melts. Ice crystals of a much finer texture than the cells themselves indicate rapid intra- and extracellular freezing (*12*) in the tissue beneath. In these circumstances it seemed likely that melting points give a reliable indication of solute concentration in life. Warming the sections to about $-4°$ produced a slight but perceptible recession of the ice from the unfrozen surface zone (Fig. 1, a to f). Above $-4°C$ the ice front generally receded more rapidly with temperature, but with some irregularity. During this stage ice crystals became larger, rounded in outline, and more transparent (Fig. 1, g to i). Tissues deeper than about 20 μm from the surface melted simultaneously at temperatures close to the melting point of bulk hemolymph samples (0.22 to 0.29 osmole per kilogram).

A quantitative survey of the position of the ice front in relation to temperature (Fig. 2) showed a consistent pattern of progressive melting away from the surface, demonstrating considerable apical dehydration and solute concentration. A superficial zone exhibits a steep linear osmotic gradient from about 2.2 os/kg to an external concentration exceeding the limit of direct measurement: 5 os/kg. It is possible to calculate the osmotic concentration at the snail's surface from known atmospheric vapor pressures immediately before freezing. A value of 28 os/kg, equivalent to a vapor pressure of 9.9 mm-Hg (50 percent relative humidity at 22°C) was obtained by using a formula relating depression of vapor pressure to osmolality (*13*). A less steep osmotic gradient appears to extend across the bulk of the epithelial cells, ranging from close to hemolymph osmolality (0.3 os/kg) at the base to about 2.2 os/kg near the apical region. Gradients were absent in control sections of mantle tissue taken from areas which were covered with the shell in life, and hence not subjected to the dehydrating effects of the atmosphere.

Under the steady-state conditions existing when the mantle was first frozen, the permeability of an osmotic barrier is, by Fick's law, inversely proportional to the osmotic gradient across it. Results in Fig. 2 show the principal barrier to be located close to the epithelial cell's free edge, although the whole cell appears to impede water flow to some extent. Further confirmation that water penetrates the apical zone with some difficulty was provided by

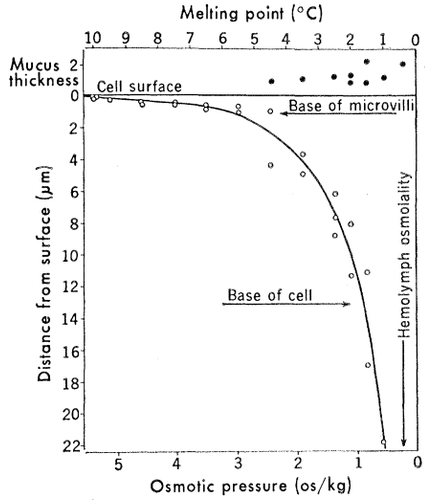


Fig. 2. Analysis of the location of melting points or osmotic pressures across the epithelium (open circles). The thickness of the unfrozen mucus layer (closed circles) at different stages of melting is also plotted. Each point is a mean of 15 measurements taken on a grid placed randomly on a photograph of the section. The points are based on data taken from several different animals.

experiments in which the intact mantle was exposed to a Ringer solution (0.3 os/kg) for varying lengths of time before freezing. It took 4 to 5 minutes to abolish dehydration, whereas most other cells reach osmotic equilibrium with external media much more quickly—of the order of a second in the case of mammalian erythrocytes (*14*). These observations, together with a steep osmotic gradient which appears to coincide with the apical microvilli, suggest that these structures might be the site of the waterproof barrier. However, microvilli are normally associated with increasing transport (*15*) and their role in retarding water movement requires further confirmation.

