Pergamon

Journal of Insect Physiology 45 (1999) 305–315

Journal of Insect Physiology

Site of synthesis, tissue distribution, and lipophorin transport of hydrocarbons in *Blattella germanica* (L.) nymphs

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Received 27 May 1998; accepted 16 July 1998

Abstract

The site of hydrocarbon (HC) synthesis and the amount of HC in various tissues were investigated in relation to developmental stage in the last larval stadium of the German cockroach, *Blattella germanica*. Abdominal integument linearly incorporated [1-^{14}C]propionate into HC for at least 6 h in vitro, whereas other body parts synthesized little or no HC. The third through sixth abdominal sternites and tergites were the principal sites of synthesis. High rates of HC synthesis resulted in a fivefold increase in internal HC during the last stadium. We examined the distribution of HC in the hemolymph, fat body, and the developing imaginal cuticle. Hemolymph HC titer was relatively constant at $\approx$ 8 μg/μl. However, as hemolymph volume increased from 5 to 11 μl in the first 4 days of the last stadium, HC content increased and then remained stable the remainder of the stadium. Lipophorin, immunoprecipitated with adult lipophorin polyclonal antibodies, was the only HC carrier protein in nymphal hemolymph and its HC profile was identical to that of hemolymph and similar to that of the epicuticle. The concentration and total amount of hemolymph lipophorin increased until 3 days before adult eclosion and declined immediately after ecdysis. The HC content of non-biosynthetic integument (legs, pronotum) doubled during formation of the imaginal cuticle, as did the HC content of sternites, which synthesize HC. HC content of fat body, however, increased threefold during the same period, suggesting that the fat body serves as a storage site for HC during cuticle formation. We conclude that in the last stadium HC is synthesized by abdominal oenocytes, loaded onto hemolymph lipophorin, and transported to fat body and both nymphal and imaginal cuticle. Hydrocarbons associate with the imaginal integument several days before eclosion.

Keywords: *Blattella germanica*; Hydrocarbons; Hemolymph; Cuticle; Lipophorin; Epidermis

1. Introduction

Epicuticular hydrocarbon (HC) is the principal waterproofing barrier of many insects, including the German cockroach, *Blattella germanica* (L.) (Hadley, 1982; Nelson and Blomquist, 1995). Disruption or removal of HC results in the rapid death of the insect (Wigglesworth, 1944, 1958; Ebeling, 1976). The stress imposed by the terrestrial environment on organisms with a high surface-to-volume ratio makes waterproofing a critical concern, even over short time spans. Moreover, immatures face a repeated challenge of HC synthesis and deposition onto the new cuticle as they pass through the series of molts (six in female *B. germanica*) to adulthood. The epicuticle of newly eclosed insects is covered with HC synthesized during the previous stadium (Guo and Blomquist, 1991; Young and Schal, 1997), but the course of this synthesis, sites of HC storage, and the transport pathway(s) of new HC to the developing epicuticle remain largely unknown (Schal et al., 1998).

The site of HC synthesis varies among insects (Romer, 1991; Schal et al., 1998). In the locust *Schistocerca gregaria*, the peripheral fat body, containing oenocytes, was the only tissue that produced HC in vitro, suggesting that the oenocytes were the HC biosynthetic site (Diehl, 1973, 1975). Oenocytes, excised from *Tenebrio* larvae, synthesized two orders of magnitude more HC than the epidermal cells (Romer, 1980). In the cockroaches *Periplaneta americana* (Nelson, 1969) and *B. germanica* (Gu et al., 1995), HC is synthesized by the abdominal integument, and the oenocytes of cockroaches are associated with the cuticle, in intimate contact with the epidermal cells (Kramer and Wigglesworth, 1950). The association of the synthetic tissue with the depo-

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0022–1910/99/$ - see front matter
PII: S0022-1910(98)00128-0

sition site of its product, the epicuticle, provides an opportunity to determine how HC synthesis and sites of HC deposition change during the last stadium and through the imaginal molt.

Once synthesized, HC must be transported to target tissues. In insects where HC is synthesized in the epidermal layer HC may be transferred directly to the cuticle, perhaps through the pore canals. This suggests that although the abdominal epicuticle of cockroaches may be directly supplied with HC, epicuticle in other body regions would have to be supplied with HC by some transport mechanism. Grooming does provide an opportunity for insects to redistribute cuticular waxes, but several studies implicate a hemolymph transport pathway (Chino, 1985; Soulages and Wells, 1994; Schal et al., 1998). HC contained in the hemolymph of many insect species, including locusts, beetles, bees, flies, and cockroaches, is qualitatively similar to the respective epicuticular HC (Katase and Chino, 1982, 1984; Blomquist et al., 1987; Pho et al., 1996; see Schal et al., 1998 for other examples). In adult *B. germanica* all of the hemolymph HC is associated with lipophorin, a hemolymph lipoprotein that transports HC to both the epicuticle and developing oöcytes (Gu et al., 1995).

In this paper we use in vitro radiotracer techniques to determine the site of HC synthesis in *Blattella* nymphs. We then determine the role of hemolymph lipophorin in HC transport and the relative importance of hemolymph, fat body, and the imaginal cuticle as sites of HC deposition. The titer and total quantity of lipophorin in the hemolymph are of interest because only lipophorin can solubilize HC in the hemolymph. We conclude that the fat body serves as a HC storage depot and that significant stores of HC associate with the newly forming imaginal cuticle several days before eclosion.

2. Materials and methods

2.1. Insects

Insects were from an American Cyanamid insecticide-susceptible strain. The colony was raised in glass battery jars at 27°C and ambient relative humidity on a 12:12 light:dark cycle. Insects were provided Purina Rat Chow #5012 (Purina Mills, St. Louis, MO) and water ad libitum. All insects used in experiments were staged to within 2 h of ecdysis into the last nymphal stadium in mid-photophase and were not allowed to eat their exuviae. Pairs of nymphs were placed in clean 100 × 15 mm plastic Petri dishes with water and chow and maintained at 27°C and 50% RH. Preliminary work showed that chow portions larger than what an insect would ingest in the last stadium contained no detectable HC.

