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Sex pheromone conversion and degradation in antennae of the silkworm moth *Bombyx mori* L.

G. Kasang*, M. Nicholls and L. von Proff

Max-Planck-Institut für Verhaltensphysiologie, D-8131 Seewiesen (Federal Republic of Germany)
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Dedicated to Professor Adolf Butenandt on the occasion of his 85th birthday

Summary. In living antennae of the silkworm moth *Bombyx mori* L. the pheromone compound (*E, Z*)-10,12-hexadecadienol and hexadecanol are enzymatically converted to their corresponding fatty aldehydes, acids and long-chain fatty acid esters. The pheromone is completely degraded at high rates in the antennae of freshly hatched moths. The polar volatile [^{3}H]metabolites exclusively consist of tritiated water. The half-life of the pheromone is about 2.5 min in males and 0.5 min in females. Drying inactivates the enzymes responsible for pheromone oxidation.
Key words. Lepidoptera; *Bombyx mori* L.; moths; antennae; pheromones; metabolites; enzymes.

Introduction

Fifty years ago, Adolf Butenandt described chemical experiments with sex attractants obtained from abdominal glands (sacculi lateralis) of female silkmoths of *Bombyx mori* L.[1]. The main pheromone component, bombykol, was identified later as a long-chain and double unsaturated fatty alcohol: (*E, Z*)-10,12-hexadecadien-1-ol (*E*10, *Z*12-16:ol)[2,3]. An historical retrospect on this pioneering work was given by Hecker and Butenandt[4]. The domestic silkworm *Bombyx mori* L. belongs to the most widely used and investigated species of silkworms, which has been cultivated in China for more than four thousand years[5].

The pheromone isomer (*E, E*)-10,12-hexadecadien-1-ol and the component (*E, Z*)-10,12-hexadecadien-1-al (bombykal: *E*10, *Z*12-16:Al) were also found in gland extracts of the female moths of *Bombyx mori* L.[6–8]. Studies on pheromone metabolism began after the synthesis of bombykol and its labeling to [^{3}H]bombykol[9].

Pheromone uptake and transformation in the antennae of *Bombyx mori* L. begins with the adsorption of the airborne molecules on the long sensory hairs of the antennae[10,11]. The adsorbed molecules reach the cuticular pores and pore tubuli by two-dimensional diffusion along the epicuticle of the sensilla surface[12,13]. It is probable that they diffuse through the pore tubuli towards the dendritic membranes of the receptor cells. Bombykol molecules also migrate along the outer hair surface or within the hair lumen towards the branches of the antennae. Presumably, some molecules penetrate through the cuticular pores of the antennal branches into the cells and hemolymph[14].

It was suggested that interaction of pheromone molecules with proposed receptor-molecules of the dendritic membranes is followed by rapid inactivation[15]. Time courses of electrophysiological responses indicate that the pheromone molecules must be inactivated within seconds after the excitatory interactions, whereas the half-lives of pheromone conversion were found in the order of some minutes[16]. Therefore, inactivation by enzymatic metabolism is not identical with the previously postulated rapid inactivation mechanism[17].

Investigations on the enzymatic conversion of bombykol in *Bombyx mori* L. were initially performed with [12,13-^{3}H] (*E, Z*)-10,12-hexadecadien-1-ol [^{3}H]bombykol. Tritiated bombykol and homologous fatty alcohols applied onto antennae of moths were oxidized to long-chain fatty acids and, to a small extent, esterified to fatty acid esters[18].

This paper reports on the pheromone conversion and degradation using [^{3}H]bombykol as the pheromone

source for male and female moths of *Bombyx mori* L. [1-^{14}C]hexadecanol was also used to test the substrate specificity of the pheromone metabolizing enzymes and the degradation pathway. The term 'pheromone conversion' denotes the first steps of pheromone transformation, the enzymatic oxidation of the pheromone alcohol to the corresponding fatty aldehyde and fatty acid. The term 'pheromone degradation' describes the total oxidation and/or decomposition of the pheromone or the newly produced fatty metabolites to short-chain polar metabolites, i.e. tritiated water, in the case of [^{3}H]-labeled pheromones.