J. MACHIN

Department of Zoology,
University of Toronto,
Toronto, Ontario, M5S 1A1, Canada

References and Notes

1. A. P. M. Lockwood, *Animal Body Fluids and Their Regulation* (Heinemann, London, 1963), p. 26.
2. N. J. Lane, *Q. J. Microsc. Sci.* **104**, 495 (1963); G. Wondrak, *Protoplasma* **66**, 151 (1968); D. C. Lloyd, *ibid.* **68**, 327 (1969); A. S. M. Saleuddin, *Can. J. Zool.* **48**, 409 (1970); D. C. Rogers, *Z. Zellforsch. Mikrosk. Anat.* **114**, 106 (1971).
3. A. R. Mead, *The Giant African Snail: A Problem in Economic Malacology* (Univ. of Chicago Press, Chicago, 1961), p. 28; J. Machin, *J. Zool. Proc. Zool. Soc. Lond.* **152**, 55 (1967).
4. J. Machin, *Naturwissenschaften* **52**, 18 (1965).
5. ———, *J. Exp. Biol.* **45**, 269 (1966).
6. ———, *ibid.* **57**, 103 (1972).
7. Mantle permeability in the snail *Otala lactea* at 25°C was found to be 0.016 mg cm^{-2} hour^{-1} per mm-Hg vapor pressure difference (*6*). This value is almost identical to the permeability of cockroach nymph cuticle (0.017 mg cm^{-2} hour^{-1} mm-Hg^{-1}) at the same temperature calculated from data in J. W. L. Beament, *J. Exp. Biol.* **35**, 494 (1968).
8. J. Machin, *Biol. Bull. (Woods Hole)* **134**, 87 (1968).
9. Active land snails are extremely sensitive to any form of mechanical disturbance and respond by secreting mucus [R. J. Ross, thesis, University of Toronto (1967)]. Great care was therefore taken to avoid disturbing the snails before dropping them into the freezing mixture.
10. B. J. Luyet, *Cryobiology* **3**, 97 (1966).
11. ———, *Biodynamica* **10**, 227 (1969).
12. H. T. Meryman, in *Recent Research in Freezing and Drying*, A. S. Parkes, Ed. (Blackwell, Oxford, 1960), p. 23; *Fed. Proc.* **22**, 81 (1963).
13. J. Machin, *Am. J. Physiol.* **216**, 1562 (1969).
14. M. H. Jacobs, *Biol. Bull. (Woods Hole)* **62**, 178 (1932); D. C. Tosteson, in *The Cellular Functions of Membrane Transport*, J. F. Hoffman, Ed. (Prentice-Hall, Englewood Cliffs, N.J., 1964), p. 5.
15. J. M. Diamond and W. H. Bossert, *J. Gen. Physiol.* **50**, 2061 (1967); I. W. Richards, V. Licko, E. Bartoli, *J. Membr. Biol.* **11**, 293 (1973).
16. Supported by grants from the National Research Council of Canada.

3 October 1973; revised 12 November 1973 ■

Biological Responses of Atta texana to Its Alarm Pheromone and the Enantiomer of the Pheromone

Abstract. S-(+)-4-Methyl-3-heptanone *is the principal alarm pheromone of* Atta texana. *The dextrorotatory form of the ketone has also been identified from* Atta cephalotes. *Both enantiomers have been synthesized in high optical purity;* Atta texana *is more responsive to the (+) enantiomer than to the (−) form. These results implicate a chiral receptor system.*

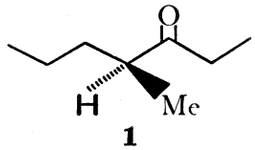
S-(+)-4-Methyl-3-heptanone (**1**) has been identified as the principal alarm pheromone of the leaf-cutting ant, *Atta texana*. Both enantiomers have been synthesized with rigorous precautions to ensure chemical and optical purity, and bioassayed for threshold activity. Identification of the pheromone without specification of chirality has previously been reported, and the bioassay has been described (*1*). We now report the (+) enantiomer to be more effective in eliciting response, at the threshold level, from all sizes of workers of *A.*

Table 1. Response values for the enantiomers of 4-methyl-3-heptanone (4-M-3-H) and standard 2-heptanone (2-H). A response value of 10^0 indicates that 1 cm³ of saturated vapor in air was the minimum concentration at which the ants were able to detect the tested compound. A value of 10^{-1} means the threshold response was observed from 1 cm³ of vapor at a concentration tenfold more dilute than the saturated vapor. The response value of 10^{+1} was assigned when 10 cm³ of saturated vapor was needed to obtain a threshold response. Response ratios of 4-methyl-3-heptanone to 2-heptanone were determined (ten ratios for each enantiomer), and a geometric average response ratio was calculated for each enantiomer.

(−)4-M-3-H	2-H	(+)4-M-3-H	2-H
10^{-2}	10^{-1}	10^{-3}	10^0
10^{-1}	10^{-1}	10^{-5}	10^0
10^{-2}	10^{-1}	10^{-4}	10^{-2}
10^{-1}	10^{-2}	10^{-3}	10^0
10^{-1}	10^{-1}	10^{-3}	10^{-1}
10^{-3}	10^0	10^{-3}	10^0
10^{-1}	10^0	10^{-2}	10^0
10^{-2}	10^{-1}	10^{-2}	10^{+1}
No response	10^0	10^{-3}	10^0
10^{-2}	10^0	10^{-3}	10^0

texana than the (−) enantiomer. The (−) enantiomer showed no inhibition of the activity of the (+) enantiomer.

