Enzymatic Conversion of Sex Pheromones in Olfactory Hairs of the Male Silkworm Moth *Antheraea polyphemus*

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The sex pheromone compound [6,7-^{3}H]-6,11-hexadecadienyl acetate is enzymatically converted to its corresponding fatty alcohol, aldehyde and acid in living and homogenized olfactory hairs and in other parts of the antennae of the male silk-moth *Antheraea polyphemus*. The pheromone is metabolized *in vitro* mainly in the inner hydrophilic compartments, but scarcely in the outer hydrophobic cuticle of the olfactory hairs.

Introduction

Enzymatic pheromone conversion and subsequent degradation, as found in antennae, wings, legs and other organs of the insect body, probably serve as cleaning mechanisms which prevent pheromone accumulation in the integument and adaptation of the highly sensitive receptor cells [1–6].

Airborne pheromone molecules are adsorbed on the long olfactory hairs [7, 8] and inactivated within seconds after interaction with the postulated receptor molecules of the dendritic membranes, as demonstrated by EAGs and single cell recordings [9]. The half-lives of pheromone conversion and degradation in intact moth antennae were found in the order of several minutes [10, 11]. Rapid non-enzymatic inactivation was attributed to the binding of the native pheromone to the binding protein in the sensillum lymph [9].

Preliminary experiments on the enzymatic conversion of pheromones in insect antennae were performed with [12,13-^{3}H](E,Z)-10,12-hexadecadienol in *Bombyx mori* L. [1, 2] and with [^{3}H](Z)-7-dodecenyl acetate in the cabbage looper, *Trichoplusia ni* [12]. Studies with [6,7-^{3}H]- and [11,12-^{3}H]-(E,Z)-6,11-hexadecadienyl acetate have provided information on pheromone uptake, its enzymatic conversion and its interaction with binding and receptor proteins in the long olfactory hairs of *Antheraea polyphemus* and *A. pernyi* [9, 13]. Recently, pheromone oxidation in antennae of *Bombyx mori* L. and *A. polyphemus* was described *in vivo* [10, 11].

This paper reports on pheromone hydrolysis and subsequent oxidation, using the labeled pheromone component [6,7-^{3}H](E/Z)-6,11-hexadecadienyl acetate, in the isolated olfactory hairs of *A. polyphemus in vitro*.

Materials and Methods

Radiochemicals: Pheromones and stereoisomers were produced by multistep synthesis *via* the Wittig reaction [10]. The three isomers: (E,Z)-, (Z,Z)- and (Z,E)-6,11-hexadecadienyl acetate were labeled at the 6,7-position as described. Specific activity of [6,7-^{3}H(E/Z)-6,11-16:Ac: 110 ± 10 mCi/mg = 31 ± 3 Ci/mmol (1 dpm = 8.78×10^6 molecules = 4.12 fg; 1 fg = 2.13×10^6 molecules).

Insects: Pupae of *A. polyphemus* (Cramer) were supplied by M. Furr, Germantown, T.N. (U.S.A.). The cocoons were kept at 4 °C with a light-dark cycle of 11:13 h. For emergence, the cocoons were warmed up to 22 °C at 65% rel. humidity in a light-dark cycle of 13:11 h for 3 weeks. The experiments were performed with batches of 20 freshly excised

Abbreviations: EAG, electroantennogram; TLC, thin-layer chromatography; LSC, liquid scintillation counting; BHT, 2,6-ditertiarybutyl-4-hydroxytoluene; PMMA, polymethylmethacrylate; [^{3}H]HDAc, [6,7-^{3}H]-6,11-16:Ac, [6,7-^{3}H]-6,11-hexadecadienyl acetate; [^{3}H]HDOl, [6,7-^{3}H]-6,11-hexadecadienol; [^{3}H]HDAl, [6,7-^{3}H]-6,11-hexadecadienal; [^{3}H]HDAcid, [6,7-^{3}H]-6,11-hexadecadienoic acid; [^{3}H]HDEster, [6,7-^{3}H]-6,11-hexadecadienoic acid ester.

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antennae which were cleaned with an air-stream and washed with distilled water.

