Regulatory steps in sex pheromone biosynthesis in *Mamestra brassicae* L. (Lepidoptera: Noctuidae)[1]

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Summary. The de novo biosynthesis of (*Z*)-11-hexadecenyl acetate, the most abundant pheromone component in *M. brassicae*, starting from acetate via palmitic acid, requires the presence of a pheromone-biosynthesis-activating neurohormone. Moreover, the conversion of palmitic acid to (*Z*)-11-hexadecenyl acetate is strongly dependent on the presence of the neurohormone. However, no significant dependence was found for the conversion of (*Z*)-11-hexadecenoic acid to (*Z*)-11-hexadecenyl acetate. This indicates that the neurohormonal control of pheromone biosynthesis in *M. brassicae* occurs at the level of palmitic acid.
Key words. Pheromone biosynthesis regulation; deuterium labeling; brain factor; neurohormone; (*Z*)-11-hexadecenyl acetate; *Mamestra brassicae*.

Most female moths are attractive to males only during certain periods. These intervals of sex pheromone release, known as calling times, usually occur during scotophase and show a diurnal periodicity. Raina and coworkers[2–5] have isolated a hormone from the brains of *Heliothis zea* that triggers pheromone production when injected into females whose pheromone production has been prevented by ligating them between head and thorax. However, according to Raina et al.[3], this hormone does not induce pheromone production when injected into ligated females during the photophase. Here we report that in *Mamestra brassicae* (Lepidoptera: Noctuidae) the injection of a brain homogenate induces pheromone production in females even during the photophase. We were also interested in finding out at what biosynthetic level this pheromone-biosynthesis-activating neuropeptide[4] (PBAN) plays a regulatory role. By using this brain factor, we were able to study pheromone biosynthesis independently of the natural rhythm, because the time of pheromone production could be induced at will.

Materials and methods

Insects were obtained from a laboratory colony maintained at Hoechst AG (Frankfurt/Main). The insects were reared under a 16-h light/8-h dark cycle, and anesthetized with CO_2 before being subjected to any treatment. Brain homogenates were prepared from females of ages ranging from newly emerged to 4 days old. The buffer saline solution for homogenization was prepared as described by Raina et al.[3]. Four parts of this solution were added to one part of 0.5 M sucrose solution and used for homogenization. The homogenate (20 µl, 0.5 brain equivalent) was injected between the 3rd thoracic and the 1st abdominal sternites of females in their first photophase. After 1 h the intersegmental membrane between the segments VIII and IX ('sex pheromone gland') was excised, sealed in a glass capillary, and analyzed by gas chromatography[6].

In a similar experiment a cut was made between the segments VII and VIII of newly emerged females and a brain homogenate (10 µl, 0.5 brain equivalent) was injected into the incision, and after 1 h the gland was prepared and analyzed as described above.

Newly emerged females (1–10 hours old) in the first photophase were used for all biosynthetic studies. A brain homogenate was injected as described above, and after 20 min a solution of 40 mg/ml sodium [$^2H_3$]acetate (Aldrich) in a 1:1 mixture of water and dimethylsulfoxide was applied (1–5 µl), by means of a pair of microtweezers, to the everted pheromone glands of four females. After 40 min the glands were analyzed by solid-sampling GC-MS. The incorporation of deuterium was monitored by selected-ion-monitoring mass spectrometry. The intensities of ions at *m/z* 226, 227, and 228, corresponding to quasi molecular ion [M^+-60]+4, +5, +6, respectively, were recorded. GC-MS analysis was performed on a Finnigan 3200 E quadrupole mass spectrometer linked to a Finnigan 9502 gas chromatograph.