Pathways of pheromone conversion were simultaneously investigated with [^{3}H]-labeled compounds in other species of Lepidoptera. Enzymatic hydrolysis of [^{3}H](*Z*)-7-dodecenyl acetate and analogues was demonstrated in the cabbage looper moth, *Trichoplusia ni*[19]. Enzymatic conversion of [7,8-^{3}H](*Z*)-7,8-epoxy-2-methyl-octadecane ([^{3}H]disparlure) was shown in the gypsy moth, *Lymantria dispar*[20,21]. Studies with [6,7-^{3}H]- and [11,12-^{3}H](*E, Z*)-6,11-hexadecadienyl acetate have provided information on the enzymatic hydrolysis and molecular interaction of pheromone acetates in *Anthereaea polyphemus* and *Antheraea pernyi*[22–24]. The lepidoptera *Heliothis virescens, Choristoneura fumiferana* and *Reticulitermes flavipes* were recently investigated with radiolabeled pheromones with respect to pheromone reception and enzymatic conversion[25].

Materials and methods

Insects

Male and female pupae of *Bombyx mori* L. (supplied by M. Trevisan, Istituto Zoologia Agraria, Padua, Italy) were kept separately at 20 °C until emergence, and the moths at 4 °C in a refrigerator.

Experiments were performed at 20 °C using 20–40 freshly excised antennae for each experimental group. The moths used were all 1 week old unless stated otherwise. In control experiments air-dried antennae were used which had been stored at −20 °C for several (up to 6) years.

Chemicals

[12,13-^{3}H](*E, Z*)-10,12-hexadecadien-1-ol was produced by multistep synthesis. The acetylenic bond at the 12,13-position of (*E*)-10-hexadecen-12-yn-1-ol was tritiated with tritium gas and the Lindlar catalyst (courtesy of P. E. Schulze, Schering, Berlin). The labeled analogue, [1-^{14}C]hexadecanol was obtained from Amersham Inc. (England). The [^{3}H]-labeled compounds were stored in hydrocarbons as described[24]. The specific activities and molecular concentrations per dpm and ng are shown (table 1).

Hydrocarbons were used as standard solvents for the radiochemicals and chloroform-methanol (2:1, by vol.) for the extraction of pheromones and metabolites. Cellosolve (glycolmonoethylether) was used as a mixing solvent for aqueous and nonaqueous solutions.

Standard solvent for TLC: hexane, diisopropyl ether, methanol, and NH_3 (25%) (100:20:10:0.15, by vol.). Scintillator: PPO and POPOP in Rotiszint 22 (Roth), PPO without POPOP in Cellosolve for wet oxidation. Oxidizers for the antennae: 30% H_2O_2 and 60% $HClO_4$. The chemicals were all of analytical grade and obtained from Merck (Darmstadt), and Roth (Karlsruhe). The radiochemicals were analyzed and purified twice by TLC to a purity of over 98%.

Thin-layer chromatography

TLC was performed with precoated silica gel plates (0.25 mm) with concentration zones (Merck)[24]. The radioactive metabolites were identified by comparison with reference compounds[18]. After TLC each radio-chromatogram was completely scraped into 30 fractions with half-automatic scrapers (Desaga, Heidelberg and Hölzel, Dorfen). LSC was carried out at 25 °C in a Minaxi 4430 counter or at 5 °C in a Tricarb 3390 counter (Packard Instrument, USA). The data were recorded on paper tapes or diskettes which were analyzed by electronic data processing (PDP 11/40, Digital Equipment or IBM Personal Computer) and plotted as described (fig. 1)[24].

Incubation and elution

The airborne pheromones were blown onto the antennae by using a current of compressed air (10 s at 50 ml/s)[24]. The amount of the adsorbed [^{3}H]bombykol per antenna was between 40 and 400 pg (10^{10}–10^{12} molecules). Groups of 20 or 40 antennae were incubated for periods of 1, 5, 10 or 30 min at 20 °C unless stated otherwise.