2.2. Chemicals

Sodium [1-^{14}C]propionate (1.9 GBq/mmol) and [carboxyl-^{14}C]inulin (63.3 MBq/g) were obtained from NEN Research Products (DuPont, Boston, MA) or American Radiolabeled Chemicals Inc. (St. Louis, MO). All other chemicals were from Sigma (St. Louis, MO), Bio-Rad (Richmond, CA), or Fisher Scientific (Pittsburgh, PA). Pansorbin Cells used in immunoprecipitation were obtained from Calbiochem (San Diego, CA).

2.3. HC extraction, purification, and quantification

All insects were killed by freezing at − 20°C overnight. We adopted procedures that were developed and validated for *B. germanica* nymphs: the epicuticular and internal extractions effectively removed 98% and 99%, respectively, of the available HC (Young and Schal, 1997). Briefly, external lipids were removed with three consecutive rinses (10 min total immersion) in hexane containing *n*-hexacosane as an internal standard. Internal lipids, including those in any developing imaginal cuticle, and hemolymph samples were extracted by a modification (Young and Schal, 1997) of the procedure of Bligh and Dyer (1959).

The solvents were evaporated under N_2 and the lipids were loaded in hexane onto silica gel (Biosil A, Bio-Rad, Richmond, CA) in a Pasteur pipette minicolumn. The HC fraction was eluted with 7 ml hexane and an aliquot was injected into a splitless injector leading to a Hewlett-Packard (Avondale, PA) HP-1 capillary column (25 m × 0.32 mm × 1 μm). A Hewlett-Packard 3890II gas–liquid–chromatograph (GLC) was interfaced with a 3365II ChemStation. The column temperature was held at 150°C for 2 min and then increased at 10°C per min to 245°C, at 1°C per min to 260°C, and at 10°C per min to 280°C. The injector and flame ionization detector were held at 280°C and 300°C, respectively.

2.4. Synthesis of HC in vitro

Abdominal sternites and tergites, the pronotum, and the digestive tract were removed with forceps and rinsed three times with saline. Great care was taken to remove all fat body from the tissues. The head and remainder of the thorax, including the legs, were each separated from adjacent tissues, bisected longitudinally with a razor, and incubated with the hemolymph they usually contained. Tissues were incubated in 0.5 ml *B. germanica* saline solution (BG-SSA, pH adjusted to 7.2, osmotic pressure 410 mOsm) (Kurtti and Brooks, 1976) at 27°C with 4657 Bq sodium [1-^{14}C]propionate. Methylmalonyl CoA, derived from propionate, can serve as a methyl-branch donor in the synthesis of methyl-branched HC in *B. germanica* (Chase et al., 1990). An orbital waving shaker (The Waver, VWR) was used to oxygenate the

tissues (Katase and Chino, 1982). After 3 h, or as indicated, 1 ml of methanol and 3 ml of hexane were added and the tissue and medium were disrupted with a sonicator probe (Micro Ultrasonic Cell Disrupter, Kontes, Vineland, NJ). Following centrifugation (2000***g*** for 10 min) a 2 ml aliquot of the hexane was subjected to column chromatography, as above, and the HC fraction was quantified by liquid scintillation spectrometry (LS5301, Beckman, Palo Alto, CA), with a counting efficiency of 97%.

2.5. Redistribution of HC during the last stadium

The fate of newly synthesized HC was tracked before and throughout the genesis of the imaginal cuticle beneath the nymphal cuticle. Methyl-branched HC was radiolabeled in 4-day-old female nymphs by injecting 4033 Bq [1-^{14}C]propionate. At this dose all label incorporation into HC occurred within 4 h (Young and Schal, 1997). Paired nymphs were removed at 2 day intervals through adult eclosion, and radiolabeled HC was quantified in hemolymph and the remaining internal tissues. Total hemolymph [^{14}C]HC, total internal [^{14}C]HC, and other internal (non-hemolymph) [^{14}C]HC were calculated. To determine hemolymph volume, insects were anesthetized on ice, and 0.6 μl of [carboxyl-^{14}C]inulin (431.3 Bq; 26 000 dpm) in *Blattella* saline was injected into the abdominal hemocoel using a 10 μl Hamilton syringe (Reno, NV) fitted with a 33-gauge needle (Gu et al., 1995). After 2 h at 27°C the insects were anesthetized with CO_2 and hemolymph was drawn from a clipped cercus into 5 μl calibrated glass capillaries which had been preloaded with 1 μl of *Blattella* saline. The hemolymph/saline was dispensed into chloroform:methanol and the capillary rinsed with three aliquots of chloroform. Total hemolymph volume per insect was calculated by dividing total dpm injected by dpm per μl of hemolymph. The hemolymph volume thus calculated was used to calculate titers and total hemolymph content of HC, proteins, and lipophorin in all subsequent experiments.

2.6. Lipophorin purification and extraction

High density lipophorin was purified from adult female *B. germanica* using KBr gradient ultracentrifugation (Gu et al., 1995; Sevala et al., 1997). Lipophorin polyclonal antibodies were raised in New Zealand White rabbits and radial imunodiffusion assays confirmed specificity to lipophorin and no cross-reactivity with other hemolymph proteins (Sevala et al., 1999). Hemolymph was collected from 6-day-old female nymphs, the hemocytes were removed (6000***g*** for 10 min), and lipophorin was immunoprecipitated by addition of excess anti-lipophorin rabbit serum followed by Pansorbin cells. Lipids were extracted and HC purified from the pelleted (10 000***g*** for 10 min) lipophorin and from the supernatant using the modified Bligh and Dyer (1959) procedure, as detailed above.

2.7. Quantification of hemolymph protein and lipophorin

Lipophorin titers were determined by indirect ELISA as described in Popham and Chippendale (1993). Aliquots (200 μl) of diluted hemocyte-free hemolymph in coating buffer (50 mM sodium carbonate–bicarbonate, pH 9.4) were bound to wells of Immunoware-96 well ELISA plates (high binding) by overnight incubation at 4°C. A series of lipophorin standards and blanks was similarly prepared and included in each plate. Plates were rinsed three times with PBS containing Tween-20 (PBST; 8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride and 0.05% Tween-20, pH 7.4) and blocked for 90 min with 2% normal rabbit serum at 27°C. Wells were then loaded with 200 μl of diluted lipophorin antiserum in PBST (1:500) containing normal rabbit serum, and incubated for 2 h at 27°C. Plates were rewashed and developed at room temperature with 200 μl of enzyme substrate ABTS, and the reaction was stopped after 30 min by adding 100 μl of 1% sodium dodecyl sulfate to each well. Absorbance was read at 405 nm in a Bio-Tek automated microplate reader. Specificity and validity of the assay have been demonstrated with serially diluted hemolymph, purified *Blattella* vitellin, and bovine serum albumin (Sevala et al., 1999). Protein titer was determined by the Bradford (1976) method (Bio-Rad, Richmond, CA), using BSA as a standard.