Microscopy: The purity of the antennae, branches and olfactory hairs was examined using light microscopy. For scanning electron microscopy (Fig. 1–3), the excised branches and extracted hairs were air-dried, mounted, sputtered with gold and viewed in a Zeiss Novascan 30. For ultrathin sectioning (Fig. 4), the isolated hairs were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2–7.4) for 3 h. The specimens were post-fixed in 1% OsO_4 in the same buffer for 3 h, stained in 1% uranyl acetate (aq.) at 60 °C for 15 h, dehydrated in ethanol, embedded in Spurr's medium, and sectioned with a diamond knife on an ultramicrotome (Reichert OmU2 or Ultracut). All preparations were examined in a Zeiss EM 10 electron microscope [14].

Antennae and branches

Washings and extraction: Each experiment was performed with 1 freshly cut antenna or with 100 branches with or without hairs, suspended in 400 µl PPS buffer: 0.15 M NaCl, 0.05 M KH_2PO_4, 0.01 M NaN_3 (pH 7.2) treated with 0.1% Tween 80 (polyoxyethylene sorbitan mono-oleate) as detergent and antioxidant. A scheme of sample preparation is shown in Fig. 5. For extractions, the antennae or branches were vortexed for 1 min with "press to mix", and vibrated using "maspo" and a Bransonic ultrasonic bath (Branson, Switzerland). Extracted branches with hairs were suspended in 400 µl buffer and homogenized with a microhomogenizer (Potter: PMMA/glass, 1 ml) at 0 °C for 6 min before resuspension and incubation. The samples were centrifuged with an Eppendorf Z 3200 centrifuge (Eppendorf, Hamburg) at 12,000 rpm for 2 min and the sediments resuspended in buffer and centrifuged in the same manner. The supernatants (extracts) were carefully removed and collected together.

Incubation and oxidation: In each experiment the antennae, branches or homogenized fragments (suspended or resuspended in 400 µl buffer) were incubated in a labshaker (Braun, Melsungen) with 14–46 pg [^{3}H]HDAc for 20 h at 20 °C. The suspensions were acidified with 1 M HCl to pH 2 and vortexed and extracted with 200 µl chloroform. After centrifugation for 2 min, the buffer layer (containing [^{3}H]water) and chloroform layer (containing ^{3}H-pheromone and lipophilic ^{3}H-metabolites) were removed and samples taken for liquid scintillation counting (LSC) with a Minaxi 4430 counter (Canberra Packard, U.S.A.) and for TLC. [^{3}H]Water and other ^{3}H-metabolites remaining in the samples (^{3}H-residues) were determined by LSC after wet oxidation of the tissues with 200 µl $HClO_4$ and 400 µl H_2O_2 for 8 h at 70 °C [15]. The oxidized residue solutions were dissolved in 5 ml Cellosolve (glycolmonoethyl ether) and mixed with 10 ml PPO scintillation cocktail for LSC. To obtain blank values (Control), 400 µl buffer samples were incubated with 11–56 pg [^{3}H]HDAc and treated as described.

Olfactory hairs

Preparation of isolated hairs: The branches of one antenna were excised and mixed in a siliconized test tube with 11 g glass beads of two diameters (∅ a: 0.10–0.11 mm, ∅ b: 0.45–0.50 mm; 1:1 by weight). The mixture was frozen by dipping the test tube into liquid nitrogen for 2 min. The hairs were broken off the branches by vigorously shaking the frozen mixture in the air 20 times, re-cooling and shaking, for a further 7 repetitions. The hairs were warmed to room temperature and isolated by differential adhesion to siliconized glass petri dishes [16].

Suspension and extraction: Each experiment was performed with an amount of olfactory hairs calculated equivalent to that on about 2.5 antennae, suspended in 400 µl PPS buffer. For extraction, the suspensions were vortexed and vibrated as described; the samples were centrifuged and resuspended and the supernatants removed and collected in the same manner. Extracted hairs were also resuspended in 400 µl buffer and homogenized with a microhomogenizer at 0 °C. For electron microscopy (Fig. 4), the extracted hairs were collected with membrane filters (∅ 5 µm, Nucleopore, U.S.A.) prior to mounting.

Incubation and oxidation: The isolated and extracted hairs or homogenized hair fragments were suspended or resuspended in 400 µl buffer. These samples and their supernatants were incubated with 26–60 pg [^{3}H]HDAc for 20 h at 20 °C. The suspensions were acidified, vortexed and eluted with 200 µl chloroform as usual. The extracts and residues were treated as described.