After pheromone incubation the antennae were extracted with 5 ml pentane and 5 ml chloroform-methanol (2:1, by vol.). The [^{3}H] or [^{14}C]activity remaining in the antennae ([^{3}H]/[^{14}C] residue) was determined after wet oxidation with 100 µl $HClO_4$ and 200 µl H_2O_2 for 4 h at 70 °C as described[24,26]. The clear residues were treated with 10 ml Cellosolve and 10 ml PPO scintillation fluid and the radioactivity measured by LSC.

Table 1. Specific activities and numbers of [^{3}H]bombykol and [^{14}C]hexadecanol molecules per dpm and ng ±SD: standard deviation.

Pheromone compound	Specific activity mCi/mg	Ci/m Mol	Number of molecules 1 dpm =	1 ng =
[12,13-^{3}H]E 10,Z 12-16:ol	242.0 ± 12.0	58.1 ± 3.0	4.67×10^6	2.49×10^{12}
[1-^{14}C]-10:ol	47.2×10^{-3}	11.4×10^{-3}	2.38×10^{10}	2.48×10^{12}

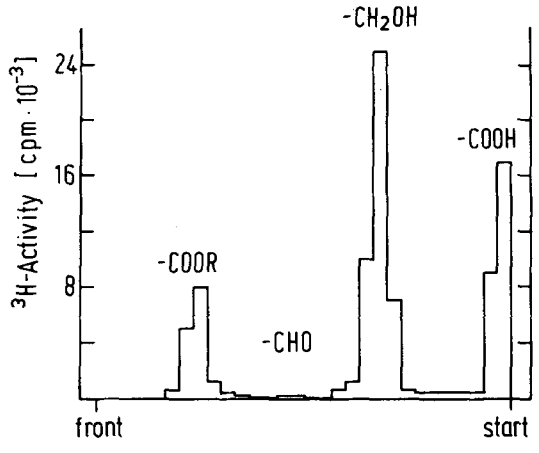

Figure 1. TLC of [^{3}H]bombykol and [^{3}H]metabolites. The metabolites were obtained by applying the pheromone in an air current to freshly excised antennae of male moths of *Bombyx mori* L. [^{3}H]Bombykol per antenna: 400 pg. The antennae were incubated (5 min) at 20 °C and eluted with chloroform-methanol (2:1, by vol.). An aliquot was chromatographed and the scraped zones were measured by LSC.

Pre-treatment and post-treatment

Freshly excised antennae were irradiated in petri dishes with UV-light (366 nm) from a low pressure mercury vapor lamp (Desaga, Heidelberg) (table 2). Dried antennae, stored for 5 years at −20 °C (freeze drying), were used as controls. Freshly excised antennae were also dried in a vacuum (desiccator, 20 mm Hg) with P_4O_{10} for up to 26 h (table 2).

After incubation with [^{3}H]bombykol, freshly excised antennae were desiccated in dry air (< 50% relative humidity) for up to 18 hours. Freshly excised antennae were also dehydrated with P_4O_{10} at normal pressure (750 mm Hg) and reduced pressure (20 mm Hg) (table 3). To determine the residual [^{3}H]/[^{14}C]activity (see elution section), the antennae were oxidized with $HClO_4$ and H_2O_2 after incubation and elution. However, to measure the total adsorbed radioactivity as a control, the antennae were similarly wet oxidized yet without prior elution.

Volatile metabolites

The evaporation of the pheromone metabolites was investigated after TLC as described [24]. Samples of the radiolabeled extracts were pipetted onto silica gel (of the TLC plates), dried, chromatographed, scraped and measured by LSC. The evaporation of the radiolabeled metabolites was calculated as the difference between the total radioactivity before scraping and the nonvolatile radioactivity measured after scraping. The polar [^{3}H]metabolite was identical with [^{3}H]water.

Errors

Errors of ±1–2% arose from the elution and wet oxidation of the antennae. Errors of ±1–3% were found after TLC and scraping. The total error of the cited operations was estimated at ±2–5%.

Table 2. Conversion and degradation of [^{3}H]bombykol in male moth antennae after pre-treatment. [^{3}H]Bombykol per antenna: 7.0–33.0 pg. Pretreatment, incubation (5 min), elution, TLC and LSC as described, drying results not corrected against blank values (fig. 1 and results section).

