12. U. Nilsson and H. J. Müller-Eberhard, *ibid.* **122**, 277 (1965).
13. These tests were performed in microtiter plates with 25 μl of dilutions of water-hemolyzed EAC1,4 or EAC1,4,2,3 prepared with the isolated components and 25 μl of human erythrocytes (8×10^7 cell/ml). This system reaches maximum reactivity at a concentration of 1×10^7 EAC1,4 or EAC1,4,2,3 per milliliter.
14. Coombs-type agglutination tests were performed in microtiter plates. Twenty-five microliters of complement bearing complexes (1×10^8 cell/ml) were added to dilutions of monospecific antiserums to C3 or C4. After being shaken intermittently for 15 minutes at 37°C, the cells were permitted to settle at 37°C for 30 to 45 minutes. The titer was appraised by inspection of the pattern of the sedimented erythrocytes.
15. The number of labeled C3 and C4 molecules per cell was obtained from ^{125}I counts; a molecular weight of 2×10^5 was used for both components.
16. D. S. Nelson and R. A. Nelson, *Yale J. Biol. Med.* **31**, 185 (1959).
17. D. S. Nelson, in *Complement*, G. E. W. Wolstenholme and J. Knight, Eds. (Churchill, London, 1965), p. 222.
18. Supported by a Dernham Junior Fellowship of the American Cancer Society, California Division (No. J-120); by PHS grant AI-07007; by American Heart Association, Inc. grant 68-666; and by AEC contract AT(04-3)-730. This is publication No. 327 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California.

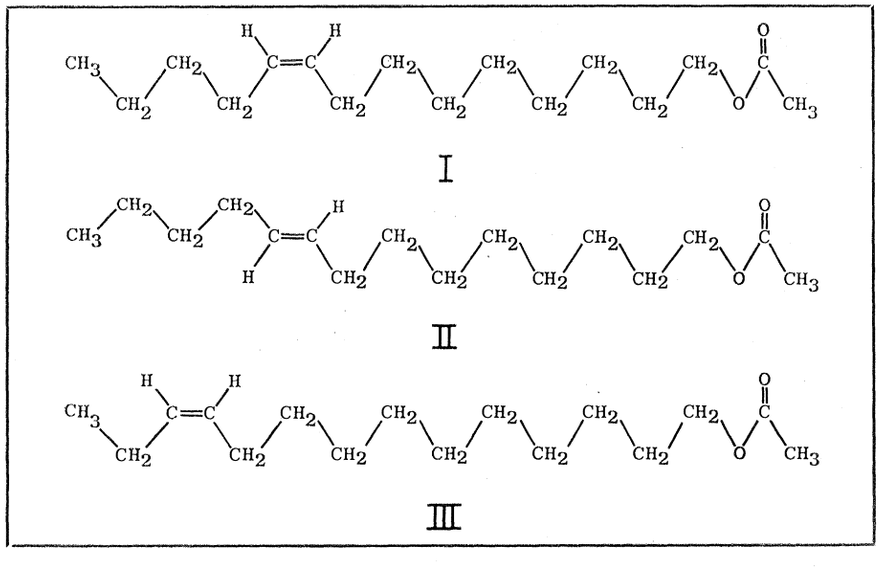
19 March 1969

Sex Pheromone Specificity:
Taxonomic and Evolutionary Aspects in Lepidoptera

Abstract. *Sex pheromone specificity is the only obvious reproduction isolating mechanism for two tortricid species and two gelechiid species. Pheromones of the gelechiid species are* cis-*9-tetradecenyl acetate and* trans-*9-tetradecenyl acetate. Male response of one gelechiid species is inhibited by the pheromone of the other species. This could be important for sympatric evolutionary saltation.*

Sex pheromone specificity is one of several ways by which insect species can achieve reproductive isolation under natural conditions. In cases where pheromones are apparently nonspecific, other factors, such as variations in mating rhythms, seasonal cycles, host plant selection, and geographical distribution, can serve to isolate the species (*1*). If several insect populations appear similar in these biological considerations and in morphological detail, the determination of sex pheromone specificity would provide excellent support for the reproductive isolation of these populations in a nondimensional system. Reproductive isolation under natural conditions in turn "supplies a completely nonarbitrary criterion for the determination of species status of a population" (*2*). We have used sex pheromone specificity as a taxonomic principle in two examples of morphologically similar species of Lepidoptera by determining specificity with crude pheromone extracts in one case, and with synthetic pheromone compounds in the other. The pheromone compounds in the latter case were previously unknown for both species in the pair, and therefore, represent two new pheromone identifications.