Thin-layer chromatography: TLC was used to separate and identify the ^{3}H-metabolites from the ^{3}H-pheromone and [^{3}H]water was determined by dif-

ference measurements [10]. Solvent I for adsorption chromatography (silica gel plates, Merck): hexane, diisopropyl ether, methanol and NH_3 (25%) (100:20:10:0.3, by vol.). Solvent II for reversed-phase chromatography (silanized silica gel RP-18 plates, Merck): dimethylformamide and water (95:5, by vol.). The chromatograms were completely scraped, as 35 zones, into 20 ml scintillation vials with a semi-automatic scraper (Hölzel, Dorfen) [17]. The LSC results were recorded on diskettes, and further analyzed and plotted by electronic data processing (IBM personal computer) (Fig. 6).

Results

Olfactory hair micrographs: The side branches of the antennae support mainly long olfactory hairs (about 70,000 sensilla trichodea per antenna) and a smaller number of shorter olfactory hairs (about 10,000 sensilla basiconica per antenna) (Fig. 1).

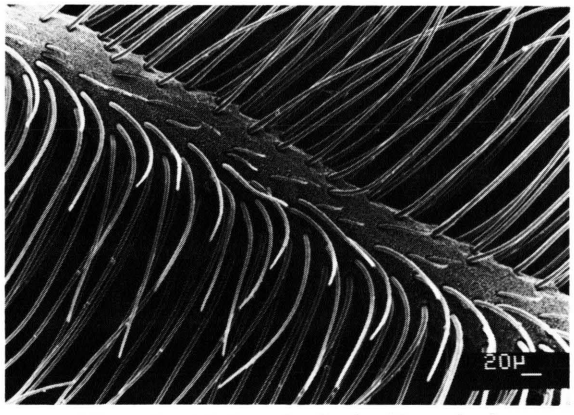

Fig. 1. View of a side-branch of a freshly excised antenna with long and short olfactory hairs (sensilla trichodea and basiconica).

After deep-freezing and shaking with glass beads, the hairs were mainly broken near the surface of the branch integument (Fig. 2). Few microtrichia were ground off or damaged by this operation. The yield of hairs was about 30% of the total olfactory hairs supported by the branches, according to hair length estimation [16].

After extraction and filtration (Nucleopore, ∅ 5 µm), the hairs were practically free from microtrichia and glass bead fragments (Fig. 3). Furthermore, these samples were shown under the electron microscope to be free from residues of the hair contents extracted (sensillum lymph, pore tubuli and receptor cell dendrites) (Fig. 4).

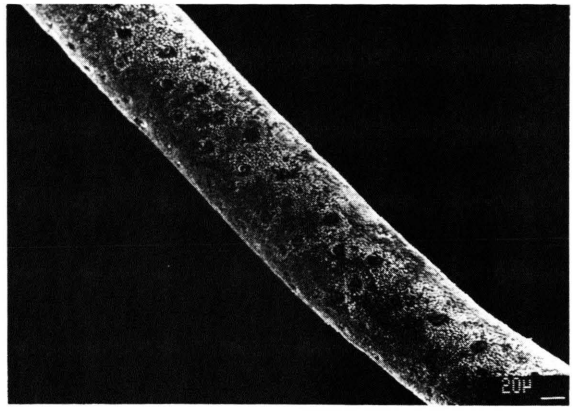

Fig. 2. Scanning electron micrograph of a deep-frozen branch after separation of olfactory hairs, showing stumps of sensilla trichodea and basiconica.

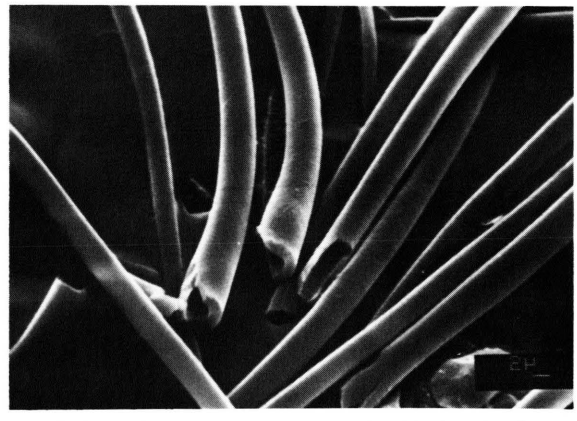

Fig. 3. Scanning electron micrograph of isolated olfactory hairs (sensilla trichodea).

