Reexamination of Tetradecenyl Acetates in Oak Leaf Roller Sex Pheromone and in Plants

Abstract. *The chemistry of the oak leaf roller sex pheromone is shown by means of microozonolysis and computerized gas chromatography–mass spectrometry to be dominated by an approximate 70 to 30 ratio of* E- *and* Z-*11-tetradecenyl acetates. Tetradecenyl acetates are undetectable in highly purified oak leaf, apple leaf, and corn extracts analyzed by gas chromatography–mass spectrometry. These results reflect negatively on previous reports and on the hypothesis that plant components might govern insect chemical communication systems.*

Recent investigations (*1–3*) in this laboratory into the possible origins of insect chemical communicants have generated considerable interest and controversy (*4*). Various behavioral associations and interactions between insects and their diet or environment were reported, and compounds previously identified as components of insect chemical communication systems were reported isolated and identified from host food plants by combined gas chromatography and mass spectrometry (GC-MS). To explain these findings, it was hypothesized (*1, 3*) that chemicals in food plants might govern in part the chemical communication signals of the herbivore. Such a hypothesis has far-reaching implications with regard to evolutionary biology, pest management, and several other fields. In further studies of the sex pheromone of the oak leaf roller (OLR) (*Archips semiferanus* Walker) we have obtained results that reflect negatively on this hypothesis. Previously, a series of isomeric tetradecenyl acetates (*5*) with varying degrees of biological activity was reported in the sexual attractant fraction of the adult OLR female (*6*); the most attractive component in field tests was reported as *Z*-10-tetradecenyl acetate (*7*). Tetradecenyl acetates were also reported (*1, 3*) in oak leaves, as well as in corn and apple foliage (*8*). Anomalies were reported in the pheromone content and in the cross-attraction of field- and laboratory-reared OLR's (*1*); furthermore, determinations of the biological activity of synthetic tetradecenyl acetate isomers as measured by the electroantennogram technique and in field traps were inconsistent (*9*). Taxonomically identical OLR males were reported to have been attracted in the field by different tetradecenyl acetate isomers on different days of the OLR flight period.

Miller *et al.* (*10*) recently found that the OLR sex pheromone was consistently a mixture (67 : 33) of *E*- and *Z*-11-tetradecenyl acetates, regardless of diet. Furthermore, no other isomers were chemically detected and no OLR males were trapped by other synthetic isomers in the field. These workers focused on the nature of the OLR sex pheromone rather than the actual presence and significance of pheromones in plants (*10*) but their results reflected negatively on the hypothesis that diet might be directly related to insect sex pheromones.

For the present studies we used high-efficiency gas chromatography (GC), microozonolysis, and computerized chemical ionization (CI) and electron impact (EI) GC-MS techniques (*11*). Crude extracts of the abdominal tips of virgin females were obtained from OLR's collected in the field during 1972, 1973, and 1974, and from OLR's reared in the laboratory on a wheat germ diet during 1972. Each sample analyzed was equivalent to extracts obtained from 200 females.

The extracts were purified by means of sequential thin-layer chromatography (TLC) on silica gel, and GC on nonpolar 3 percent OV-1 and polar 10 percent DEGS (*12*). All OLR female extracts as well as standard tetradecenyl acetates and solvent blanks were subjected to this isolation procedure. The purifications were monitored by GC and GC-MS analysis on OV-1, DEGS, and polar 10 percent XF-1150 (*13*). After purification by TLC or on OV-1, the extracts were analyzed on XF-1150 and DEGS and they showed a characteristic triplet of peaks in the 14-carbon acetate region at precisely the retention times of standard tetradecyl acetate and *E*- and *Z*-11-tetradecenyl acetates. The last two peaks generally appeared in an approximate 70 to 30 ratio. This GC pattern was well defined and consistent for all field-collected samples. However, the GC trace was very weak for the sample from the laboratory-reared females, hence the detailed 14-carbon acetate pattern and retention times were approximate for this case.

