tion is also available to the follower bees. The capacity of the follower bees to form averages of the distances and directions indicated in the individual wagging runs is not well understood. In this connection it is important whether the total scatter in the pooled data is caused by different bees indicating different average directions, or whether a similar amount of scatter is present in the consecutive wagging runs of a single dance. The latter appears to be true. In Fig. 3, an example is given of the directions indicated by 11 consecutive wagging runs in a single dance announcing food 1 m from the hive. This is the message received by the follower bees that stay with a single dancer for some time.

In conclusion, an observation of the directions of individual wagging runs in a reasonable number of dances does not result in any significant directional indication at the distances below 15 m, where typical round dances occur in *Apis mellifera carnica*. Fairly similar data have been obtained in the Italian race (*Apis mellifera ligustica*), which has sickle-shaped transition dances rather than the direct transition dances used by *carnica*. Our results thus support the interpretations offered by von Frisch (1967). The significant indication of direction already at a distance of 1 m reported by Kirchner et al. (1988) was obtained by a two-step averaging of at least 60 dances. We do not believe this to be a likely strategy for follower bees, but we are aware that only little is known about the number of round dances actually followed and about the capacity of bees to make averages. Studies on such behavioral aspects are in progress.

The Center for Sound Communication is financed by the Danish National Research Foundation.

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Naturwissenschaften 84, 454–457 (1997) © Springer-Verlag 1997

In Vitro Production of the Pheromone Intermediates Ipsdienone and Ipsenone by the Bark Beetles *Ips pini* (Say) and *I. paraconfusus* Lanier (Coleoptera: Scolytidae)

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Received: 1 April 1997 / Accepted: 21 July 1997

The pine engraver *Ips pini* (Say) and the California five spined ips *I. paraconfusus* Lanier are North American bark beetles that share a zone of sympatry in California and Oregon. Male *Ips* spp. release an aggregation pheromone that attracts conspecific males and females, and this chemical signal facilitates colonization of the host tree [1]. The two most common aggregation pheromone components for *Ips* spp. are the acyclic monoterpene alcohols ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol; produced most abundantly by *I. pini*) and ipsenol (2-methyl-6-methylene-7-octen-4-ol; produced most abundantly by *I. paraconfusus*) [2]. Both species produce ipsdienol, but California and Oregon populations of *I. pini* produce 94–98% (–)-ipsdienol [3], while California populations of *I. paraconfusus* produce 86–98% (+)-ipsdienol [4, 5]. De novo production of ipsdienol was first demonstrated for the Eurasian species *I. duplicatus* (Sahlberg) [6, 7]. Radiochemical studies have shown that *I. pini* and *I. paraconfusus* synthesize ipsdienol and ipsenol (only *I. paraconfusus*) de novo from acetate and mevalonate [8]. The pheromone in male *Ips* spp. accumulates in the hindgut [2, 9] and is released as the beetles feed on the phloem of their host tree [10]. However, studies over the past 30 years have failed to demonstrate the presence of a hindgut-associated pheromone gland in male *Ips* spp. [11, 12]. This paper reports an in vitro assay for pheromone production in *I. pini* and *I. paraconfusus* that can be used to localize the site of pheromone synthesis. This method, which

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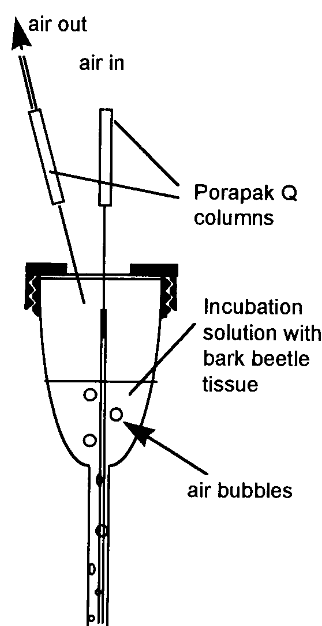

Fig. 1. Setup for collection of volatiles produced in the in vitro assay

monitors the incorporation of ^{14}C from acetate or mevalonate into the ketone derivatives of the alcohol pheromone components, can also be applied to studies of the regulation and intermediates in the de novo biosynthesis of ipsdienol and ipsenol.

