerably in their structure (Ismail and Zachary, 1984). The dorsal atrichial areas have a normal epidermis and an unperforated cuticle, whereas the ventral atrichial areas have a cuticle perforated by numerous tubules and no epidermal cells. Oenocytes, which produce sex pheromone, are located only beneath the ventral atrichial areas, and sex pheromone from oenocytes is believed to be transported through the hemolymph to the ventral porous area where it is released.

Many questions arise from the present work regarding the different roles that lipophorin plays at different hydrocarbon deposition sites, and its apparent dual role as a shuttle as well as a maternal contribution to embryos. We are particularly interested in the mechanisms involved in the internalization of epicuticular lipids, and whether lipophorin contributes to this reverse process. In the cabbage looper and the house cricket, extrusion of newly synthesized hydrocarbons to the cuticle was well correlated with insecticide uptake, including carbaryl, parathion, DDT and methoxychlor, which were layered on the cuticle of the insect (Theisen *et al.*, 1991; de Renobales *et al.*, 1991). The authors suggested that the extrusion of cuticular lipids to the surface might occur through the same route by which contact insecticides were taken up into the insect. Perhaps the lipid extrusion and internalization is an exchange process between lipophorin and its epidermal target tissues, although our results (not shown) indicate that extrusion is greatly favored.

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