The first example involves two tortricids, *Archips mortuanus* and *Archips argyrospilus*. Although Freeman (*3*) considered them sibling species, Powell (*4*) later suggested that the former is merely a darker form of the latter. In addition to possessing indistinguishable morphological adult characters, the two forms have the same seasonal cycles, feed on the same hosts (apple trees), and have indistinguishable egg masses and young larvae. An intensive comparative study (*5*), however, revealed differences in the ultimate larval instar that enables the two forms to be separated in field collections. Histological study (*5*) of the adult female genitalia showed that the gland producing pheromone is very similar in both forms; the glands are situated dorsally in the intersegmental membrane between the eighth and ninth abdominal segments as a convoluted, eversible sac of highly secretory, cuboidal-to-columnar, prismatic epithelium.



Studies of sex pheromone specificity (*6*) were conducted with crude pheromone extracts obtained by pulverizing the two terminal abdominal segments from 180 female *A. argyrospilus* moths in one batch and 80 female *A. mortuanus* in another. The moths were reared from ultimate instar larvae collected in stands of wild apple trees. A sample containing ten female equivalents of pheromone was placed on a 4-cm dental wick located within each trap (355.2 ml drinking cups lined with sticky polybutene). Sets of traps, each set consisting of an *A. argyrospilus* and an *A. mortuanus* trap located within 1 m of each other, were hung in the apple trees during moth flight. The traps with *A. argyrospilus* extract attracted 203 male *A. argyrospilus* and no *A. mortuanus* adults on the same nights that traps with *A. mortuanus* extract attracted 82 male *A. mortuanus* and 10 *A. argyrospilus* adults. The occurrence of these ten strays may be due to the high level of *A. argyrospilus* infestation encountered, and to the proximity of the *A. argyrospilus* traps and wild females. Clock-traps (*7*) charged with crude pheromone extracts were used to determine the circadian rhythm of male activity for each form. The two forms were attracted at essentially the same hours, so the only obvious isolating factor would be sex pheromone specificity. Our evidence for this specificity supports the conclusion that these are separate species.

The second example involves two species of Gelechiidae. The two species are morphologically similar and exhibit identical four-dot wing patterns, although one form, *Bryotopha similis* (B1), appears grayish and the other form (B2) more yellowish (*8*). The

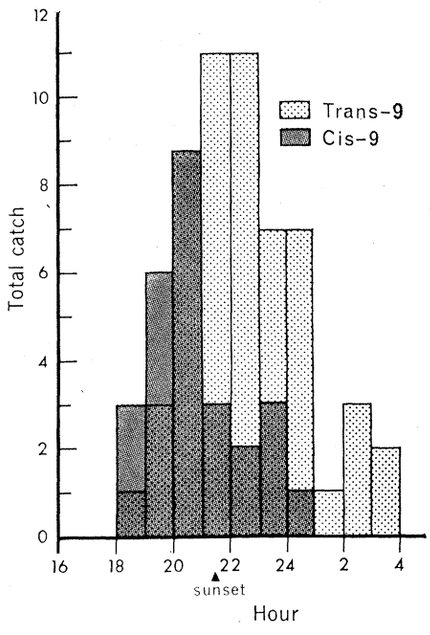


Fig. 1. Circadian rhythm response of male gelechiid moths to sex pheromones.

moths were first observed when males were attracted to two traps used in an experiment testing a large number of potential attractant compounds for the red-banded leaf roller, *Argyrotaenia velutinana* (*9*). The grayish form (B1) was always attracted to traps charged with 10 μg of *cis*-9-tetradecenyl acetate (I), whereas the yellowish form (B2) was always attracted to the geometrical isomer, *trans*-9-tetradecenyl acetate (II) (*10*). Further field tests over a period of almost 2 months showed that the attraction of these two forms to these compounds was highly specific even though the traps were spaced only 30 cm apart. A total of 240 B2 males were attracted to *trans*-9-tetradecenyl acetate, and 45 B1 males were attracted to *cis*-9-tetradecenyl acetate without a single cross-over in either case.