Ips pini and I. paraconfusus were collected as immatures; *I. pini* from *Pinus jeffreyi* Grev. & Balf. and *P. ponderosa* Laws. logging debris in the Lassen National Forest, Lassen Co., California and *I. paraconfusus* from standing, infested *P. radiata* D. Don near Briones Reservoir, Contra Costa Co., California. Both species were reared to the adult stage and collected upon emergence [8]. Male and female *I. pini* were prefed on *P. jeffreyi* phloem, while male and female *I. paraconfusus* were prefed on *P. radiata* and *P. jeffreyi* phloem. Both of the latter tree species are natural hosts for *I. paraconfusus* [13]. The prefed beetles were topically treated on their ventral side with 1 µl (7.5 µg/µl in acetone) juvenile hormone (JH) III/ beetle (Sigma). After 48 h in film cannisters the beetles were frozen at –80 °C.

The beetles were either manually homogenized or cut longitudinally with a scalpel and then immersed in a saline solution (154 mM NaCl, 2.7 mM KCl, 2.7 mM $CaCl_2$, 0.9 mM $NaHCO_3$, and 83 µM NaH_2PO_4 in double-distilled water) at 25 °C. Each incubation consisted of 20 beetle equivalents in 1.5 ml solution. Phenylmethylsulfonic acid (0.5 mM; PMSF; Sigma), a protease inhibitor, was added to minimize protein degradation. A fatty acid synthesis inhibitor (2-octynoic acid; Aldrich) was added (20 µM) to minimize the conversion of radiolabeled acetate to fatty acids. Finally, NADPH (5.6 µM) and sodium [1-^{14}C]acetate (1 MBq; ICN) or *RS*-[2-^{14}C]mevalonate (2 MBq; DuPont) were added. In some experiments 150 µg JH III was added to the incubation mixture. Porapak Q (50/80 mesh; Supelco) purified air was bubbled at a rate of 30 ml/min through the solution and volatiles were trapped on a Teflon column (Fig. 1) containing 300 mg Porapak Q during the 6-h incubation. Incubations were replicated 30 times for male *I. pini* and 6 times for male *I. paraconfusus*. Incubations with female beetles were replicated 3 times for each species.

A 2-ml pentane eluate of the Porapak column was fractionated by gradient normal-phase HPLC on an HP Series 1050 Instrument with UV detection at 227 nm (based on the ε_{max} of ipsdienone). Fractions (400 µl collected every 30 s) were divided into 50 µl aliquots for liquid scintillation analysis and 350 µl aliquots for chemical analysis. Heptyl acetate was added to the latter aliquots as an internal standard, and the aliquots were analyzed with a Hewlett Packard 5890 Series II gas chromatograph (GC) on a 60×0.25 mm (ID) HP-wax fused silica column, temperature programmed from 70 °C (5 min) to 210 °C at 5 °C/ min and held at this final temperature for 5 min. The carrier gas was helium with a flow rate of 31 cm/s and detection was by flame ionization. Injection temperature was 210 °C and detector temperature was 230 °C. To further confirm the presence of ipsenol, ipsenone, ipsdienol, and ipsdienone in various fractions, samples were analyzed on a Varian model 3400 GC coupled to a Finnigan MAT ssQ 710 mass spectrometer (MS). The GC was equipped with a DB-5 fused silica column (J&W; 30×0.25 mm) temperature programmed as described above. Compounds were identified by their retention times, electron impact spectra and chemical ionization (CI; methane and ammonia) spectra. The level of ^{14}C-associated radioactivity was determined by assaying 50 µl aliquots using Ecolume scintillation cocktail on a Beckman LS-1701 liquid scintillation counter. The counting efficiency was 97% for ^{14}C.

To demonstrate its association with radiolabel, ipsdienone in the radioactive fractions was reduced to ipsdienol with $LiAlH_4$ and subsequently fractionated by normal-phase radio-HPLC (Fig. 2C). The radioactive fractions were also separated by preparative GC on a HP 5710A equipped with a thermal conductivity detector. Fractions were collected in a coldtrap consisting of a glass tube lowered into dry ice-cooled ethanol and assayed by liquid scintillation counting (Fig. 2D). The structure of ipsdienol was further confirmed by GC-MS. Association of radiolabel with ipsenone was also demonstrated by reversed-phase HPLC (data not shown).

Large amounts of radiolabel were incorporated into ipsdienone (male *I. pini*) and ipsenone (male *I. paraconfusus*) from acetate and mevalonate (background approx. 30 dpm). A radio-HPLC chromatogram from male *I. pini* (Fig. 2B) shows a typical level of radioactivity in the ipsdienone peak [mean = 12 500 dpm SE = 2500 dpm N = 30 (ipsdienone)]. The radioactivity in the ipsenone peak (same retention time for ipsdienone and ipsenone) for *I. paraconfusus* was mean = 17 000 dpm SE = 2000 dpm N = 6 (ipsenone). Radiolabel from acetate or mevalonate was not incorporated into myrcene, ipsdienol, or ipsenol (Fig. 2B), but both the monoterpene and alcohols were present in the respective Porapak extracts as demonstrated by HPLC retention time on the UV trace (Fig. 2A) and confirmed by GC-MS. The absence of radiolabel in these alcohols suggests that they were produced during prefeeding and/or JH treatment, but not synthesized during the in vitro incubation. The association of radiolabel with ipsdienone was demonstrated by partial reduction to the alcohol and reanalysis by normal phase radio-HPLC (Fig. 2C). Prepara-