In order to investigate the regulatory role of PBAN in pheromone biosynthesis, the following experiments were performed: Females (n = 10) were decapitated 1–10 h after eclosion, and kept for 17 to 24 h before the application of [13,13,14,14,15,15,16,16,16-$^2H_9$]-palmitic acid ([$^2H_9$]16:acid) (~1 µl, 2.2 mg/ml DMSO) onto the gland. Afterwards, some females (n = 5) received a brain homogenate as described above and the others (n = 5) received a buffer solution. After 1 h each gland was prepared and analyzed by gas chromatography. A similar experiment was carried out (n = 16) with a solution of [13,13,14,14,15,15,16,16,16-$^2H_9$]-(*Z*)-11-hexadecenoic acid ([$^2H_9$]Z-11-16:acid) (~1 µl, 0.8 mg/ml DMSO).

Results and discussion

Most adult females of *M. brassicae* exhibit calling behavior about 5 h after the beginning of the scotophase, from the second night onwards[6]. Newly emerged females of

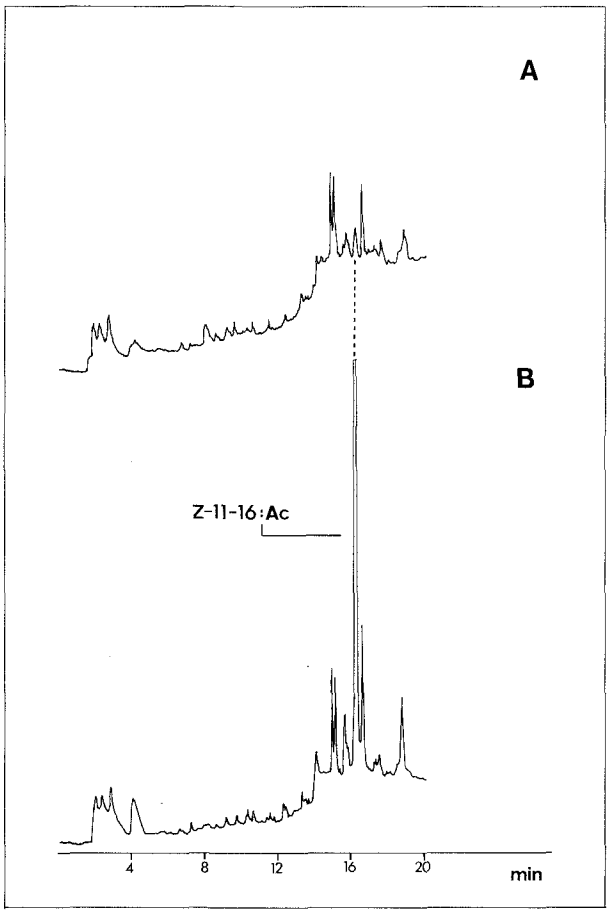

Figure 1. Gas chromatograms of volatiles from the intersegmental membrane between the abdominal segments VIII and IX obtained from PBAN-untreated (*A*) and PBAN-treated (*B*) *Mamestra brassicae* females. The samples were introduced by a solid-sample injection technique[6,17] onto a 25 m × 0.22 mm fused-silica column coated with SP-2340. The oven temperature was held at 60 °C for 2 min and programmed to 195 °C at 10 °C/min.

M. brassicae contain only insignificant amounts (less than 1 ng) of (*Z*)-11-hexadecenyl acetate (Z-11-16:Ac) during their first photophase (fig. 1 A). However, 1 h after injecting a brain homogenate obtained from any other *M. brassicae* into newly emerged females in their first photophase, we were able to detect Z-11-16:Ac in quantities as large as 100–200 ng (fig. 1 B). According to our results the introduction of the brain homogenate induces production of Z-11-16:Ac, even during the photophase, in amounts similar to those usually found only during the periods of natural calling. Moreover, we found that an intact abdomen is not necessary for pheromone production; when a cut was made between segments VII and VIII, and the posterior end of the abdomen was separated from the rest of the body, this excised abdominal tip was still able to produce Z-11-16:Ac, in amounts similar to those found under normal conditions, when a brain homogenate was injected into the incision. The control abdominal tip which was not treated with brain homogenate was unable to produce Z-11-16:Ac in significant quantities.