Pretreatment	[^{3}H]activity (%)		Nonvolatile [^{3}H]metabolites (%)					n
	Volatile	Nonvolatile	Alcohol	Aldehyde	Acid	Ester	Residue	
–	43.1 ± 4.4	56.9 ± 5.2	30.8 ± 6.0	0.2 ± 0.1	14.8 ± 1.4	3.0 ± 0.6	8.1 ± 1.0	8
Irradiation UV: 366 nm	42.0 ± 3.0	58.0 ± 3.2	28.0 ± 3.6	0.5 ± 0.1	17.1 ± 0.2	3.9 ± 0.5	8.5 ± 1.1	3
Freeze drying: −20 °C	9.8 ± 2.8	90.2 ± 5.0	70.2 ± 4.5	5.2 ± 0.8	8.3 ± 0.6	3.5 ± 0.2	3.0 ± 0.3	3
Vacuum drying: P_4O_{10} + 20 °C	9.0 ± 0.7	91.0 ± 7.1	73.2 ± 1.2	1.6 ± 0.4	6.4 ± 0.5	6.7 ± 0.4	3.1 ± 0.2	3

n, number of experiments; ±SD, standard deviation.

Table 3. Desiccation of male and female antennae after incubation with [^{3}H]bombykol. The antennae were either (a) dried in air or (b) dehydrated with P_4O_{10} at reduced or (c) normal pressure. [^{3}H]Bombykol per antenna: 11.3–30.5 pg. Incubation, post-treatment, elution, TLC and LSC as described (fig. 1 and methods section).

	Drying time (min)	Loss of [^{3}H]activity by drying (%)	Volatile [^{3}H]activity (%)	Nonvolatile [^{3}H]activity (%)	[^{3}H]residue (%)
Male antennae	1[a]	1.7 ± 0.4	18.4 ± 3.8	73.0 ± 4.6	6.9 ± 1.2
	5[a]	2.0 ± 0.2	42.0 ± 5.9	45.7 ± 1.9	10.3 ± 1.0
	30[a]	5.6 ± 1.4	62.3 ± 4.0	25.5 ± 3.8	6.6 ± 1.8
	180[b]	60.1 ± 4.3	6.4 ± 1.0	10.6 ± 0.9	22.9 ± 4.0
	1080[b]	60.0 ± 4.7	6.9 ± 3.4	18.3 ± 2.8	14.8 ± 1.6
Female antennae	1[a]	2.2 ± 0.5	33.1 ± 3.2	51.2 ± 4.8	13.5 ± 2.5
	5[a]	2.6 ± 0.6	63.9 ± 8.8	28.0 ± 3.7	5.5 ± 0.9
	30[a]	6.6 ± 0.7	64.2 ± 0.7	24.0 ± 1.9	5.2 ± 1.3
	180[a]	32.4 ± 2.0	33.3 ± 1.5	25.4 ± 2.3	8.9 ± 2.6
	1080[a]	63.6 ± 3.6	7.1 ± 0.5	24.2 ± 3.0	5.1 ± 0.5
	180[c]	47.9 ± 3.6	21.7 ± 0.7	20.9 ± 2.1	9.5 ± 1.1
	1080[c]	66.4 ± 4.0	7.0 ± 1.2	21.4 ± 3.6	5.2 ± 0.7

n = 3, number of experiments; ±SD, standard deviation.

Results

Time dependency

Freshly excised antennae of male moths of *Bombyx mori* L. incubated with [^{3}H]bombykol for 5 min, yielded mainly fatty acids and, to a lesser extent, fatty acid esters [18] with traces of [^{3}H]bombykal (fig. 1). [^{3}H]Bombykol was partly converted to less volatile but mainly degraded to highly volatile [^{3}H]metabolites. With increasing incubation time (0.1–30 min) the amounts of [^{3}H]bombykol in fresh antennae decreased while those of the [^{3}H]metabolites increased. The production of [^{3}H]water increased parallel to that of the [^{3}H]fatty acids. The overall half-life was reached at an incubation time of 2.5 ± 0.3 min (fig. 2).