Metabolism in antennae and branches: The freshly excised intact antennae (Fig. 5, Table I, A1) metabolized the pheromone acetate ([^{3}H]HDAc) to the corresponding alcohols, aldehydes, acids and esters. The tritium of the applied ^{3}H-pheromone was partly oxidized to [^{3}H]water. Pre-washed antennae (Table I, A2a) produced similar amounts of ^{3}H-metabolites. In the washing fraction (Table I, A2b), all the ^{3}H-metabolites remained below 2%, as in the control experiments, except [^{3}H]HDOl which increased up to 5%. The "unpaired" t-test at the 95% significance level rejects the two [^{3}H]HDOl means (washing fraction: 5.1% and control: 2.6%) as being similar. The test values of all experiments with an-

Fig. 4. Transmission electron micrograph (cross-section, 9,000×) of empty olfactory hairs (sensilla trichodea) after washing.

tennae, branches and isolated olfactory hairs were not corrected by subtraction of the control values. The small amounts of ^{3}H-metabolites of the control tests were mainly autoxidation products and not identical with those of enzymatic pheromone oxidation.

Suspended branches with hairs (Table II, B1) gave similar amounts of [^{3}H]residue and [^{3}H]water as whole antennae. In the homogenized sediment of extracted branches with hairs (Table II, B2a), the tritium of [^{3}H]HDAc was scarcely oxidized to [^{3}H]water and only 9% transformed into [^{3}H]residue; the ^{3}H-metabolites were mainly [^{3}H]HDOl. In the extract (supernatant) (Table II, B2b), only small amounts of the [^{3}H]HDAc were metabolized – the highest rate was [^{3}H]HDOl at 20%. [^{3}H]HDAc was also metabolized similarly in branches without hairs (Table II, B3), yet the fractions of [^{3}H]HDOl and [^{3}H]HDEsters were higher, and the [^{3}H]residue was much lower.

Metabolism in olfactory hairs: In untreated suspended hairs (Fig. 5, Table III, C1), [^{3}H]HDAc was mainly metabolized to [^{3}H]HDOl (8%), [^{3}H]HDAl (20%) and [^{3}H]HDAcid (41%). Less than 5% [^{3}H]-HDEster, [^{3}H]residue and [^{3}H]water was formed.

The sediment of extracted hairs (Table III, C2a), yielded a maximum of 31% ^{3}H-metabolites: 15% [^{3}H]HDAl and relatively small amounts of other ^{3}H-metabolites, but the extract I (supernatant) (Table III, C2b) gave much higher amounts (65%) of ^{3}H-metabolites: [^{3}H]HDAcid (25%) and [^{3}H]HDOl (21%). In both experiments [^{3}H]residue and [^{3}H]-water were below 2%. The results from hairs which were repeatedly washed (Fig. 6a: extremely small amounts of ^{3}H-metabolites) barely differed with those from hairs washed only once (*cf.* Table III, C2a).

In the incubated sediment of extracted and homogenized hairs (Table III, C3a), only small amounts of ^{3}H-metabolites were found; 15% of the [^{3}H]-HDAc was converted. In the second supernatant (extract II) (Table III, C3b), the [^{3}H]HDAc was also scarcely metabolized (16%) to the corresponding [^{3}H]HDOl (8%), [^{3}H]HDAl (5%) and [^{3}H]HDAcid (2%). The amounts of [^{3}H]residue, [^{3}H]water and [^{3}H]HDEster were negligible in this experiment. Comparing the total amounts of ^{3}H-metabolites produced in the empty hairs with those from the hair extracts, it can be seen that a minimum of 15% of the [^{3}H]HDAc was metabolized in the hair shells and fragments.

Discussion

These investigations yielded similar patterns of pheromone metabolites in isolated olfactory hairs

Table I. Pheromone conversion and degradation in untreated and pre-washed whole antennae incubated for 20 h at 20 °C in PPS buffer with 14–38 pg [^{3}H]HDAc per antenna. Chloroform extracts were analyzed by TLC. $^3H_{rec}$: recovered ^{3}H-activity, n = number of experiments. ± SD = standard deviation.