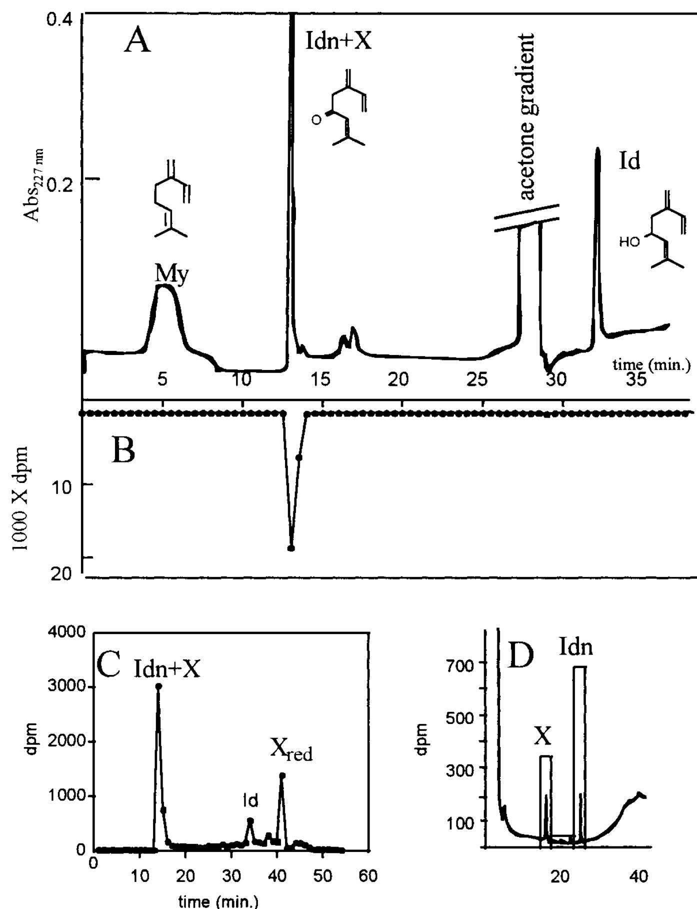

Fig. 2. Normal-phase radio-HPLC separation of volatiles collected from 20 homogenized male *I. pini* incubated with 1 MBq [1-^{14}C]acetate for 6 h at 25 °C. The peaks in the UV-trace (A) are marked with myrcene (*My*), ipsdienone (*Idn*), unidentified compound (*X*) and ipsdienol (*Id*). Male *I. pini* does not produce ipsenone or ipsenol in detectable amounts. B) Radioactivity. Column, partisil PXS 5/25 (Whatman); 25×5 cm. Mobile phase, hexane:acetone 99:1 for 15 min, 1% acetone/minute for 5 min and then isocratic for 30 min; flow rate 0.8 ml/min. C) Normal-phase radio-HPLC of reduced ipsdienone fraction from A/B. D) Preparative GC of radiolabeled ipsdienone fraction. *Unshaded bars*, radioactivity in collected fractions. Steel column (2 m×6 mm ID) packed with 3% Dexsil 300 on Supelcoport and compounds were detected with thermal conductivity detector. Carrier gas was helium and the oven was held at 50 °C for 4 min and then programmed at 4 °C/min to 200 °C

tive GC fractionation and liquid scintillation counting (Fig. 2D) also confirmed that ipsdienone was radiolabeled. In addition to the monoterpene ketones, a smaller and slightly more polar unidentified radiolabeled compound (marked as X in Fig. 2) eluted with the ketones on normal-phase radio-HPLC.