Previous studies of chemical perception by insects involved structural isomers (skeletal or functional group isomers) [for example, (*1*) and (*2*)] or diastereomers (geometric isomers) (*3*). To our knowledge, this is the first report dealing with perception by insects of enantiomers, which have identical physical (including spectral) and chemical properties in an achiral medium. They can be distinguished only by interaction with a chiral physical agent, such as plane polarized light, or with a chiral chemical reagent.



1

Our results, therefore, implicate a chiral receptor system. The only difference between the (+) and (−) enantiomers of 4-methyl-3-heptanone lies in the configuration of four different groups tetrahedrally disposed about the chiral center; interchange of any two of these groups converts one enantiomer to the other without any change in physical and chemical properties in an achiral medium. It seems reasonable to postulate sites on the receptor of the ant that fit the spatial disposition of only one of the enantiomers. A minimum of three points within the site is required for discrimination (*4*).

Our results and conclusions are in accord with those discussed by Friedman and Miller (*5*) who showed that the human olfactory system can discriminate between rigorously purified (+) carvone and (−) carvone. Chiral structure of receptor sites thus appears to be widespread throughout the animal kingdom—at least in species as diverse as the ant and man.

S-(+)-4-Methyl-3-heptanone was synthesized as follows. Alkylation of diethyl methylmalonate with allyl chloride in sodium ethoxide and ethanol yielded diethyl methylallylmalonate. Saponification of the diester in an ethanol-water solution of potassium hydroxide followed by acidification yielded the diacid, which was decarboxylated at 160°C at 20 mm-Hg yielding (±) methylallylacetic acid (2-methyl-4-pentenoic acid). This acid was converted to its quinine salt, which was recrystallized five times, to a constant rotation, from acetone. Decomposition of the quinine salt in 2*N* HCl yielded *S*-(+)-methylallylacetic acid $[\alpha]_D^{25} = +10.5° \pm 0.2°$ (1.0, $CHCl_3$) (*6*), which was converted to *S*-(+)-2-methylpentanoic acid $[\alpha]_D^{27} = +15.5° \pm 0.3°$ (1.0, $CHCl_3$) by hydrogenation over platinum oxide in ethanol. Reaction of *S*-(+)-2-methylpentanoic acid with ethyl lithium in ether gave *S*-(+)-4-methyl-3-heptanone $[\alpha]_D^{27} = +21.0° \pm 0.4°$ (1.0, hexane).

R-(−)-4-Methyl-3-heptanone was synthesized by the following procedure: (±)-2-Methylpentanoic acid was converted to its quinine salt and recrystallized 13 times, to a constant rotation, from acetone and water. Decomposition of the quinine salt in 3*N* HCl yielded *R*-(−)-2-methylpentanoic acid $[\alpha]_D^{25} = -15.7° \pm 0.3°$ (1.0, $CHCl_3$) (*7*). The acid was reacted with ethyl lithium in ether to give *R*-(−)-4-methyl-3-heptanone $[\alpha]_D^{25} = -21.5° \pm 0.4°$ (1.0, hexane).

The absolute configurations of the enantiomers of 2-methylpentanoic acid have been established (*8*), thus establishing the absolute configurations of the ketones. Prior to bioassay and spectral analysis, each synthetic enantiomer of 4-methyl-3-heptanone was purified by gas liquid chromatography (*9*). Prior to spectral analysis, each optically active synthetic precursor acid (*10*) and samples of the naturally occurring ketones were also purified by gas liquid chromatography (*11*).

The pheromone isolated from *Atta*

Table 2. Response ratios relative to 2-heptanone for both enantiomers of 4-methyl-3-heptanone. Comparison of the two geometric average response ratios showed *S*-(+)-4-methyl-3-heptanone to be detectable on the average at a concentration about 100 times more dilute than the (−) enantiomer. Similarly, the geometric average response ratio for racemic 4-methyl-3-heptanone was calculated to be 3.98×10^{-3}; thus, the (−) enantiomer was not inhibiting the response to the (+) enantiomer.

(−)4-M-3-H	(+)4-M-3-H
2-H	2-H
10^{-1}	10^{-3}
10^0	10^{-5}
10^{-1}	10^{-2}
10^{+1}	10^{-3}
10^0	10^{-2}
10^{-3}	10^{-3}
10^{-1}	10^{-2}
10^{-1}	10^{-3}
	10^{-3}
10^{-2}	10^{-3}
Geometric average response ratios	
1.32×10^{-1}	1.26×10^{-3}

texana had $[\alpha]_D^{25} = +22.1° \pm 0.4°$ (1.0, hexane); that from *Atta cephalotes*, $[\alpha]_D^{27} = +22.0° \pm 0.4°$ (1.0, hexane) (*12*).