The first of these three characteristic peaks was identified as tetradecyl acetate by retention time (spectrum number) and total spectra methane CI-GC-MS; *m/e* (mass to charge) 257 $(M + 1)^+$, 37 percent; 285 $(M + 29)^+$, 15 percent; 297 $(M + 41)^+$, 10 percent; 197 $(M + 1 - CH_3COOH)^+$, 69 percent; and 61 $(CH_3COOH_2)^+$, 37 percent. The two peaks occurring at the unique retention times of *E*- and *Z*-11-tetradecenyl acetates were, in fact, shown to be unsaturated 14-carbon acetate isomers. As shown in Fig. 1A, the characteristic CI ions were *m/e* 255 $(M + 1)^+$, 7 percent; 283 $(M + 29)^+$, 3 percent; 295 $(M + 41)^+$, 2 percent; 195 $(M + 1 - CH_3COOH)^+$, 17 percent; and 61 $(CH_3COOH_2)^+$, 25 percent.

Extracts purified on DEGS showed on-

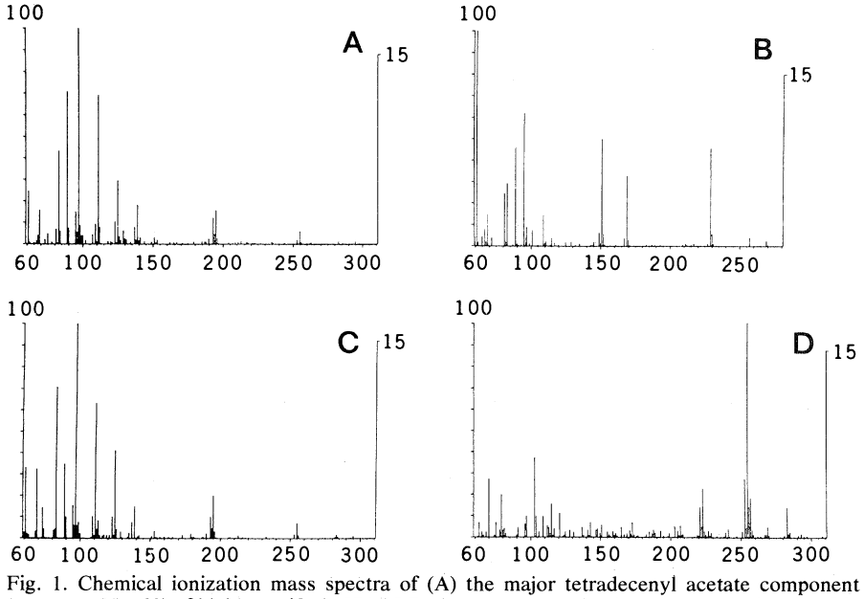

Fig. 1. Chemical ionization mass spectra of (A) the major tetradecenyl acetate component (spectrum No. 82) of highly purified 1974 OLR pheromone extract chromatographed on DEGS; (B) the major component (identified as acetoxyundecanal; spectrum No. 223) of OLR ozonolysis products temperature programmed at 100° to 170°C at a rate of 6°C per minute on OV-1; (C) *Z*-11-tetradecenyl acetate (spectrum No. 79) chromatographed on DEGS; and (D) the single component (spectrum No. 70) eluting within the tetradecenyl acetate retention region from highly purified oak leaf extract chromatographed on DEGS.

ly two peaks, and these had the same retention times as *E*- and *Z*-11-tetradecenyl acetates when analyzed on both OV-1 and DEGS (*14*). Analogous procedures on standard 14-carbon acetates verified that the entire tetradecenyl acetate region had been collected from the DEGS column. Again, the exception was the sample from the laboratory-reared females. Levels of 14-carbon acetates in this purified extract were below facile detectability, presumably because of the overall dearth of material in this sample. For this reason it is impossible to state whether tetradecenyl acetates were present or absent in extracts from the 1972 laboratory-reared females (*15*).

The OLR tetradecenyl acetate components highly purified by TLC, OV-1, and DEGS collection were subjected to microozonolysis in methylene chloride solution (*16*) according to a technique similar to that of Beroza and Bierl (*17*). In addition, identical reactions were conducted on blanks that had been subjected to the preparative procedure (*18*) as well as microgram and 50-ng amounts of available standard tetradecenyl acetates (*19*) with double bonds in positions 2, 3, 4, 5, 8, 9, 10, and 11. Ozonolysis products were analyzed by GC on OV-1 (*20*). Little could be interpreted from the extremely weak chromatogram of the sample from laboratory-reared females. However, gas chromatograms of microozonolysis products from purified extracts of females collected in the field in 1972, 1973, and 1974 were all dominated by a prominent peak eluting at the standard acetoxyundecanal (11-hydroxyundecanal acetate) retention time. Other than recognized trace impurities found in the blanks and a small peak at approximately the retention time of tetradecanol, there was no evidence of other ozonolysis products in amounts greater than a few percent of the major 11-carbon acetoxyaldehyde peak.