Exp. No.	Antennae	$^3H_{rec}$ [dpm]	^{3}H-Labeled compounds [%] Acetate	Alcohol	Aldehyde	Acid	Ester	Residue	Water	n
A1	Untreated	9580	14.1 ± 4.5	4.8 ± 2.2	1.3 ± 0.7	13.8 ± 2.5	2.3 ± 0.4	43.9 ± 2.8	19.8 ± 2.4	3
A2a	Pre-washed	8740	7.4 ± 1.4	17.4 ± 5.8	2.3 ± 1.0	11.4 ± 0.8	2.9 ± 0.5	50.1 ± 2.6	8.5 ± 4.7	3
A2b	Washings	4120	88.0 ± 0.6	5.1 ± 0.4	0.8 ± 0.1	1.9 ± 0.2	2.0 ± 0.2	0.7 ± 0.2	1.5 ± 0.2	3

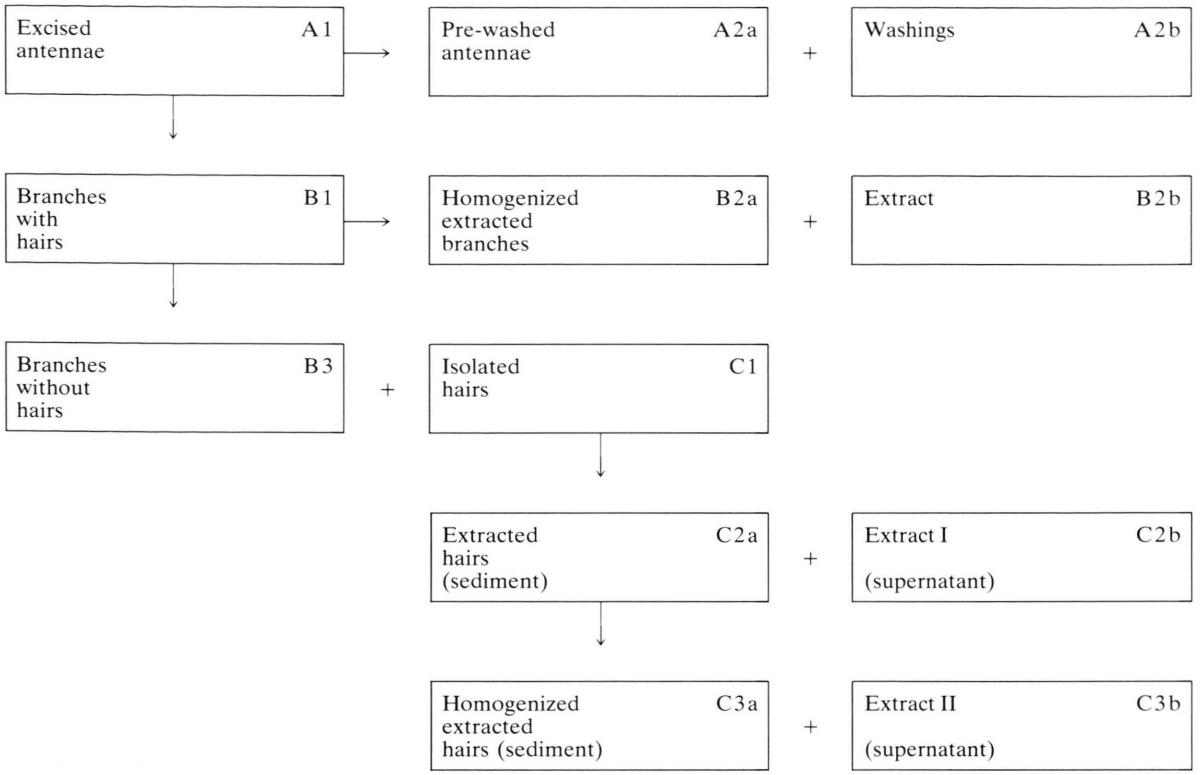

Fig. 5. Scheme of sample preparation of antennae, branches and olfactory hairs.