The bioassays were performed by one of us (J.C.M.) in his laboratory, well removed from the synthesis laboratory. Each sample was coded, kept in a separate refrigerator, and handled individually at different times. Only one enantiomer was bioassayed on any single day, and the bioassay apparatus was thoroughly purged with air after each run. Comparison of geometric average response ratios for each enantiomer showed *S*-(+)-4-methyl-3-heptanone to be about 100 times more active than the (−) enantiomer (*13*) (Tables 1 and 2). The possibility that the (−) enantiomer might inhibit the response of the (+) enantiomer was tested by bioassay, ten times, of a twofold concentration of racemic 4-methyl-3-heptanone and comparison of each bioassay with a corresponding bioassay of 2-heptanone. A comparison of these data with the data for the (+) enantiomer showed that the (−) enantiomer was not inhibiting the response to the (+) enantiomer.

No chemical impurity in either enantiomer could be detected by spectroscopy or gas chromatography. Whether the (−) enantiomer is slightly active or totally inactive, its presence to the extent of 2.5 percent in the more active (+) isomer would have only the

effect of a much less active component or an inert impurity on the total response of the more active (+) enantiomer. However, the presence of 1.3 percent of the (+) enantiomer in the (−) enantiomer makes it impossible to determine whether or not the (−) enantiomer has some activity of its own (*12*).

ROBERT G. RILEY
ROBERT M. SILVERSTEIN
State University of New York, College of Environmental Science and Forestry, Syracuse 13210
JOHN C. MOSER
Southern Forest Experimental Station, U.S. Department of Agriculture, Alexandria, Louisiana 71360

References and Notes

1. J. C. Moser, R. G. Brownlee, R. M. Silverstein, *J. Insect Physiol.* **14**, 529 (1968).
2. J. E. Amoore, G. Palmieri, E. Wanke, M. S. Blum, *Science* **165**, 1266 (1969).
3. W. L. Roelofs and A. Comeau, *J. Insect Physiol.* **17**, 435 (1971).
4. A. G. Ogston, *Nature (Lond.)* **162**, 936 (1948).
5. L. Friedman and J. G. Miller, *Science* **172**, 1044 (1972).
6. The synthesis of *S*-(+)-methylallylacetic acid was similar to that employed by S. Stallberg-Stenhagen [*Ark. Kemi Mineral. Geol.* **23A**, 1 (1946)].
7. Resolution similar to that employed by P. A. Levene and R. E. Marker [*J. Biol. Chem.* **98**, 1 (1932)].
8. S. Stallberg-Stenhagen and E. Stenhagen, *Ark. Kemi Mineral. Geol.* **24B**, 1 (1947).
9. Each synthetic enantiomer of the ketone was isolated in pure form from the reaction mixture on the following gas liquid chromatography (GLC) column: 4 percent TCEP [1,2,3 tris(2-cyanoethoxy)propane] on Chromosorb G, 60/80 mesh, 7.3 m by 3 mm (outside diameter); He flow rate, 20 cm³/min; column temperature, 88°C. Purity was further checked on a Carbowax 20M capillary column, 30.5 m by 0.5 mm, with a He flow rate of 3 cm³/min; the column temperature was 80°C.
10. The optically active synthetic precursor acids were purified from the reaction mixture on the following GLC column: FFAP (free fatty acid phase), 14 percent, was placed on Chromosorb W, 80/100 mesh; the column was 1 m by 3 mm (outside diameter); the He flow rate was 30 cm³/min; the column temperature was 108°C.
11. The natural ketone from *A. texana* and *A. cephalotes* was isolated and the purity was confirmed by GLC on the following columns. (i) Five percent high efficiency DEGS (diethylene glycol succinate) on Chromosorb G, 60/80 mesh, 3 m by 6.3 mm; He flow rate, 30 cm³/min; column, 95°C. (ii) Four percent Carbowax 20M on Chromosorb G, 60/80 mesh, 7.3 m by 3 mm; He flow rate, 30 cm³/min; column, 95°C. (iii) Four percent TCEP on Chromosorb G, 60/80 mesh, 7.3 m by 3 mm; He flow rate, 20 cm³/min; column, 90°C. (iv) Carbowax 20M capillary column, 30.5 m by 0.5 mm; He flow rate, 3 cm³/min; column, 80°C. (v) DEGS capillary column, 30.5 m by 0.5 mm; He flow rate, 3 cm³/min; column, 70°C. (vi) Apiezon L capillary column, 15.2 m by 0.5 mm; He flow rate, 3 cm³/min; column, 87°C.
12. Details will be forthcoming on the isolation and identification of *S*-(+)-4-methyl-3-heptanone from *A. texana* and *A. cephalotes* (R. G. Riley, *Tetrahedron*, in press). If we accept the optical rotation of the enantiomer produced by two species of ants as representing 100 percent optical purity, the optical purity of the synthetic enantiomers can be calculated to be 95.0 (± 1.9) percent [97.5 parts (+), 2.5 parts (−)] for the (+) enantiomer and 97.3 (± 1.9) percent [98.7 parts (−), 1.3 parts (+)] for the (−) enantiomer. The limit of error on the measurement of optical rotation was determined by comparing the optical rotations of three individually prepared standards of a 0.05 percent aqueous solution of (+)-10-camphorsulfonic acid at 292 nm. Each standard was within 2 percent of the known value of rotation; thus all measurements of optical rotation are within 2 percent of their measured or calculated values. A temperature difference of 2°C has a negligible effect on optical rotation. C. Djerassi, *Optical Rotatory Disperson* (McGraw-Hill, New York, 1960), p. 27; M. K. Hargreaves, *J. Chem. Soc.* (1953), p. 2953.
13. One milliliter of head space vapor of each sample, whose concentration had been determined (*1*), was drawn into a 10-ml syringe and serially diluted in the syringe in tenfold increments until the threshold concentration of response for each sample was achieved. Each sample was injected into a slow air stream leading to the test chamber. The minimum concentration at which 50 percent of the ants (workers of all sizes) present in the chamber at any given time responded by characteristic antennae raising was designated as the threshold response. Each enantiomer was bioassayed ten times, and each time its response value was compared with that of a sample of 2-heptanone, another less active component of the mandibular gland (*1*) (Tables 1 and 2).
14. We thank Dr. J. E. Amoore for pointing out the need to interpret the biological response data in terms of geometrical averages. Supported by the Environmental Protection Agency and the Rockefeller Foundation.