Microozonolysis products were also analyzed by total spectra methane CI-GC-MS on OV-1. Mass spectral patterns of standard acetoxyaldehydes evidenced the usual methane addition products $(M + H)^+$, $(M + C_2H_5)^+$, $(M + C_3H_5)^+$, and loss of the two functional groups $(M + 1 - CH_3COOH)^+$, $(M + 1 - CH_3COOH - H_2O)^+$. Analysis of the extract ozonolysis products verified that the dominant peak in the chromatogram corresponded in retention time and mass spectrum (Fig. 1B) to the 11-carbon acetoxyaldehyde: *m/e* 229 $(M + 1)^+$, 45 percent; 257 $(M + 29)^+$, 5 percent; 269 $(M + 41)^+$, 4 percent; 169 $(M + 1 - 60)^+$, 32 percent; 151 $(M + 1 - 78)^+$, 50 percent; 61 $(CH_3COOH_2)^+$, 100 percent. Furthermore, reconstructed mass chromatograms (*21*) were examined for pseudomolecular ions of acetoxyaldehyde and aldehyde ozonolysis products of all tetradecenyl acetate isomers. As computed by $(M + 1)^+$ chromatogram peak areas, the other products present were at most on the order of 1.0 percent of the dominant acetoxyundecanal. In general, ozonolysis products indicating other tetradecenyl acetates could not be confirmed by total CI spectra. For example, ozonized 1973 field OLR pheromone (Fig. 2A) demonstrates the presence of the 11-carbon acetoxyaldehyde, *m/e* 229 $(M + 1)^+$ at spectrum number 66, and the apparent absence of the 10-carbon acetoxyaldehyde, *m/e* 215 $(M + 1)^+$ which should occur at spectrum number 42.

Retention times and total CI mass spectra of highly purified female extract before and after microozonolysis clearly indicate that the chemical composition of the OLR sex pheromone is overwhelmingly dominated by an approximate 70 to 30 mixture of *E*- and *Z*-11-tetradecenyl acetates. These chemical results substantiate the data presented by Miller *et al.* (*10*).

An examination of the presence of tetradecenyl acetates in oak leaves, apple leaves, and corn was initiated because of the above results and because the 14-car-

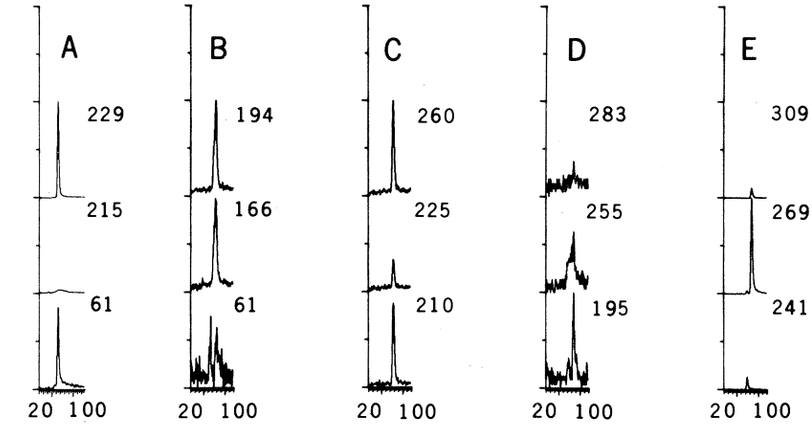

Fig. 2. Reconstructed mass chromatograms. (A) Ozonolysis products for highly purified 1973 OLR pheromone analyzed on OV-1 at 140°C, isothermal: *m/e* 229 $(M + 1)^+$ for acetoxyundecanal, *m/e* 215 $(M + 1)^+$ for acetoxydecanal, and *m/e* 61 (CH_3COOH_2). (B to E) Reconstructed mass chromatograms of highly purified oak leaf extract. (B) EI of *m/e* 194, 166, 61, chromatographed on DEGS; (C) EI of *m/e* 250, 225, 210, chromatographed on DEGS; (D) CI of *m/e* 283, 255, 195, chromatographed on OV-1; and (E) Cl of *m/e* 309, 269, 241, chromatographed on OV-1.