The sex pheromones of Lepidoptera appear to be produced exclusively by de novo biosynthesis[7–9], starting from acetate. The observation that we are able to induce pheromone production independently of the light-dark cycle was used to study de novo biosynthesis of pheromones.

The glands of newly emerged *M. brassicae* females were treated with deuterated sodium acetate ($CD_3CO_2^-Na^+$). The incorporation of deuterium into Z-11-16:Ac, with or without the presence of the brain factor, was followed by selected-ion-monitoring mass spectrometry. Due to natural isotope abundance, signals for *m/z* [M-60]+1, +2, and +3 are also seen in the mass spectrum of Z-11-16:Ac; however, the contribution from ions above [M-60]+4 is considered to be negligible. Therefore an increase of the intensities of the ions *m/z* 226, 227, and 228, representing [M-60]+4, +5, and +6, respectively, signifies an incorporation of at least four or more deuterium atoms into Z-11-16:Ac. As shown in figure 2 A, within 40 min of application of deuteroacetate substantial increases were observed in the intensities of the three ions of interest. In the control experiment carried out with animals not treated with brain homogenate, no incorporation was observed (figure 2 B). The results shown in figure 2 A confirm that de novo synthesis takes place in the gland under the influence of PBAN. The origin of the deuterium-labeled product is not a result of a direct acetylation of the precursor alcohol, and subsequent proton scrambling in the mass spectrometer[10]. The label is incorporated into the hydrocarbon part of the molecule. This was verified by comparing the results with those obtained from a standard solution of (*Z*)-11-hexadecenyl trideuteroacetate (fig. 2 C).

As we have described earlier,[11] the final step of Z-11-16:Ac biosynthesis, the acetylation of (*Z*)-11-hexadecen-1-ol (Z-11-16:OH), is independent of the light-dark cycle. When [13,13,14,14,15,15,16,16,16-$^2H_9$]Z-11-16:OH was applied topically to the gland, it was readily converted into [$^2H_9$]Z-11-16:Ac. The enzymes controlling this step show a low degree of substrate specificity.

In the present study, we used [$^2H_9$]16:acid in order to determine whether palmitic acid is the precursor of Z-11-16:OH. We found that the deuterium-labeled acid is readily converted to labeled Z-11-16:Ac when applied to the gland. It was presumed that palmitic acid is converted to Z-11-16:acid and subsequently reduced to Z-11-16:OH. Similar biosynthetic routes are already known for other species of Lepidoptera[12]. Although the involvement of PBAN in pheromone biosynthesis has been known for some time[2,13,14], it was not known at which step of pheromone biosynthesis it plays a regulatory role. In order to find out whether PBAN is involved in the regulation of pheromone biosynthesis in a step before or after the intermediate palmitic acid is produced, we performed the following experiment. A few fe-