Freshly excised antennae of female moths, incubated with [^{3}H]bombykol under the same conditions as the male moth antennae, gave 70% volatile [^{3}H]water after 30 min incubation. As with the males, the amount of aldehyde was below 1%. The overall half-life was reached at an incubation time of 0.5 ± 0.2 min (fig. 3).

Freshly excised antennae of male moths incubated with [^{14}C]hexadecanol for periods of 1–30 min gave small amounts of fatty acids and fatty acid esters but high amounts of non-extractable [^{14}C]residues (fig. 4). At much higher concentrations of [^{14}]hexadecanol per antenna, (10–20 ng: about 10^3 higher than that of [^{3}H]bombykol) the overall half-life was reached at 3.3 ± 0.3 min. This is slightly slower than that of the metabolism of [^{3}H]bombykol.

Pre-treatment effects

Freshly excised antennae of male moths exposed to daylight and incubated with [^{3}H]bombykol for 5 min in air gave about 43% volatile [^{3}H]water (table 2). Similar results were obtained from antennae irradiated with UV-light of 366 nm. Freeze drying of the antennae reduced the amount of volatile [^{3}H]water obtainable to below 10% of the total initial [^{3}H]activity. The amount of the nonvolatile [^{3}H]metabolites (including the [^{3}H]residue) remained 20%. However, in this case, the relatively high amount of [^{3}H]aldehyde (5%) should not be overlooked (table 2).

Vacuum drying of the antennae with P_4O_{10} gave similar amounts of volatile and nonvolatile [^{3}H]metabolites after 5 min incubation (table 2). Control experiments with cotton wool have shown that, in this case, the volatile compounds were [^{3}H]bombykol and not [^{3}H]water.

Intensively dried antennae of male moths similarly incubated with [^{3}H]bombykol (0.1–30 min; n = 18 (number of experiments)) yielded only small amounts (average = 7%) of nonvolatile [^{3}H]metabolites but no volatile [^{3}H]water. These results were obtained after subtraction of the blank values (cotton wool experiment).

Post-treatment effects

After incubation of freshly excised male and female antennae with [^{3}H]bombykol the amounts of the