2.8. Relative quantities of HC associated with early- and late-stadium tissues

In order to estimate the relative HC contents of early-stadium (day 4) and pre-eclosion (day 10) epidermis, cuticle was removed from various body parts of hexane-washed insects and extracted for HC. The second and third abdominal sternites and the pronotum were excised, separately washed in saline, and adhering fat body and tracheae carefully removed. The sternites and pronotum were then separately homogenized and extracted and the purified HC fractions were quantified by GLC. The distal portion of all six legs was removed at the trochanter, the hemolymph expressed onto a filter paper, and the remaining internal cuticular HC quantified. Fat body was cleared of as much tracheae as possible, rinsed in saline, dried, weighed and extracted for HC analysis. The tracheae so removed were placed in vials, their total length measured with an ocular micrometer, and their HC quantified.

2.9. Preparation of histological sections of cuticle

Longitudinal sections of the third abdominal sternite were dissected, cleaned of other tissues, fixed in alcoholic Bouin's solution, dehydrated and mounted in Spurr's medium (Spurr, 1969); 5 μm sections were stained with 1% methylene blue: Azo II, 1:1, in 1% sodium borate solution.

2.10. Statistical analysis

Statistical analyses were performed using either Microsoft Excel version 5.0 (Microsoft, 1995) or Abacus Statview version 4.5 (Abacus Concepts, 1995). All data are presented as means ± SEM. Multiple-range comparisons were made by Fisher's Protected Least Significant Difference if sample sizes were equal, and by the Games–Howell test if they were not.

3. Results

3.1. Landmark events during the last stadium

Females fed intensively during the first 5 days of the last stadium and synthesized HC until day 8; 55.7% eclosed on day 13 (Young and Schal, 1997). Histological sections of sternites showed only the dark-staining lamellate nymphal cuticle on day 4 whereas day 10 insects also showed a thick (17 μm), convoluted, lightly stained imaginal cuticle separated from the nymphal cuticle by the apolytic space (data not shown). Radioimmunoassays of hemolymph ecdysteroids (data not shown) indicated a hemolymph ecdysteroid peak on day 8, preceded by slowly rising titers on days 4–7, suggesting that endocuticle synthesis occurred on or immediately after day 8 (Nijhout, 1994).

3.2. In vitro determination of the site of hydrocarbon synthesis

Cuticle, dissected from female nymphs 6 days post-ecdysis, synthesized HC in vitro at a constant rate over a range of incubation times from 1 to 6 h (Fig. 1). The 3 h incubation time was used in subsequent experiments. Incubation of other tissues, dissected from 6-day-old female last instars, showed that only the abdominal integument synthesized HC (Fig. 2). Incubation of individual sclerites showed that sternites 3–6 and tergites 3–6 had the greatest biosynthetic activity (Fig. 3). The sternum synthesized HC at a slightly higher rate than the tergum, roughly in proportion to their relative apparent surface areas.

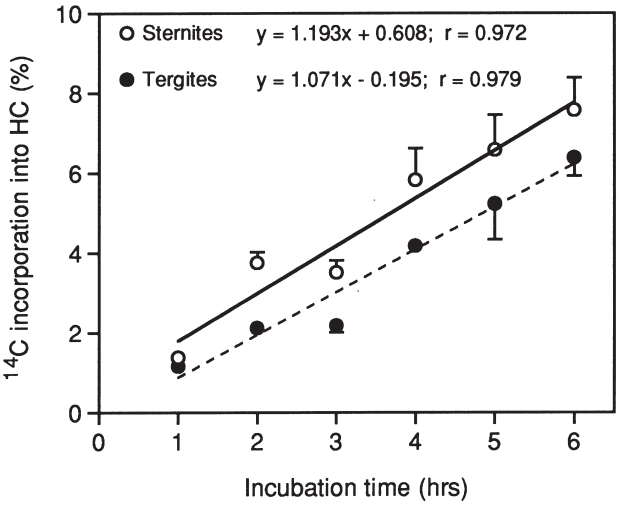

Fig. 1. Time course of ^{14}C uptake into methyl-branched hydrocarbons by abdominal sternites and tergites of *B. germanica* nymphs. Sclerites were incubated with 4043.7 Bq [1-^{14}C]propionate in *Blattella* saline. Each point represents the mean ± SEM of four to five determinations at 1, 2, 4, 5 and 6 h and of 15 determinations at 3 h.

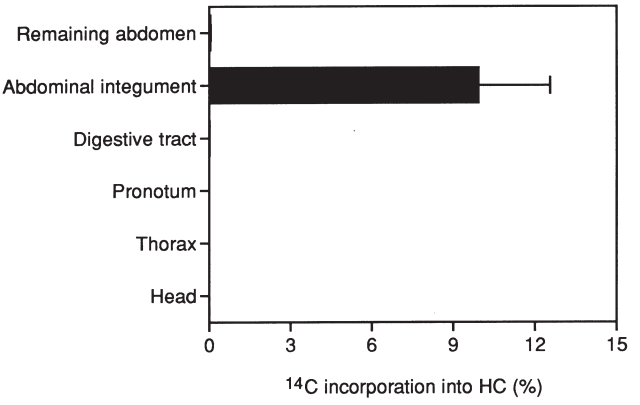

Fig. 2. Hydrocarbon biosynthesis in vitro by dissected tissues of *B. germanica* nymphs. Dissected tissues were incubated for 3 h with 4043.7 Bq [1-^{14}C]propionate. Data represent the means + SEM of 10 insects.