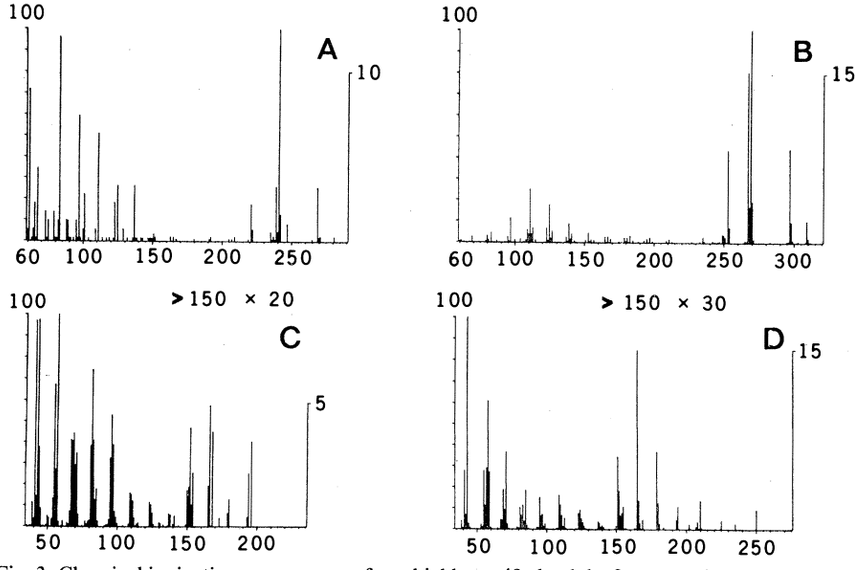

Fig. 3. Chemical ionization mass spectra from highly purified oak leaf extract chromatographed on OV-1: (A) *m/e* 241 component at spectrum No. 75; (B) *m/e* 269 component at spectrum No. 85; (C and D) Electron impact mass spectra from highly purified oak leaf extract chromatographed on OV-1: (C) *m/e* 241 component at spectrum No. 91; (D) *m/e* 269 component at spectrum No. 107.

bon acetate content of plant extracts was not as unequivocal as previously reported (*1*). Extracts were purified by chromatography and analyzed by GC-MS (both EI and CI) on a variety of GC columns.

The purification procedure described for OLR pheromone tetradecenyl acetates (TLC, OV-1, and DEGS) was applied to methylene chloride extracts of corn, apple leaves, and oak leaves. When these purified extracts were analyzed on DEGS a single peak occurred in the proper retention region for tetradecenyl acetates. However, the CI and EI mass spectra of this peak were entirely different from those of standard tetradecenyl acetates. Examination by CI on OV-1 revealed two components: (i) *m/e* 241, apparent $(M + 1)^+$ (Fig. 3A) eluting virtually at the tetradecenyl acetate retention time, and (ii) *m/e* 269, apparent $(M + 1)^+$ (Fig. 3B) eluting slightly after the tetradecyl acetate retention time.

Examination of these same purified extracts by total spectra EI-GC-MS on OV-1 and DEGS showed that this ionization mode does not readily reveal the true molecular weights of these components. Electron impact mass spectra, as demonstrated in Fig. 3, C and D, are far more equivocal than the CI spectra of the same components shown in Fig. 3, A and B (*22*). Indeed, these two components which clearly are not tetradecenyl acetates by CI spectra, evidence EI peaks at the same diagnostic ions used for the tetradecenyl acetates: *m/e* 194, 166, and 61. These ions occur in ratios vaguely similar to those of tetradecenyl acetates and could be mistaken for tetradecenyl acetate ions by EI analysis (*23*), especially if one were relying heavily on mass fragmentography. For example, reconstructed mass chromatograms clearly indicate that EI analysis of *m/e* 194, 166, 61 (Fig. 2B) could give a totally different impression than analysis of *m/e* 250, 225, 210 (Fig. 2C) for the same purified oak leaf extract. The last three ions are absent in EI spectra of all standard tetradecenyl acetates. Examination of a limited number of CI ions instead of total spectra could also lead to faulty interpretation. Reconstructed (CI) mass chromatograms of *m/e* 283, 255, and 195 (Fig. 2D) appear to indicate the presence of characteristic tetradecenyl acetate ions in oak foliage. However, reconstructed mass chromatograms (Fig. 2E) of the actual significant CI ions (*m/e* 309, 269, and 241) in the same sample must be interpreted entirely differently.