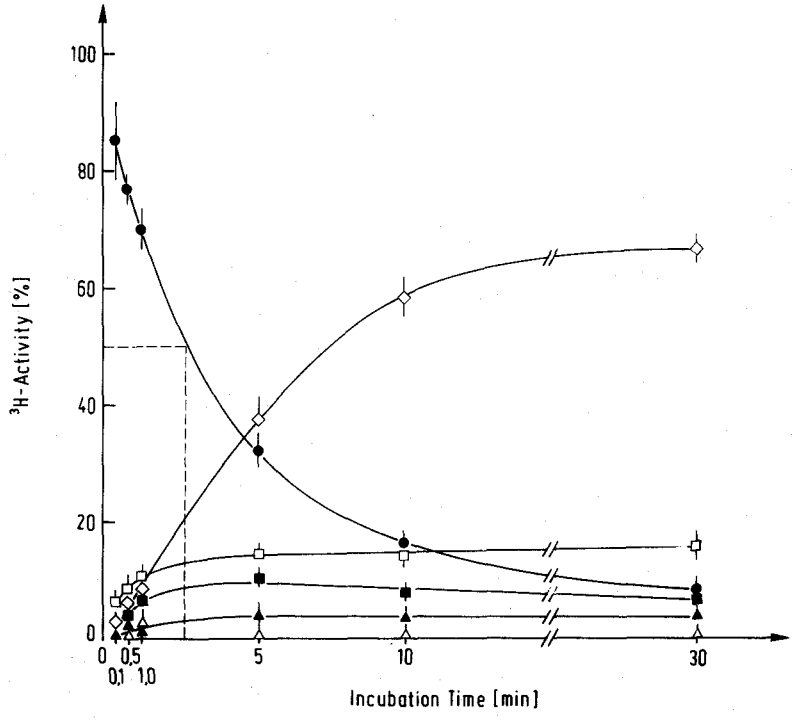

Figure 2. Time course of pheromone conversion and degradation in fresh male antennae. The [^{3}H]metabolites were recovered from antennae which were applied and incubated with [^{3}H]bombykol in air. [^{3}H]Bombykol per antenna: 12.2–33.0 pg. Incubation, elution, TLC and LSC as described (fig. 1). Pheromone half-life: 2.5 ± 0.3 min. Symbols: [^{3}H]bombykol (●), [^{3}H]bombykal (△), [^{3}H]acid (□), [^{3}H]ester (▲), [^{3}H]residue (■), volatile [^{3}H]water (◇), n = 3 (number of experiments), error bars: ±SD.

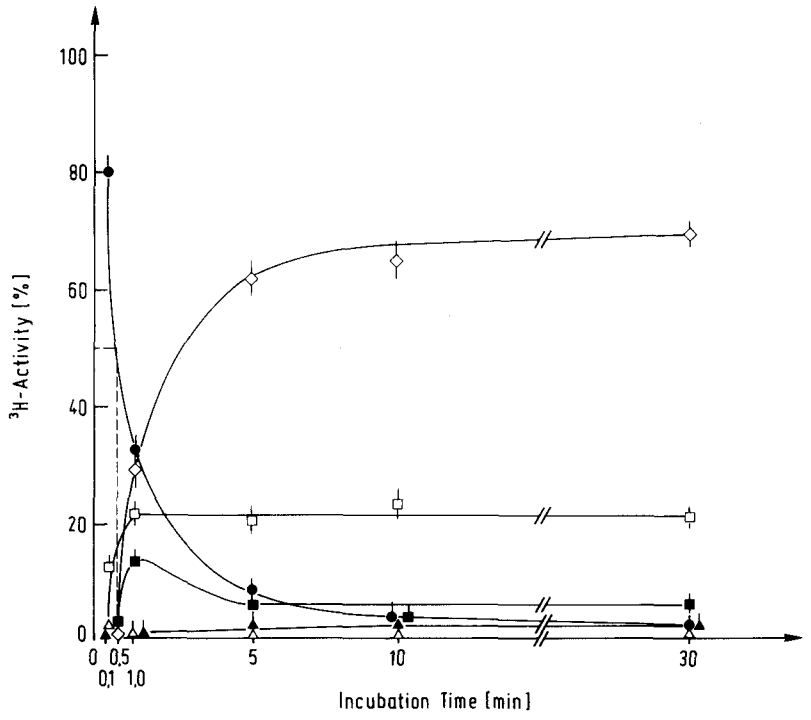

Figure 3. Time course of pheromone conversion and degradation in freshly excised female antennae. [^{3}H]Bombykol per antenna: 13.0–21.5 pg. Incubation, elution, TLC and LSC as described (fig. 1). Pheromone half-life: 0.5 ± 0.2 min. Symbols: see fig. 2. n = 3; error bars: ±SD.