3.3. Sites of accumulation of internal HC

We sought to determine what pathways were involved in HC transport and which tissues accumulated HC. Epicuticular HC doubled during the course of the last stadium (Young and Schal, 1997). By contrast, total internal HC increased nearly fivefold between days 2 and 12 of the last stadium (Fig. 4(b)). Hemolymph HC accounts for a significant fraction of internal HC. The HC concentration of the hemolymph ranged from a low of 7.6 ± 0.4 μg/μl on day 8 to a high of 9.0 ± 0.4 μg/μl on day 6; it thus remained constant at ≈ 8 μg/μl throughout the last larval stadium, except for day 6 (ANOVA, $F_{6,44} = 4.704$, $p = 0.0009$, Games–Howell test) (Fig. 4(a)). Hemolymph volume, as determined by dilution of radiolabeled inulin, was 5.2 ± 0.25 μl 2 days

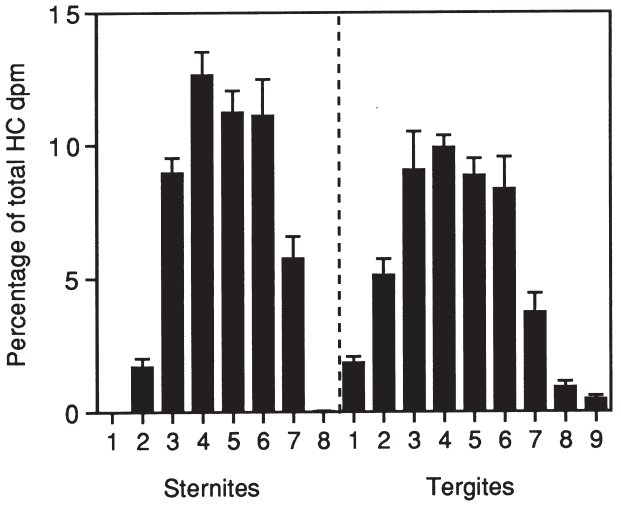

Fig. 3. Hydrocarbon biosynthesis in vitro by sternites and tergites of *B. germanica* nymphs. Sclerites were individually incubated for 3 h with 4043.7 Bq [1-^{14}C]propionate in saline. Data represent mean + SEM percentage of the total recovered hydrocarbons (n = 10 insects).

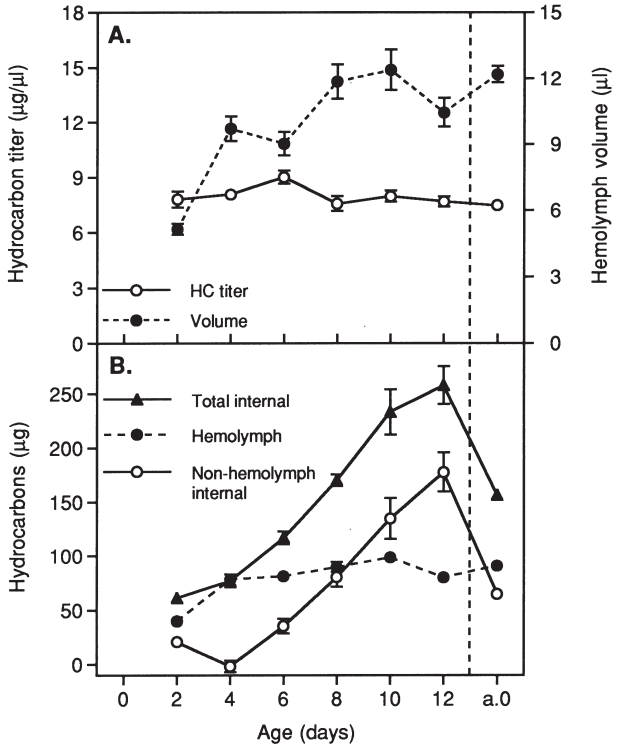

Fig. 4. Tissue distribution of internal HC in last instar *B. germanica* females: (a) hemolymph volume and hemolymph HC titer (n = 11–15 insects for volume determinations; 5–9 insects for HC titers); HC titer on day 6 was significantly higher than on other days; (b) total internal hydrocarbon content, hemolymph HC (titer × volume) and non-hemolymph HC (total internal HC minus hemolymph HC) (n = 5–9 insects). Results are expressed as means ± SEM. Dashed vertical line indicates the time of imaginal ecdysis. a.0 = adult day 0.

post-ecdysis and increased significantly to double that, 10.7 ± 0.73 µl, 2 days later (Fig. 4(a)). Hemolymph volume continued to increase gradually to 12.4 ± 0.9 µl on day 10, but values between day 4 of the last stadium and day 0 of the adult stage were not significantly different ($p > 0.05$, Games–Howell test).

Total hemolymph HC content is the arithmetic product of hemolymph volume and HC titer values of a given day. Hemolymph of 2-day-old nymphs contained 40.2 ± 2.3 µg HC, but only 2 days later the HC content of the hemolymph doubled to 86.2 ± 2.8 µg (ANOVA, $F_{6,44} = 17.725$, $p < 0.0001$) (Fig. 4(b)). However, much like hemolymph volume, total hemolymph HC changed little after day 4 ($p > 0.05$, Games–Howell test). Then, like hemolymph volume, HC content of the hemolymph increased gradually to ≈ 100 µg on day 10 ($p < 0.05$; Games–Howell test). The arithmetic difference of total internal and total hemolymph HC is also represented in Fig. 4(b). These non-hemolymph HC pools increased from 0 on day 4 to 177.7 ± 18.0 µg immediately before eclosion. Thus, hemolymph HC accounted for 100% of internal HC on day 4, 53% on day 8, and only 32% on day 12. Relatively constant hemolymph HC suggested that after an initial accumulation of HC in the hemolymph, the blood might serve a transport role, delivering HC to other tissues. We hypothesized that the fat body and the developing adult cuticle accumulated HC after day 4.