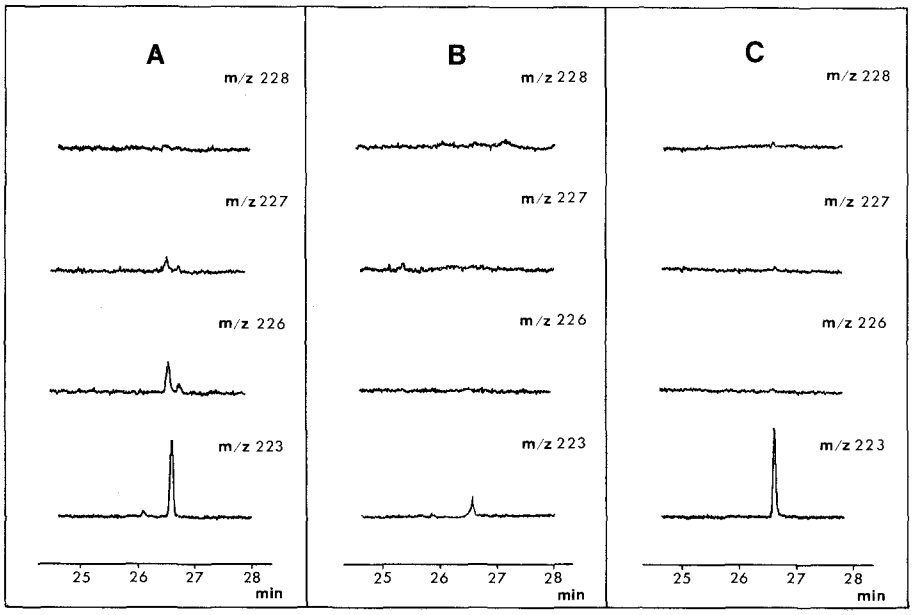

Figure 2. Mass chromatograms obtained for the ions of *m/z* 223, 226, 227, and 228 by selected-ion-monitoring mass spectrometry. *A* The volatiles from the intersegmental membranes treated with $[^2H_3]$sodium acetate and brain homogenate; *B* as for *A*, but without brain homogenate; *C* authentic Z-11-hexadecenyl trideuteroacetate. The samples were introduced by solid-sampling, onto a 25 m×0.22 mm fused-silica column coated with SE-54. The oven temperature was held at 50 °C for 2 min and programmed to 260 °C at 6 °C/min.

males were decapitated to prevent PBAN entering the hemolymph[13]. In this way the natural pheromone production was decreased to a minimum[15]. Decapitated females produce only trace quantities of Z-11-16:Ac (less than 0.5 ng/female). We found that the decapitated females were unable to convert deuterium-labeled palmitic acid into labeled Z-11-16:Ac in significant quantities (fig. 3A). However, after the injection of a brain homogenate, the pheromone production in decapitated females was restored to natural levels. Consequently, brain-homogenate-treated females were able to convert $[^2H_9]$palmitic acid to $[^2H_9]$Z-11-16:Ac. This result indicates that the presence of PBAN is absolutely necessary for the conversion of palmitic acid to Z-11-16:Ac.

Ando et al.[16] have found in *Bombyx mori* that the incorporation of radioactive label from ^{14}C-palmitic acid into the pheromone bombykol followed the same circadian rhythm as the bombykol titer. Presumably, this is due to the fact that PBAN concentration in the hemolymph also follows a circadian rhythm. Therefore, according to our findings with *M. brassicae*, we can expect that the circadian control of bombykol production in *B. mori* may also occur after palmitic acid acts as an intermediate.

Presumably, palmitic acid is desaturated to Z-11-16:acid, which is reduced to Z-11-16:OH before being acetylated to yield Z-11-16:Ac. In order to verify whether any biosynthetic step, after Z-11-16:acid participates as an intermediate, depends on PBAN, $[^2H_9]$Z-11-16:acid was