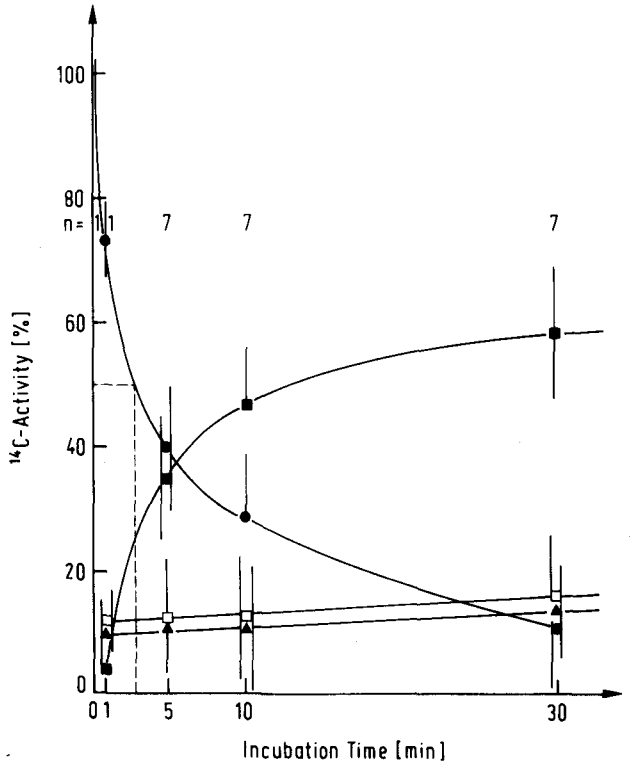

Figure 4. Time course of [^{14}C]hexadecanol conversion and degradation in freshly excised male antennae. [^{14}C]hexadecanol per antenna: 10–20 ng. Incubation, elution, TLC and LSC as described (fig. 1). [^{14}C]hexadecanol half-life: 3.3 ± 0.3 min. Symbols: [^{14}C]hexadecanol (●), [^{14}C]acid (□), [^{14}C]ester (▲), [^{14}C]residue (■), *n = number of experiments, error bars:* ±SD.

[^{3}H]pheromone decreased and the amounts of the [^{3}H]metabolites increased with incubation time (figs 2 and 3). In dried air (< 50% relative humidity) the total amounts of nonvolatile [^{3}H]activity sharply decreased and the [^{3}H]loss by drying slightly increased with time (table 3). This effect dramatically increased after longer drying times (3–18 h). A significant desiccation was measured not only after drying in air but also after dehydration with P_4O_{10} under normal and reduced pressure (table 3).

Discussion

These new findings of bombykol and hexadecanol metabolism conclude that insect pheromones are inactivated firstly by conversion and secondly by degradation. Pheromone conversion includes the oxidation of the pheromones by oxidases and/or dehydrogenases to the corresponding fatty acids according to the following scheme:

$$RCH_2OH \rightarrow [RCHO] \rightarrow RCOOH$$

Fatty aldehydes were found only in trace amounts after the pheromone oxidation suggesting that they are produced as intermediate metabolites. Only a small proportion of the pheromone alcohols and their newly produced fatty acids was esterified to long-chain fatty acid esters [27].

Enzymatic oxidations have been described in a number of plant and animal tissues where long-chain fatty alcohols are irreversibly converted to fatty acids [28]. In insects it is the primary and secondary hydroxyl groups of long-chain fatty alcohols that are preferably oxidized [29]. Oxidases and/or dehydrogenases were also found in antennae and female pheromone glands of *Choristoneura fumiferana* [30] and *Heliothis virescens* [25, 31] which oxidize long-chain fatty alcohols and/or aldehydes to the corresponding fatty acids. (*E*)-10-hexadecen-1-ol and hexadecanol are converted in the same pathway in male moths of *Bombyx mori* [10].

Recently it was demonstrated that isomers of (*E, Z*)-6,11-hexadecadienyl acetate are also enzymatically converted in intact antennae of male moths of *Antheraea polyphemus*. Soluble pheromone hydrolyzing esterases were isolated and identified in the receptor lymph of *Trichoplusia ni* [19] and *Antheraea polyphemus* [22, 32]. Cuticle bound esterases which preferably hydrolyze pheromone acetates were detected in adults of *Trichoplusia ni* [33], *Choristoneura fumiferana* [34] and *Antheraea polyphemus* [35].

The results of this paper show that [^{3}H]bombykol is metabolized in living antennae of *Bombyx mori* L. with an increase of volatile metabolite appearance with increasing incubation time. Pheromone degradation can be interpreted as the oxidation of the tritium at the carbon double bonds and/or the total decomposition of the [^{3}H]-labeled pheromone to tritiated water. The dehydration experiments have shown that [^{3}H]loss from the antennae increased with drying time and followed the decrease of volatile metabolite appearance. We must conclude that the total volatile [^{3}H]activity exclusively consists of [^{3}H]water. Studies with isomers of [6,7-^{3}H] (*E/Z*)-6,11-hexadecadienyl acetate in living antennae of *Antheraea polyphemus* have also revealed a substantial production of tritiated water [24]. Results obtained from both species show that the increase in volatile metabolites closely follows that of the fatty acids.