3.4. Transport of HC through the hemolymph

Two routes of transport of HC to the imaginal cuticle are possible: (1) direct transfer from the oenocytes to the newly forming cuticle, or (2) indirect transport through the hemolymph. Thus, HC that is synthesized before the imaginal cuticle is formed (< 8 days) is either stored in the integument or transported via the hemolymph to internal storage sites. This model predicts a redistribution of internal HC during the stadium. On day 4, before the adult cuticle formed, injected [1-^{14}C]propionate was incorporated into methyl-branched HC and 98.9 ± 4.77% of the newly synthesized internal HC appeared in the hemolymph within 6–8 h that elapsed between the injection of propionate and removal of the hemolymph sample (Fig. 5). The [^{14}C]HC subsequently declined in the hemolymph and by day 8 60% of the internal [^{14}C]HC appeared in other internal tissues. Throughout the stadium some [^{14}C]HC appeared on the nymphal epicuticle, but this quantity was small (< 25% of the total) and did not increase or decrease after day 4 (data not shown). Also, total internal [^{14}C]HC remained relatively constant, confirming that we were tracking a finite pool of previously synthesized HC.

Lipophorin was immunoprecipitated from hemolymph of day 6 female nymphs. All hemolymph HC was associated with the pelleted lipophorin, and none with other

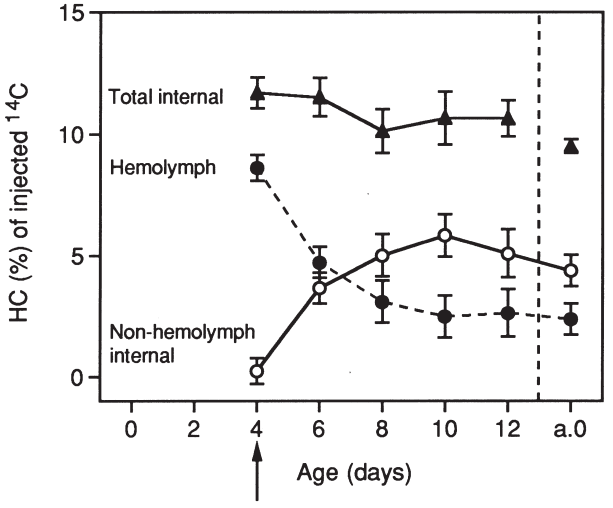

Fig. 5. Internal HC resolved into two pools in last instar females of *B. germanica*. [1-^{14}C]Propionate was injected into the hemocoel of all insects on day 4 (arrow); it was incorporated into methyl-branched HC within 4 h. Hemolymph-borne labeled HC and total internal labeled HC were determined, and non-hemolymph internal values were calculated as in Fig. 4. Data are means ± SEM (n = 7–10 insects). Dashed vertical line indicates the imaginal ecdysis. a.0 = adult day 0.

hemolymph proteins (data not shown). Moreover, lipophorin HC was qualitatively identical to hemolymph HC and, with only minor quantitative differences in individual peaks, the pattern was the same on the epicuticle (Fig. 6). These data suggest that the hemolymph and epicuticular HC share a common origin and that lipophorin accepts HC from the oenocytes, the presumptive site of HC synthesis.

An ELISA was used to determine the concentration of lipophorin throughout the last stadium. Lipophorin was detected in hemolymph samples from all stages. Its concentration increased steadily from 2.9 ± 0.29 μg/μl on day 1 to 14.8 ± 0.67 μg/μl on day 10, 2–3 days before eclosion (Fig. 7(a)). Lipophorin remained relatively constant during the last 3 days of the stadium, followed by a sharp decrease in the lipophorin titer during ecdysis. HC, which is carried by lipophorin, remained constant through eclosion (Fig. 4(a)). Total hemolymph lipophorin, a product of hemolymph volume and lipophorin concentration, followed a pattern almost identical to lipophorin titer, given the stable blood volume after day 4 (Fig. 7(b)).

In contrast, the concentration of all hemolymph proteins (including lipophorin) increased much more rapidly from 10.9 μg/μl to 37.1 μg/μl on day 7 (Fig. 7(a)). A gradual decline in both protein concentration and protein content followed, culminating in a large decline through ecdysis.

3.5. Accumulation of HC in the fat body and the imaginal integument

In the second half of the last stadium, after HC synthesis had ceased, stored HC could be translocated from

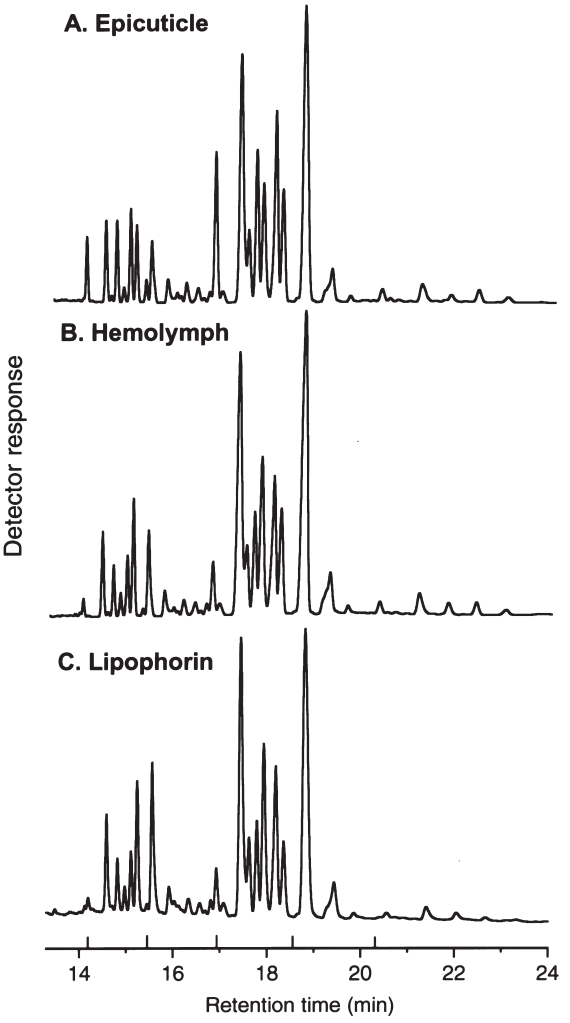

Fig. 6. Gas liquid chromatograms of HC from last instar *B. germanica* females: (a) epicuticle, day 4; (b) hemolymph, day 4; (c) lipophorin, day 6. Tick marks above the x-axis denote retention times of *n*-C27 (R_t = 14.18 min), 28 (15.42), 29 (16.93), 30 (18.54), and 31 (20.32).

the site of biosynthesis directly to the new cuticle. However, because all newly synthesized HC on day 4 is delivered to the hemolymph (Fig. 5), an alternative model would suggest that HC is stored internally, possibly in the fat body, and late in the stadium is delivered, through the hemolymph, to the new imaginal cuticle. We tested these alternatives by comparing, on days 4 and 10, the internal HC content of the legs, fat body, and the pronotum, which do not synthesize HC, and the sternites, which do. A hemolymph transport pathway late in the stadium would result in accumulation of HC throughout the integument, including the legs and pronotum, and not just in HC synthesizing integument. Moreover, greater accumulation of HC in the fat body and in the sternites would suggest that both tissues might store HC.