Apparent temporal, spatial, or species variations in the inferred tetradecenyl acetates in plants could have been due to the effect of collecting different quantities of the $(M + 1)^+$ 269 component from the OV-1. The retention time of this compound on OV-1 is at the borderline of our collected 14-carbon acetate region, and this component gives a significant *m/e* 194 in EI spectra. These findings, which fail to support the presence of tetradecenyl acetates in plants, have been confirmed in triplicate for extracts of corn, apple, white oak, and black oak from foliage collections at various times over the growing season and various locations in Pennsylvania. Because of interference from these other components (*24*), it was impossible to achieve sound quantitation of the presence or absence of tetradecenyl acetates in plants (*25*). However, if they are present, their amounts are considerably less than previously interpreted.

To address more accurately the question of quantitation, another isolation scheme was devised to separate the leaf compounds from any possible tetradecenyl acetates. In this case the purification sequence was TLC, OV-1, and 10 percent QF-1 (*26*), followed by total spectra CI-GC-MS on DEGS. Separate extracts of white and black oak foliage collected on several dates, along with standards and blanks, were subjected to such isolation and analysis.

All extracts purified by this last scheme evidenced the same chromatographic pattern consisting of saturated tetradecyl acetate (at spectrum No. 59), new compounds outside the tetradecenyl acetate DEGS retention region (at spectrum Nos. 50, 63, and 108), and a tiny peak eluting within this region (at spectrum No. 70). The mass spectrum of the latter chromatographic peak (Fig. 1D) contained an intense pseudo-molecular ion of *m/e* 255, 100 percent, and a major fragment *m/e* 223, 23 percent (*27*). Although having the same molecular weight, this component is clearly not a tetradecenyl acetate. Figure 1C shows a representative tetradecenyl acetate CI spectrum with no *m/e* 223. In addition, all tetradecenyl acetates contain prominent peaks at *m/e* 195 $(M + 1 - CH_3COOH)^+$ and *m/e* 61 $(CH_3COOH_2)^+$; as well as a strong hydrocarbon splitting pattern. These are not present in the oak leaf component. Based on quantitative analysis of standards taken through the purification procedure, if any tetradecenyl acetates were present in these oak leaf extracts they would be present on the order of one to ten parts per trillion or less (*28*).

The hypothesis regarding the possible direct effect of dietary compounds on insect chemical communication systems is at least a logical possibility worthy of consideration. The sequestering proposal seems to be consistent with studies on allomone (*29*) and kairomone (*2*) communication systems. However, our present results indicate that the earlier data and derived hypotheses should be reconsidered. The OLR sex pheromone has been identified as *E*- and *Z*-11-tetradecenyl acetates occurring in a specific ratio. Tetradecenyl acetates have not, in fact, been found in oak leaf, apple leaf, or corn extracts. Thus, we do not deem it appropriate to advance a hypothesis regarding a direct association between plant chemistry and insect sex pheromones. Furthermore, we retract previous reports and interpretations of data suggesting such an association.