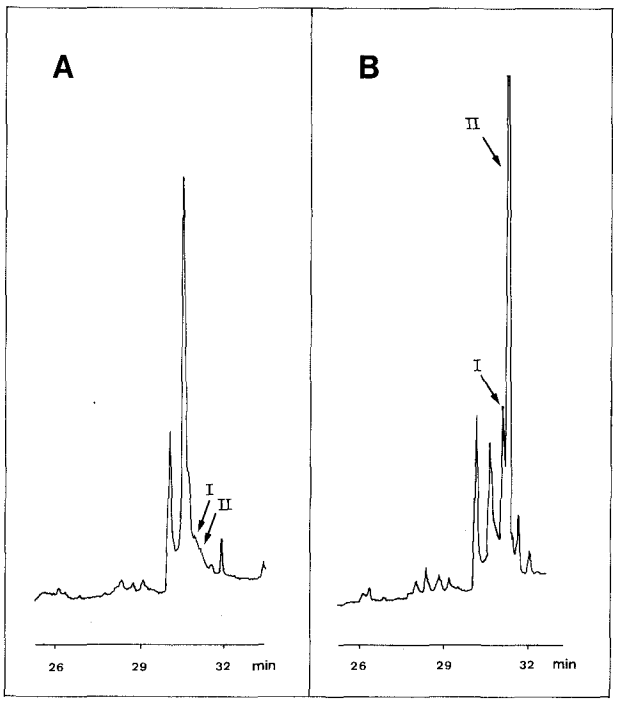

Figure 3. Partial gas chromatograms of volatiles obtained from the glands of decapitated females treated with $[^2H_9]$-16:acid; *A* without brain homogenate; *B* with brain homogenate. The samples were introduced by solid-sample injection onto a 12 m×0.22 mm fused-silica column coated with SE-54. The oven temperature was kept at 60 °C for 2 min and programmed to 260 °C at a rate of 4 °C/min.
I = $[^2H_9]$Z-11-16:Ac II = Z-11-16:Ac.

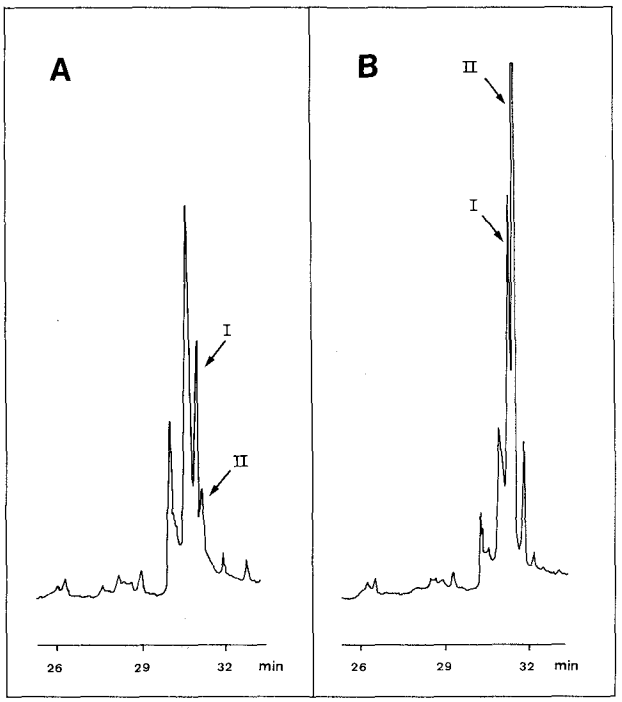

Figure 4. Partial gas chromatograms of volatiles obtained from the glands of decapitated females treated with $[^2H_9]$Z-11-16:acid, *A* without brain homogenate; *B* with brain homogenate. GC conditions were identical to those described in fig. 3.

applied to the glands of newly emerged, decapitated females. In contrast to the results obtained from the application of $[^2H_9]$16:acid, a ready conversion of $[^2H_9]$Z-11-16:acid to $[^2H_9]$Z-11:Ac was observed in PBAN-untreated insects (fig. 4A). The area ratio of $[^2H_9]$Z-11-16:Ac peak to Z-11-16:Ac peak was 3.34 ± 1.83 ($n = 8$). This value was significantly larger than the value 0.92 ± 0.07 ($n = 5$) obtained for the $[^2H_9]$Z-11-16:Ac to Z-11-16:Ac area ratio from the $[^2H_9]$16:acid-treated insects. A slight increase in $[^2H_9]$Z-11-16:Ac production, observed in brain-homogenate-treated insects (fig. 4B) compared to that of PBAN-untreated insects (fig. 4A), was considered to be due to a carrier effect.

The present results show that the presence of PBAN is not vital for the conversion of Z-11-16:acid to Z-11-16:Ac. The final step, the acetylation of Z-11-16:OH, is highly unlikely to show any PBAN dependence. According to our results, the key steps in the pheromone biosynthesis of *M. brassicae* controlled by PBAN are the biosynthetic transformation that occurs immediately following the participation of palmitic acid as an intermediate. Although we have no evidence at present, it may not be surprising if further experiments show that the biosynthetic steps which convert acetate to palmitate have no significant dependence on PBAN.

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