Internal HC in the distal portion of the legs and pronotum, both of which lack the ability to synthesize HC, increased significantly (t-test, p = 0.016 and 0.012, respectively) between days 4 and 10 (Fig. 8). Because

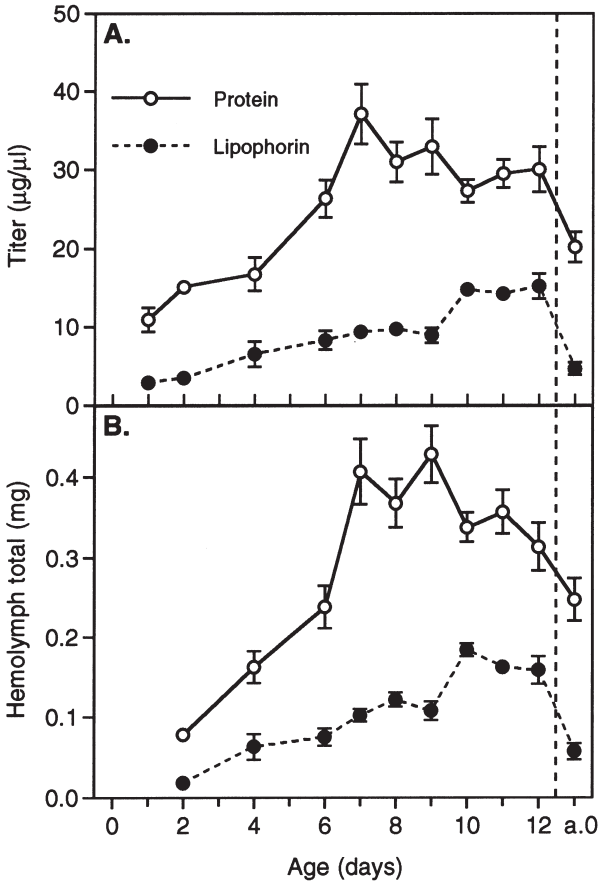

Fig. 7. Titers of lipophorin and protein concentration in the hemolymph of last instar female *B. germanica*: (a) titers of protein, as determined by Bradford assay, and lipophorin, as determined by ELISA; (b) total protein and total lipophorin in the hemolymph, calculated from titers in (a) and hemolymph volumes (Fig. 4(a)). Hemolymph volumes for days 7, 9, and 11 are averages of preceding and following days. Dashed line indicates the imaginal ecdysis. Data are means ± SEM (n = 4–12 insects per age).

the external surface of each insect was extracted prior to the dissection, and hemolymph was removed by gentle expression and a saline rinse, all the extracted HC was associated with the inner portion of the nymphal integument on day 4 and with the inner portion of the nymphal integument and the imaginal cuticle in day 10 females. The second and third abdominal sternites, which synthesize HC, showed a similar twofold increase between days 4 and 10 (p = 0.0025), suggesting that no appreciable HC was stored in the oenocytes.

In contrast, the HC content of the fat body increased threefold, from 2.0 ± 0.25 to 6.1 ± 1.02 µg/mg of dry fat body (p = 0.0004) (Fig. 8). Tracheae that were dissected from the fat body, however, did not change in HC content per unit length during the 6 days interval, indicating that stored HC associated with fat body and not with tracheal cuticle. These data support the idea that late in the stadium the fat body may serve as a source of HC for the developing adult cuticle.

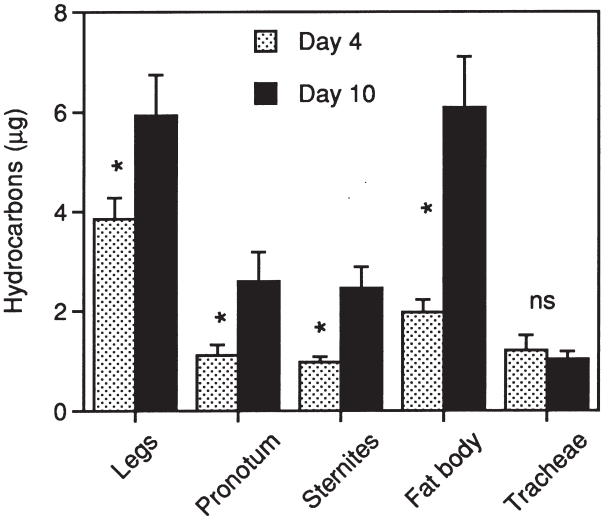

Fig. 8. Internal HC content, as determined by GLC, of the pronotum, second and third abdominal sternites, pronotum, fat body, and tracheae of day 4 and day 10 last instar *B. germanica* females. Epicuticular HC were removed from the nymphal cuticle with hexane prior to extraction. Data for fat body are per mg of dried tissue and for tracheae per linear cm. Data are means + SEM (n = 5 insects for day 4; 4 for day 10). Asterisk indicates significant differences (t-test; $p < 0.05$) between days 4 and 10 for each tissue; ns = not significant ($p > 0.05$).

4. Discussion

4.1. The sites of HC synthesis

The abdominal integument is the site of HC synthesis in *Blattella germanica* nymphs (Figs. 1–3), as previously shown for adult females (Gu et al., 1995). The different levels of HC synthesis displayed by the sternites and tergites (Fig. 3) appear to correspond roughly to the their respective surface areas, based on visual inspection. For instance, the first and last segments are much reduced in both surface area and HC production. This would suggest that the HC synthesizing cells, presumably oenocytes, are evenly distributed within the abdominal integument.