Studies of circadian rhythm indicate the average hours for male activity to be modally different, but overlapping broadly (Fig.1). Again, sex pheromone specificity appears to be the most salient reproductive isolating mechanism, and it follows that B1 and B2 are separate species.

Evidence that the two compounds were identical to the nautral sex pheromones of these moths was obtained by two different means. In one, minute quantities of compound (10 μg) were placed on wicks in traps, and male response was observed for several days. Residues present on the second night were still attractive and paralleled the physiological activity of synthetic and natural red-banded leaf roller pheromone at the same low concentrations. Another test was based on studies of pheromone perception with red-banded leaf roller moths (*11*). These studies suggested that the sex attractant, a chemical with the correct spatial arrangement of active sites, possesses the affinity and intrinsic activity for antennal receptor sites to elicit proper behavioral responses, including flight to the location of the sex attractant. Closely related isomers which do not exhibit intrinsic activity by attracting males could possess a strong affinity for the receptor sites, thus acting as inhibitors or modifiers of the sensory input. Field attractant tests with red-banded leaf roller attractant, *cis*-11-tetradecenyl acetate (III), in combination with various homologs and analogs, showed that the *trans* geometrical isomer of compound III and other primary 14-carbon acetates or alcohols with unsaturation at C-11 can inhibit males from responding to the attractant. In our experiment, we tested for inhibitory effects of geometrical isomers by hanging up a series of traps in which some traps contained wicks charged with 10 μg of one pure attractant, and other traps contained wicks of both attractants. Each of the two compounds is very inhibitory to males of the species not attracted by it (Table 1). Data on the effect of other homologs and isomers, such as *cis*-11-tetradecenyl acetate, *trans*-11-tetradecenyl acetate, *trans*-7-tetradecenyl acetate, and *cis*-9-hexadecenyl acetate show that these compounds are not inhibitory or only weakly so. Strong inhibition of male response in both species by a mixture of compounds I and II suggests that each compound is a geometrical isomer of the natural sex pheromone of one species and conversely the pheromone itself for the other species.

From an evolutionary standpoint it appears important that not only does sex pheromone specificity exist in these closely related gelechiid species, but that females of one species are inhibitory to males of the other species. Although certain criteria must be met for compounds to be inhibitory to males of any one species, apparently varying degrees of inhibition can exist with different molecular modifications (*11*). This could be related to varying degrees of affinity that the compounds have for the protein receptor sites. Mutations altering the conformation or stereospecificity of an enzyme involved in the biosynthetic pathway of the pheromone compound could cause production of a compound

Table 1. Field tests for attraction and inhibition of gelechiid males.

Trap charge	Males trapped	
	B1	B2
Compound I	10*	0
Compound II	0	39
Compounds I + II	1*	0†

* Sign test indicates significant difference at the 5 percent probability level. † Significant difference at the 1 percent probability level.

with strong affinity but no intrinsic activity for the receptor sites. The female involved would become unattractive or repulsive to males of the parent species. A mutation in a nearby male, causing proper modifications of the receptor site stereospecificity, could result in a mating pair isolated by pheromone specificity. Although the probability of this sequence of events must be minute, it becomes plausible when one considers a large population of genotypes with the high turnover rates typical of insects. In this sympatric evolutionary saltation, the new species may never need to evolve any other reproductive isolation mechanism.

A different model in accordance with the classical allopatric and gradual evolution pattern would require the existence of a more complex emitter and receptor system to allow for a gradual transition from one attractant structure to the other. In this model inhibitory effects of related structures would also evolve gradually, so that incipient species producing two related compounds do not inhibit their own males from responding. Few reasons could be found for the evolution of inhibition in addition to pheromone specificity itself unless inhibition evolution follows pheromone evolution to assure the evolving species that its pheromone will never be modified back to one used by previous populations.