In dried antennae of *Bombyx mori* L. [^{3}H]bombykol was partly converted to the corresponding aldehyde and fatty acid but scarcely degraded to [^{3}H]water. Probably, the oxidases and dehydrogenases were destroyed or inactivated by drying and freezing. In air-dried male antennae, after 5 min of incubation, about 5% of the bombykol was oxidized to bombykal. As yet it is not possible to interpret this relatively high amount of aldehyde, c.f. *Antheraea polyphemus* [24].

Experiments using [^{14}C]hexadecanol in intact antennae of *Bombyx mori* L. did not show a significant production of polar volatile [^{14}C]metabolites. Probably, the [^{14}C]-labeled degradation products were mainly incorporated into insoluble biopolymers of the antennae. The total absorbed [^{14}C]activity ([^{14}C]residue) was determined after wet oxidation of the antennae. Additional experiments with [^{3}H]- and [^{14}C]-labeled pheromones (in preparation) will give more detailed information on the mechanism of pheromone degradation.

The enzymatic conversion and degradation with half-lives of 2.5 min for *E*10,*Z*12-10:ol in male moths of *Bombyx mori* L. and 3 min for the isomers of *E*6,*Z*11-16:Ac in male moths of *Antheraea polyphemus* is strikingly similar. Furthermore, because the metabolic curves are not linear in half-logarithmic plots [23], the processes can be interpreted as the sum of at least two different inactivation processes. It appears that in *Antheraea polyphemus* most of the pheromone molecules were metabolized in the sensory hairs whereas in *Bombyx mori* L. the main conversion took place in the branches [10]. The very short half-life (0.5 min) for *E*10,*Z*12-16:ol in female moths of *Bombyx mori* L. may be due to pheromone molecules reaching the antennal branches much earlier, as the females have shorter antennal hairs.

In male moths of *Bombyx mori* L., the half-lives for (*E*)-10-hexadecenol and for hexadecanol were very similar, 3.5 min and 3.3 min respectively. Because the concentration of hexadecanol was about 1000-fold higher than that of bombykol and the stimulus is about 10^6 times less effective than bombykol for the olfactory receptor cells [16], the responsible degradative enzymes cannot be very substrate specific.

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* Reprint requests to Dr G. Kasang, Max-Planck-Institut für Biochemie, D-8033 Martinsried.

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Quantitative analysis of absorption spectra and application to the characterization of ligand binding curves

R. G. Duggleby and D. B. Northrop[a]

Department of Biochemistry, University of Queensland, St. Lucia (Queensland 4067, Australia), and [a] School of Pharmacy, University of Wisconsin, Madison (Wisconsin 53706, USA)
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Summary. The spectrum of a chromophore may change as a result of perturbations in its environment. The spectral changes resulting from the perturbation are often followed by measurements at just one or two wavelengths but it is usually no more difficult to collect entire spectra. The problem comes in analysing the data from such a series of spectra. In this paper we will suggest a simple procedure in which the spectrum observed under any particular set of conditions may be considered to consist of the sum of two distinct spectral forms. The method, which is free of any assumptions regarding the quantitative relationship between the perturbation and the extent of spectral change, defines any given spectrum in terms of an apparent molar fraction of the contributing spectral forms. The variation of this apparent molar fraction provides information from which a quantitative relationship can be developed to describe the dependence of the spectral change on the perturbant. The method is illustrated using the model system of phenol red protonation and is applied to the characterization of the binding of azide ions to cobalt-substituted carbonic anhydrase.
Key words. Spectral titrations; absorption spectra; fluorescence spectra; binding curves; carbonic anhydrase.

Many compounds contain a chromophore with spectral properties which depend on its environment. Perturbations of the environment result in spectral changes which can be used to obtain a quantitative description of the effect of the perturbant. For example, the chromophore might be a group in a protein and the perturbant a ligand which binds to the protein; by following the spectral change resulting from ligand addition, the binding curve may be determined.

In these types of titration, it is a relatively simple matter to collect many complete spectra, each representing a different amount of added perturbant. The resulting spectra contain a bewildering amount of information, so much so that it is common practice to ignore most of it.