Several related studies have implicated oenocytes in HC synthesis in both hemimetabolous and holometabolous insects. In the cockroach *P. americana* the oenocytes are distributed below the epidermal cells of the abdominal tergites and sternites and are separated from the hemocoel by a basal membrane (Kramer and Wigglesworth, 1950), as in *B. germanica* (Liang and Schal, 1993). In both species the abdominal integument synthesizes HC in vitro (Nelson, 1969; Katase and Chino, 1982; Gu et al., 1995; Figs. 1–3) but not the integument of the thorax, legs, head, and alimentary canal. Similarly, in the housefly, *Musca domestica*, cuticular HC is synthesized in the abdominal integument (Dillwith et al., 1981), and microsomal preparations from the abdominal integument synthesize HC, whereas preparations from the fat body do not (Blomquist et al., 1993). The oenocytes have also

been implicated in HC biosynthesis in the locusts *S. gregaria* and *Locusta migratoria* (Diehl, 1973, 1975; Katase and Chino, 1984); in the fifth instar females of *S. gregaria*, the oenocytes are found only in the abdominal peripheral fat body situated beneath the epidermis (Diehl, 1975). HC synthesis by isolated oenocytes has been documented only in the beetle *Tenebrio molitor* in which the oenocytes are grouped along the upper side of the tracheal trunks, separated from the fat body (Romer, 1980). In both locusts and the beetle, the epidermis synthesizes less than 1% of the amount simultaneously synthesized by the oenocytes. Thus, the location of the oenocytes varies considerably among insects, and the site of HC synthesis in insects presumably varies with the location of the oenocytes. Recently, complete feminization of male HC was induced in *Drosophila melanogaster* by targeted expression of the *transformer* gene in adult oenocytes (Ferveur et al., 1997), in support of the idea that sex-specific oenocytes synthesize sex-specific HC.

That the integument of nymphs is the site of HC synthesis is interesting in light of the fact that, in the second half of the stadium, the epidermis is also elaborating the imaginal cuticle in the space between the nymphal cuticle and the epidermal cells. This suggests a separation of biosynthetic tasks between the oenocytes and epidermal cells, and possibly a temporal separation of tasks within the oenocytes. Ultrastructural studies support the idea that differences between oenocytes and epidermal cells are related to the type of products they biosynthesize (Wigglesworth, 1970, 1973). Oenocytes are large cells characterized by regular eosinophil cytoplasm that is rich in smooth endoplasmic reticulum and mitochondria, and they synthesize lipids, including HC and ecdysteroids (Locke, 1969b; Romer, 1991). HC synthesis occurs in the first half of the stadium (Young and Schal, 1997), and while the oenocytes synthesize HC, the epidermal cells undergo mitosis (Kunkel, 1975). The epidermal cells, on the other hand, contain large amounts of rough endoplasmic reticulum and are involved in protein synthesis (Locke, 1969a, 1970). It will be of interest to determine whether late in the last nymphal stadium of *B. germanica* the oenocytes switch from synthesizing HC to secreting other products, including ecdysteroids.

4.2. Hydrocarbon transport through the hemolymph

The HC in last instar females of *B. germanica* are long-chain normal and methyl-branched alkanes, primarily composed of two homologous series of C27 and C29 compounds. The major components are 3,11-, 3,9-, and 3,7-dimethylnonacosane, 9-, 11-, 13-, and 15-methylnonacosane, 3-methylnonacosane, and *n*-nonacosane. The types of HC and their relative composition are remarkably similar to those of adult males and females (Jurenka et al., 1989). Moreover, epicuticular, internal, and hemolymph extracts exhibit similar chromatographic HC profiles, as previously shown in other insects (see Schal et al., 1998).

The internal HC pools in *B. germanica* adults and nymphs are substantially larger than the respective epicuticular HC, up to fourfold larger late in the last instar (Schal et al., 1994; Young and Schal, 1997). In both life stages large amounts of internal HC reside within the hemolymph and all hemolymph HC is associated with a high density lipophorin (Fig. 4; Gu et al., 1995). Similarity of hemolymph (lipophorin) HC to epicuticular HC (Fig. 6; Schal et al., 1998) suggests not only a common synthetic origin but also that the hemolymph serves as a transport medium for HC. In the adult female German cockroach large quantities of HC accumulate in the hemolymph, and only a small fraction is delivered to the epicuticle (Schal et al., 1994). As the oöcytes begin to mature, HC is then deposited in the developing oöcytes (C.S., unpublished).

We have now found a similar situation in last instars. The HC concentration in the hemolymph remains in a narrow range (7.6–9.0 μg/μl) throughout the stadium and through ecdysis (Fig. 4(a)), as it does in adult females through the reproductive cycle (Sevala et al., 1999). An early increase in nymphal hemolymph HC content (Fig. 4(b)) is the result of an increase in hemolymph volume (Fig. 4(a)) at a time when the nymphs feed and drink intensively (Young and Schal, 1997). After hemolymph volume reaches a plateau of $\approx$ 11 μl, HC content of the hemolymph remains relatively constant.

Because all hemolymph HC is carried by lipophorin, we measured the lipophorin concentration throughout the last instar: in *B. germanica* nymphs lipophorin represents 25–54% of total hemolymph protein, levels higher than those found in either adult female *B. germanica* (Sevala et al., 1999) or nymphs of the cockroach *Diploptera punctata*, where it represents only 10–40% of total hemolymph protein (King and Tobe, 1993). The developmental pattern of lipophorin concentration throughout the last instar was similar to that of SP2, presumably lipophorin, previously reported in the cockroach *Blatta orientalis* (Duhamel and Kunkel, 1978). In contrast, in *D. punctata* the lipophorin concentration appears to variably increase through the stadium (King and Tobe, 1993). Total hemolymph protein exhibited a different pattern, increasing dramatically during the first half of the stadium and declining steadily thereafter (Fig. 7).