WENDELL L. ROELOFS
ANDRE COMEAU
Department of Entomology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456

References and Notes

1. P. Marler and W. J. Hamilton, *Mechanisms of Animal Behavior* (Wiley, New York, 1966), p. 302; H. B. D. Kettlewell, *Entomol. Rec.* **54**, 62 (1942); A. W. Ewing and A. Manning, *Annu. Rev. Entomol.* **12**, 471 (1967); H. H. Shorey, L. K. Gaston, J. S. Roberts, *Ann. Entomol. Soc. Amer.* **58**, 600 (1965).
2. E. Mayr, *Animal Species and Evolution* (Harvard Univ. Press, Cambridge, 1963), p. 19.
3. T. N. Freeman, *Can. Entomol.*, Suppl. 7, (1958), p. 26.
4. J. A. Powell, *Univ. Calif. Publ. Entomol.* **32**, 162 (1964).
5. J. F. Bronskill, W. L. Roelofs, P. J. Chap-

man, S. E. Lienk, *Int. Entomol. Soc. Abstr.*, in press.
6. W. L. Roelofs and K. C. Feng, *Ann. Entomol. Soc. Amer.* **61**, 312 (1968).
7. Modification of clock-traps used by B. Götz, [*Umsch. Wiss. Tec.* **45**, 779 (1941)].
8. Identifications made by Ronald Hodges, U. S. National Museum, Washington, D.C. Form B2 apparently is an undescribed species.
9. W. L. Roelofs and H. Arn, *Nature* **219**, 513 (1968).
10. The test compounds were forwarded to us by Drs. M. Jacobson and D. Warthen, USDA, Beltsville, Maryland. They contained less than 5 percent impurities. *cis*-9-Tetradecenyl acetate has been reported to be the sex pheromone of the fall armyworm, *Spodoptera frugiperda*, although attraction activity in the field has not been demonstrated [A. A. Sekul and A. N. Sparks, *J. Econ. Entomol.* **60**, 1270 (1967)].
11. W. L. Roelofs and A. Comeau, *Nature* **220**, 600 (1968).
12 Supported by PHS research grant CC-00287, from the National Communicable Disease Center, Atlanta, Ga. We thank Drs. P. J. Chapman and S. E. Lienk for assistance on this subject. We thank Drs. W. L. Brown, Jr. (Cornell Univ.) and T. H. Hamilton (Univ. of Texas) for their comments on the manuscript.

24 March 1969; revised 5 May 1969

Isopentenyladenosine Stimulates and Inhibits Mitosis of Human Lymphocytes Treated with Phytohemagglutinin

Abstract. *The plant cytokinin isopentenyladenosine, a component of yeast and mammalian transfer ribonucleic acid, is both a potent inhibitor and stimulator of DNA synthesis, transformation, and mitosis of the phytohemagglutinin-stimulated lymphocyte. The stage of the cell cycle and the concentrations used are critical for these effects. The addition of isopentenyladenosine within the first 12 hours after phytohemagglutinin at a concentration above 10^{-6} molar results in inhibition, while lower concentrations (between 10^{-7} and 10^{-6} molar), added at 24 hours or later, have a stimulatory effect.*

Studies of biochemical events leading to and possibly controlling mammalian cell mitosis have been conveniently studied with the phytohemagglutinin-stimulated lymphocyte system. Mature human lymphocytes cultured in vitro generally do not synthesize DNA or divide; however, addition of phytohemagglutinin (PHA) results in a morphologic transformation to a lymphoblastoid cell capable of DNA synthesis and leads to eventual mitosis (*1*). These events are preceded by an increase in RNA and protein synthesis (*2*).