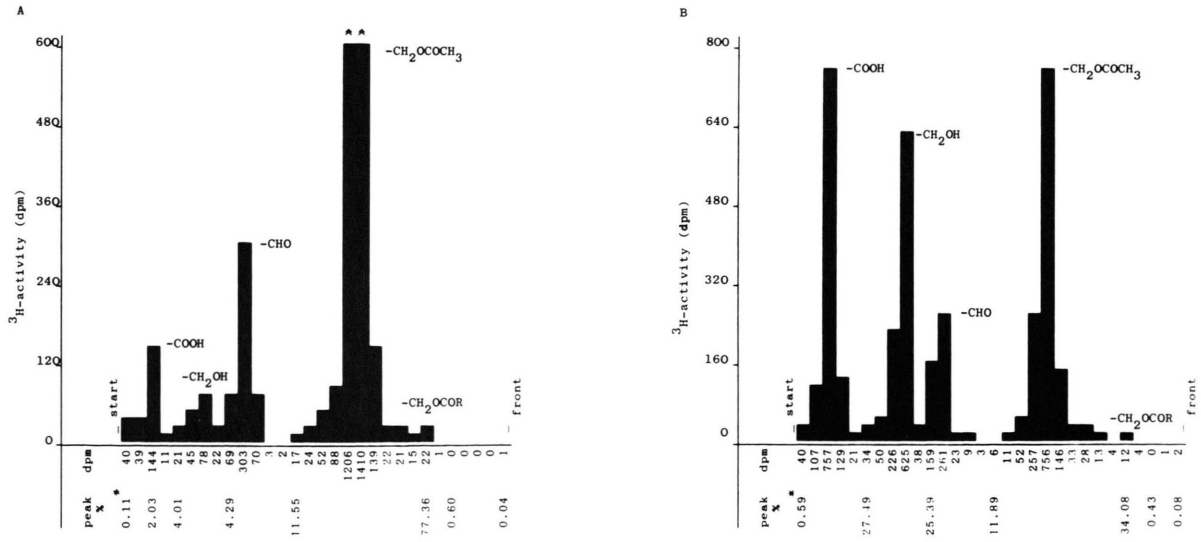

Fig. 6. TLC of ^{3}H-pheromone and ^{3}H-metabolites; [^{3}H]HDAc conversion in (A) extracted hairs and (B) extract supernatant (Table III, C2a and C2b). The ^{3}H-metabolites were obtained by incubating the samples for 20 h at 20 °C in PPS buffer with (A) 31.2 pg and (B) 27.6 pg [^{3}H]HDAc. Chloroform extracts were analyzed by TLC. *:^{3}H-activity of the concentration zone.

Table II. Pheromone conversion and degradation in freshly excised branches (a) with and (b) without hairs. The branches were suspended in PPS buffer and incubated as described (Table I). Branches with hairs were also homogenized, centrifuged, resuspended and then incubated; 14–46 pg [^{3}H]HDAc per 100 branches. Co = control tests: 400 µl buffer incubated with 11–56 pg [^{3}H]HDAc. Chloroform extracts were analyzed by TLC. $^3H_{rec}$: recovered ^{3}H-activity, n = number of experiments. ± SD = standard deviation.

Exp. No.	Branches	$^3H_{rec}$ [dpm]	^{3}H-Labeled compounds [%] Acetate	Alcohol	Aldehyde	Acid	Ester	Residue	Water	n
B1	Untreated[a]	7820	11.3 ± 1.1	7.5 ± 2.2	1.3 ± 0.3	22.8 ± 2.1	4.1 ± 1.7	38.1 ± 2.0	15.0 ± 6.0	3
B2a	Homogenized[a]	8230	39.2 ± 2.7	44.7 ± 4.6	0.6 ± 0.3	3.7 ± 1.4	1.0 ± 0.3	9.3 ± 0.8	1.5 ± 1.0	3
B2b	Extract[a]	5720	59.9 ± 2.9	20.6 ± 1.6	0.9 ± 0.6	6.6 ± 3.8	1.5 ± 0.7	2.5 ± 0.8	2.0 ± 0.5	3
B3	Untreated[b]	6260	14.1 ± 1.8	38.2 ± 4.5	2.5 ± 0.2	7.4 ± 0.6	17.3 ± 4.2	5.4 ± 1.6	15.1 ± 1.0	4
Co	Control	7960	93.7 ± 1.8	2.6 ± 0.9	0.7 ± 0.3	1.5 ± 0.7	1.1 ± 0.4		0.4 ± 0.2	13

Table III. Pheromone conversion in isolated olfactory hairs. Untreated, extracted and homogenized hairs were suspended in PPS buffer and incubated as described (Table I); 26–60 pg [^{3}H]HDAc per hairs of 2.5 antenna. Chloroform extracts were analyzed by TLC. $^3H_{rec}$: recovered ^{3}H-activity, n = number of experiments. ± SD = standard deviation.