Lipophorin shuttles multiple lipids through the hemolymph (Chino, 1985; Soulages and Wells, 1994) and it also functions as a plasma coagulagen and hemostatic agent. Because its principal functions change during development, it is unlikely that the lipophorin titer will correspond with any one of its ligands. For example, lipophorin carries juvenile hormone (Trowell, 1992;

Sevala et al., 1997), but its titer in the hemolymph has no relation to the juvenile hormone concentration (King and Tobe, 1993; Sevala et al., 1999). This is supported by our current work with last instars. During most of this stage juvenile hormone titer is extremely low and juvenile hormone synthesis in vitro is undetectable (C.S., unpublished), yet lipophorin titers remain high. However, our data indicate some functional congruence between the lipophorin and hydrocarbon contents of the hemolymph (see Figs. 4 and 7), suggesting that lipophorin might covary with the amounts of lipid ligands that occur at high concentrations and require extensive shuttling through the hemolymph, rather than with any one of its cargoes.

4.3. Dynamics of HC transport: interactions among the oenocytes, hemolymph, fat body, and the imaginal cuticle

Contrary to what one might expect, the epicuticle is generally not a major target tissue for HC accumulation during any stadium or in adult females. Large amounts of internal HC are found in other tissues, most notably the hemolymph, fat body, and gonads (Gu et al., 1995; Schal et al., 1994, 1998). Quantitative radio-inulin determinations of hemolymph volume in nymphs showed that early in the last stadium all internal HC is associated with hemolymph lipophorin (Fig. 4). However, as more HC is synthesized during mid-stadium, and the imaginal cuticle begins to form (about day 8), hemolymph HC content remains relatively constant and the HC which is accumulating internally as the stadium progresses is increasingly allocated to other, non-hemolymph, tissues (Fig. 4(b)). This net movement of HC through the hemolymph is confirmed by the radio labeling experiment in Fig. 5. In this long-term experiment HC synthesized in a 4 h period on day 4, at the end of the intermolt period (Kunkel, 1975), were tracked through the remainder of the stadium. On day 4 the hemolymph, specifically lipophorin, was a major internal site available for HC uptake (Fig. 5). As the nymphs continued to synthesize unlabeled HC, the labeled HC was swept from the hemolymph into other internal site(s) of deposition. After 4–6 days of this transport of HC there was less than half (43.9 $\pm$ 3.5%) of the labeled HC remaining in the hemolymph (Fig. 5).

As internal HC becomes associated with non-hemolymph tissues, where does internal HC accumulate? Two obvious sites are the fat body and the imaginal cuticle. Fig. 8 shows that the fat body accumulated significantly more HC between days 4 and 10 than did the integument. After analysis of tracheal HC, we dismissed the possibility that this result was due to deposition of HC on newly formed tracheae (Fig. 8). The decline in internal HC during adult ecdysis was comparable to the amount of HC found on the adult epicuticle (Fig. 4(b); Young and Schal, 1997), suggesting that some internal HC associated with the adult cuticle. That the developing adult cuticle accepts HC late in the stadium, before apolysis, is shown by doubling of HC in the interior of the sternites between days 4 and 10 (Fig. 8). We could not discern whether HC remained within the biosynthetic oenocytes or was delivered to the sternites by hemolymph lipophorin. However, examination of the pronotum and legs, which do not synthesize HC, clearly showed that HC accumulated within these tissues, suggesting that HC was delivered to the developing adult cuticle. Although the design of our experiment did not allow us to differentiate between deposition upon the exterior of the imaginal cuticle and accumulation within the cellular integument, it is unlikely that HC would be deposited on the adult epicuticle before apolysis because the highly hydrophobic HC might impede the resorption of the molting fluid within the apolytic space.

In conclusion, we have shown that HC is synthesized by the abdominal integument in *B. germanica* nymphs, as in adults (Gu et al., 1995). Presumably, oenocytes are the main cell type involved in this process in both stages and multiple tissues appear to receive their products: a fraction of the newly synthesized HC is delivered to the nymphal epicuticle (Young and Schal, 1997), but most is transported to internal tissues, notably the hemolymph and fat body. The transport vehicle in nymphs, as in adults (Gu et al., 1995), is hemolymph lipophorin. Early in the stadium the fat body stores little HC while lipophorin accounts for nearly all internal HC. As the insect feeds and synthesizes new HC, its hemolymph volume and lipophorin titer increase. Thus, while the HC titer in the hemolymph changes little, the content of HC in the hemolymph increases early in the stadium. Perhaps, however, far more HC is synthesized than can be accommodated by hemolymph lipophorin. We suggest that around mid-stadium, before the imaginal cuticle develops but while HC is still being biosynthesized, the bulk of the new HC is deposited in the fat body (which also increases in mass). HC may be stored within the fat body cells or within lipophorin associated with the fat body. During cuticle formation, between mid- and late stadium, the internal HC begins to associate with the adult cuticle. Early during this stage newly synthesized HC may transit from the oenocytes, through lipophorin, to the epidermal cells. However, late in adult cuticle formation no new HC is synthesized and we suggest that the bulk of the adult cuticular HC is derived from the nymph's fat body. Lack of a discernible change through ecdysis in either the HC titer or the HC content of the hemolymph (Fig. 4) and a concomitant decline in non-hemolymph internal HC suggests that the fat body delivers HC to the imaginal cuticle through the hemolymph, but without any major changes in lipophorin.

We thus suggest that in both nymphs and adults the hemolymph and the fat body serve as temporary HC

storage depots for later delivery of HC to their terminal destinations. This allows for HC synthesis and accumulation to occur during an early feeding stage (Schal et al., 1994; Young and Schal, 1997) before the tissue(s) to which HC is destined (imaginal cuticle in the nymph, ovaries in the adult) is ready to accept HC.

The hormonal regulation of larval events associated with HC processing, namely feeding, cuticle neogenesis, and the biosynthesis, storage, and mobilization of HC and lipophorin, is the subject of our continuing research. The subcellular localization of HC and lipophorin in biosynthetic sites, fat body, and the newly formed imaginal cuticle remains unknown and will also receive further attention.

Acknowledgements

We thank G. Barthalmus, R.J. Kuhr, R.M. Roe and G.L. Holbrook for critical comments on the manuscript, and E. Armstrong and B. Deasy for technical assistance. This work was supported in part by the Blanton J. Whitmire Endowment at North Carolina State University and by NSF (IBN-9407372) and USDA/NRI (9501922) grants to C.S.

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