We have used this in vitro system in the study of the possible regulatory influence of transfer RNA (tRNA) and some of its minor bases. One of the minor bases, N^6-(Δ^2-isopentenyl)adenosine, is of unusual interest for the following reasons. (i) It is a potent plant hormone (cytokinin), stimulating both cell division and cell differentiation in plant systems (*3*). (ii) As a component of yeast (*4*) and mammalian (*5*) serine tRNA and yeast tyrosine tRNA (*6*) it may be essential for protein synthesis. Evidence has already been presented indicating that isopentenyladenosine is necessary for binding of seryl-tRNA to the ribosomal messenger RNA complex (*7*). (iii) It has antitumor activity in animals (*8*) and in man (*9*). We report here the results of our experiments demonstrating that, depending on conditions, isopentenyladenosine either inhibits human lymphocyte transformation and mitosis or has stimulatory effects.

Peripheral blood was obtained from healthy volunteers, and the plasma containing the leukocytes was separated from erythrocytes by sedimentation at 37°C. A portion (2 ml) of this plasma was suspended in 4 ml of Eagle's minimum essential medium containing 50 units each of penicillin, streptomycin, and glutamine (4 m*M*) gently mixed and incubated in 10-ml sterile screw-cap vials at 37°C without agitation. The leukocyte suspension had been adjusted to a final concentration of approximately 1×10^6 cells per milliliter. Phytohemagglutinin M (Difco), 0.3 mg, was added at the start of the incubation. Measurements of DNA synthesis were determined from the incorporation of deoxythymidine-2-^{14}C (0.5 μc/ml) on duplicate samples, and the results were averaged.

The cells were harvested by placing the vials in ice and immediately centrifuging at 4°C; the cell pellet was washed three times with 5 to 8 ml of cold isotonic saline. The cells were then lysed by repeated freezing and thawing, and the proteins and nucleic acids were precipitated by the addition of cold 5 percent perchloric acid. After 20 minutes, the samples were washed three times with 5 percent perchloric acid, dried, solubilized with NCS (Nuclear-Chicago), and counted in a Packard liquid scintillation counter (90 percent efficiency) after being transferred to counting vials containing 10 ml of toluene phosphor. Results for DNA synthesis were for 6×10^6 cells. Protein synthesis was determined in a similar manner with ^{14}C-leucine (0.5 μc/ml) added at the start of incubation. Total protein was measured by the method of Lowry *et al.* (*10*). The RNA synthesis was determined from incorporation of ^{3}H-uridine (1 μc/ml) added at the start of culture. The percentage of lymphocytes transformed with PHA was determined by microscopic examination of a Giemsa-stained, air-dried slide 48 hours after start of the incubation with PHA. The mitotic index was also measured at 48 hours by counting the number of cells in mitosis per 1000 nucleated cells (*11*). Chromosome preparations of the cells were made according to the technique of Moorhead *et al.* (*12*).

In initial experiments lymphocytes were separated from granulocytes by the nylon-column technique (*13*). The results of these experiments did not significantly differ from results obtained with unfractionated leukocytes. Therefore, we used unfractionated leukocytes. The addition of isopentenyladenosine (8 μmole/liter) at the initiation of the culture inhibited lymphocyte DNA synthesis, transformation, and mitosis (Fig. 1). The inhibition was not accompanied by visible chromosomal aberrations or cellular morphological alterations as judged by either electron or light microscopy. The addition of isopentenyladenosine at increasing intervals after PHA resulted in a decrease in the inhibition of transformation and mitotic figures (Fig. 1). Thus, the effects of isopentenyladenosine are critically dependent upon time of addition. Once PHA-stimulation has developed, and the cells are triggered to enter into S phase, inhibition by isopentenyladenosine is reduced. The addition of isopentenyladenosine (0.8 μmole/liter) at the start of incubation had no effect on the PHA-stimulated lymphocyte. However, later addition (24 to 36 hours after PHA) produced a twofold stimulation of DNA synthesis (Fig. 1C). In the absence of the triggering effect of PHA, isopentenyladenosine alone did not stimulate lymphocyte DNA synthesis.

In Fig. 2 the results of experiments showing the effects of varying concentrations of isopentenyladenosine on DNA synthesis, transformation, and mitosis are presented. In all cases, isopentenyladenosine was added 24 hours after PHA. Although there was variation in the concentrations of isopentenyladenosine required for stimulation and inhibition with lymphocytes from different donors, the curves in Fig. 2 are typical. The concentration of PHA was critical for obtaining stimulation with