Exp. No.	Olfactory hairs	$^3H_{rec}$ [dpm]	^{3}H-Labeled compounds [%] Acetate	Alcohol	Aldehyde	Acid	Ester	Residue	Water	n
C1	Untreated	10,440	21.7 ± 7.5	7.8 ± 7.6	19.6 ± 8.9	40.5 ± 15.8	1.3 ± 0.5	5.0 ± 3.1	4.1 ± 4.4	5
C2a	Extracted	9,850	68.9 ± 6.4	6.1 ± 3.3	14.4 ± 2.8	7.8 ± 1.4	1.4 ± 0.4	0.5 ± 0.1	0.9 ± 0.4	4
C2b	Extract I	10,470	34.2 ± 17.4	21.1 ± 4.8	16.0 ± 11.4	25.2 ± 10.9	0.6 ± 0.3	0.9 ± 1.0	2.0 ± 1.0	6
C3a	Homogenized	12,820	83.3 ± 3.8	4.6 ± 0.9	4.0 ± 2.5	1.7 ± 0.3	5.6 ± 2.2	0.4 ± 0.1	0.4 ± 0.1	3
C3b	Extract II	9,360	84.3 ± 11.5	7.9 ± 6.8	4.6 ± 3.3	2.1 ± 1.4	0.7 ± 0.3		0.4 ± 0.1	3

and whole living antennae. The experiments with antennae and branches support recent results with *Bombyx mori* L. and *Antheraea polyphemus in vivo* [10, 11].

Pheromone inactivation in the olfactory hairs of male *A. polyphemus* is due to the first three steps of enzymatic conversion found in branches, legs, wings and other parts of the insect:

$$RCH_2OCOCH_3 \rightarrow RCH_2OH \rightarrow RCHO \rightarrow RCOOH$$

There is no evidence that the pheromone is completely degraded in olfactory hairs. Probably, the fatty acids produced are the end products of pheromone conversion.

These results confirm that pheromone hydrolyzing enzymes exist mainly in the hydrophilic interior of the olfactory hairs [5, 19]. Pheromone metabolites produced by oxidases and/or dehydrogenases were found up to 80% in the extracted inner compartments, which contain pore tubuli, dendrites and sensillum lymph. A minimum of 20% metabolites was determined in the sediments of the empty hairs. These may be the products of enzymes embedded in the cuticle of the hairs. Insoluble cuticle-bound esterases were found in scales and other tissues of *A. polyphemus* and other Lepidoptera species [6]. Soluble esterases were identified in the sensillum lymph of *Trichoplusia ni* [18] and *A. polyphemus* [13, 19]. The relatively high amounts of the labeled aldehydes in the olfactory hairs, compared with the trace amounts in whole antennae and branches, may result from a lack of the oxidizing enzymes. These enzymes were detected in whole antennae of *A. polyphemus, Bombyx mori* L., *Choristoneura fumiferana* and *Heliothis viriscens* [20]. The oxidation of tritium from the labeled pheromone to [^{3}H]water *in vivo,* was established in living antennae and excised branches from *A. polyphemus* and *Bombyx mori* L. [10, 11]. In these new *in vitro* experiments, the [^{3}H]water found in the untreated antennae was much lower than obtained earlier after incubation in air and extraction with chloroform–methanol [10]. Probably, the large amount of [^{3}H]residue contained further [^{3}H]water which was not eluted.

In contrast to untreated branches, the tritium of the pheromone was minimally oxidized to [^{3}H]water in homogenized branches. Possibly, the cellular compounds released by tissue destruction inhibited the degrading enzymes and, as a consequence, the whole oxidizing system.

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