20 August 1973; revised 3 December 1973

Temporal Pattern Shifts to Avoid Acoustic Interference in Singing Birds

Abstract. *Two species of forest birds, the least flycatcher and the red-eyed vireo, when breeding in the same season in the same habitat, adjust their temporal pattern of singing to avoid the overlapping of songs. The avoidance of acoustic interference is more marked in the flycatcher, which has a briefer song than the vireo.*

The acoustic signals of different species communicating in the same habitat may suffer mutual interference and masking (*1*, *2*). We report here evidence that bird species adjust the temporal patterning of their singing with respect to the singing of another species in such a way as to avoid temporal overlap between the two kinds of songs.

In North America the red-eyed vireo (*Vireo olivaceus*) and the least flycatcher (*Empidonax minimus*) breed in similar habitats at similar times. Neither species is brightly colored, and each communicates extensively with acoustic signals, particularly short advertising songs given repeatedly throughout the day while foraging. There is considerable overlap of frequency (pitch) in the songs of the two species. Five vireo-flycatcher pairs were

Table 1. Timing of least flycatcher songs relative to singing and silent periods of a red-eyed vireo in the same area.

Flycatcher number	Total songs (f)	Number of songs begun during vireo song		Number of songs begun during vireo silence		χ^2	Probability of chance difference
		Predicted (F_v)	Actual (f_v)	Predicted ($F_{\bar{v}}$)	Actual ($f_{\bar{v}}$)		
1	275	66.7	23	208.3	252	37.796	$<.001$*
2	519	130.1	23	388.9	496	117.729	$<.001$*
3	226	49.0	18	177.0	208	25.035	$<.001$*
4	43	11.3	1	31.7	42	12.690	$<.001$*
5	146	35.5	11	110.5	135	22.336	$<.001$*

* Highly significant ($\alpha = .001$, where α represents the level of significance chosen by the experimenter).

Table 2. Timing of red-eyed vireo songs relative to singing and silent periods of a least flycatcher in the same area.

Vireo number	Total songs (v)	Number of songs begun during flycatcher song		Number of songs begun during flycatcher silence		χ^2	Probability of chance difference
		Predicted (V_f)	Actual (v_f)	Predicted ($V_{\bar{f}}$)	Actual ($v_{\bar{f}}$)		
1	195	26.4	22	168.6	173	0.854	$>.3$
2	512	56.9	36	455.1	476	8.617	$<.01$*
3	173	19.0	10	154.0	163	4.753	$<.05$*
4	59	4.6	6	54.4	53	0.483	$>.3$
5	135	14.6	3	120.4	132	10.283	$<.01$*

* Significant ($\alpha = .05$, where α represents the level of significance chosen by the experimenter).