Treatments were tested in pairs using beetles of the same age and origin to study ketone synthesis. Tissue from male *I. pini* produced mostly ipsdienone, while tissue from male *I. paraconfusus* produced mostly ipsenone. Female tissue of both species produced ipsdienone, but only 25% of the amount produced by male tissue. Centrifugation of homogenized beetles at 1000 g to obtain a cell-free preparation did not interrupt ketone synthesis. Addition of antioxidant (DTT) or a mixed function oxidase inhibitor (piperonylbutoxide) did not result in labeled alcohol formation, suggesting that the ketones are not oxidation artifacts. JH III and its analogs have long been known to induce pheromone production in *Ips* spp. [7, 14, 15]. Ketone formation was enhanced when male *I. pini* were JH treated prior to incubation, but addition of JH to the incubation solution had no effect. Induction of pheromone production by JH or its analogs takes 5–20 h [7, 15], and ketone synthesis is most abundant during the first 6 h of the assay (data not shown). Thus in vitro production from tissue assays likely declined before JH added to the incubation solution could exert its effect. Previous in vivo studies have shown that ipsdienone can be converted to ipsdienol by *I. paraconfusus* [4, 16] and *I. pini* [16]. In the in vitro experiments ipsdienol was not labeled regardless of whether the beetles were homogenized, or the ventral cuticle was cut longitudinally. Incubation with intact insects did not produce any radiolabeled ketones, presumably because ^{14}C-acetate could not reach the pheromone-producing tissue.

One possible explanation for the synthesis of labeled ketone, but not labeled alcohol pheromone, is that the ketone is formed in one tissue and then transported to another tissue (possibly the hindgut or the Malpighian tubules) for the final reduction step to the alcohol. Thus a functioning circulatory system may be required for ipsdienone to reach the site of enantioselective reduction to ipsdienol.

Our results do not support the hypothesis of Fish et al. [4], that ipsenol is formed from reduction of the C-2 double bond in ipsdienol by male *I. paraconfusus*. In the in vitro assay male *I. pini* produced mainly ipsdienone while male *I. paraconfusus* produced mainly ipsenone. The most abundant pheromone components of these species are the corresponding alcohols. This suggests that male *I. paraconfusus* reduces the double bond at C-2 in ipsdienone to form ipsenone which is further reduced to ipsenol. Further data to support this hypothesis were provided by Byers and Birgersson [17], who found substantial amounts of ipsenone in the hindguts of male *I. paraconfusus* regardless of the host tree they colonized. Other *Ips* spp. that produce ipsdienol and ipsenol also contain the corresponding ketones [18].

Females of both species produce ipsdienone but in much lower amounts than males. JH also boosts the pro-

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duction of labeled ipsdienone in females, indicating that a hormonally regulated de novo pathway to ipsdienone exists in both sexes. In vivo radiotracer studies have shown that females of neither species emit the pheromone alcohols [8]. Apparently females lack the enzyme that reduces ipsdienone to ipsdienol. Furthermore, *I. paraconfusus* females produce ipsdienone and not ipsenone, which is produced by males. It is possible that ipsdienone produced by females has no behavioral function or is further converted to behaviorally relevant compounds that are released after mating.
The method described in this paper presents a powerful tool to study the anatomical localization, the intermediates, and the endocrine regulation in pheromone biosynthesis in bark beetles.

We are grateful to Dr. Désirée Vanderwel, University of Winnipeg, Canada, for advice on reduction of ipsdienone and for revising this manuscript. We thank Drs. D.L. Wood, University of California at Berkeley, and J.P. Vité, Universität Freiburg for *Lippia* plant oil, from which ipsdienone was purified and used as standard. Göran Birgersson, Göteborgs Universitet, helped improve the manuscript. This work was in part supported by Swedish Council for Forestry and Agricultural Research Grant 96.1035/94 and United States Department of Agriculture NRI-CGP Grant 9502551.

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Note added in proof
Mass spectral data for ipsdienone m/r 83 (100%); 55 (35); 150 (6)

Naturwissenschaften 84, 457–459 (1997) © Springer-Verlag 1997

How To Derive Steady-State Rate Laws for Enzyme-Catalyzed Reactions from Macroscopic Probabilities

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Received: 30 June 1997 / Accepted: 28 July 1997

The concept of conditional probabilities is shown to allow a rigorous probabilistic interpretation of the kinetics of enzyme-catalyzed reversible chemical reactions. All notions involved in this treatment are macroscopic in the same sense as concentration or chemical reaction rate.
As pointed out by Beveridge and Schechter (1970), if transition probabilities are regarded as stationary parameters of chemically reacting systems, one can no longer distinguish the final results of a probabilistic kinetic analysis from that of a deterministic one. Based on this notion, I suggest that the steady-state rate laws of enzyme-catalyzed reactions, which are generally thought of as being described in terms of deterministic rate constants, do have a simple and straightforward probabilistic derivation. I point out in advance [1] that in steady-state systems all reactant concentrations are assumed to be constant and that, therefore, all transitions are described by first-order parameters, and [2] that the line of reasoning presented below applies directly to all reversible chemical reactions in which the total concentration of intermediates is limited (because of a limiting amount of the catalyst) by a law of conservation.
The kinetic model used below for illustration (Fig. 1) has a linear mecha-