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References and Notes

1. L. B. Hendry, J. K. Wichmann, D. M. Hindenlang, R. O. Mumma, M. E. Anderson, *Science* **188**, 59 (1975).
2. L. B. Hendry, J. K. Wichmann, D. M. Hindenlang, K. M. Weaver, S. H. Korzeniowski, *J. Chem. Ecol.*, in press.
3. L. B. Hendry, J. G. Kostelc, D. M. Hindenlang, J. K. Wichmann, C. J. Fix, S. H. Korzeniowski, *Recent Adv. Phytochem.* **10**, 1 (1975).
4. P. C. Murray, *Chem. Eng. News* **52** (47), 17 (1974); *ibid.* **53** (51), 22 (1975); K. Frazier, *Sci. News* **106** (32), 343 (1974); J. A. Behnke, *BioScience* **25** (1), 60 (1975); J. R. McLaughlin and L. L. Sower, *ibid.*, (6) p. 353.
5. L. B. Hendry, M. E. Anderson, J. Jugovich, R. O. Mumma, D. Robacker, Z. Kosarych, *Science* **187**, 355 (1975); L. B. Hendry and D. M. Hindenlang, *Finnigan Spectra* **5** (1), 1 (1975); L. B. Hendry, S. H. Korzeniowski, D. M. Hindenlang, Z. Kosarych, R. O. Mumma, J. Jugovich, *J. Chem. Ecol.* **1** (3), 317 (1975).
6. J. O. Nichols and J. W. Quimby, *Pa. For. Pest Rep.* **49**, 1 (1972); L. B. Hendry, L. Roman, R. O. Mumma, *Environ. Entomol.* **2**, 1024 (1973).
7. L. B. Hendry, J. Jugovich, L. Roman, M. E. Anderson, R. O. Mumma, *Experientia* **30**, 886 (1974); L. B. Hendry, L. Capello, R. O. Mumma, *Melsheimer Entomol. Ser.* **16**, 1 (1974).
8. Corn and apple foliage are food sources of related Tortricidae also reported to use tetradecenyl acetates as sex pheromones.
9. L. B. Hendry, J. Jugovich, R. O. Mumma, D. Robacker, K. Weaver, M. E. Anderson, *Experientia* **31**, 629 (1975).
10. J. R. Miller, T. C. Baker, R. T. Cardé, W. L. Roelofs, *Science* **192**, 140 (1976).
11. The GC-MS was conducted on a Finnigan 3200/3300 dual-stack EI/CI quadrupole mass spectrometer with a model 6000 interactive digital computer data system. Chromatographic columns and conditions were as described for GC analyses, He carrier gas being used for EI and methane carrier-reagent gas for CI analyses.
12. Thin-layer chromatography was conducted on 20 by 20 cm glass plates prepared with Merck silica gel H. The developing solvent was methylene chloride and hexane (2.5 : 1). Preparative gas chromatography was conducted on a Finnigan 9500 series gas chromatograph equipped with a thermal gradient collection apparatus (DEGS, diethylene glycol succinate). Carrier gas (He) flow was 30 ml per minute at 155°C isothermal oven temperature. Retention times were calibrated with standard hydrocarbons on all preparative GC columns.
13. Analytical GC was also conducted on a Finnigan 9500. The carrier gas was He for OV-1 (30 ml/min at 155°C) and DEGS (30 ml/min at 130°C) and N_2 for XF-1150 (30 ml/min at 165°C).
14. Small peaks on the order of 10 percent of the major component were present at tetradecanol and tetradecyl acetate retention times in the highly purified OLR extracts used for microozonolysis.

15. It is presumed that volatile components of the extracts of the 1972 laboratory-reared females were lost because of evaporation during storage. These are the same samples that we analyzed previously and reported in (*1*).
16. The ozonide was decomposed with a small excess of triphenylphosphine in methylene chloride solution, in order to avoid chromatographic interference from trace contaminants in this reagent. The reaction mixture was maintained at 0°C until analysis, then methylene chloride was carefully evaporated (not to dryness) under N_2, reconstituted in hexane, and concentrated to approximately 10 μl.
17. M. Beroza and B. A. Bierl, *Anal. Chem.* **39**, 1131 (1967); *Mikrochim. Acta* **4**, 720 (1969).
18. Ozonolysis blanks were clean except for trace impurities eluting from OV-1 well before the region of interest.
19. Microozonolyses at levels below 50 ng per component yield product mixtures in which background and artifact chromatographic peaks become significant. Analysis of a natural extract for minor components would be uninformative at this level.
20. The carrier gas for GC analysis of ozonolysis products was He (30 ml/min) for OV-1 programmed from 100° to 170°C at a rate of 6°C per minute, or at a constant temperature of 140°C. GC-MS analyses of ozonolysis products were conducted with methane (30 ml/min) as the carrier-reagent, as above. The temperature programming technique allowed practical detection of aldehydes of chain length eight or greater and acetoxyaldehydes of chain length six or greater. Thus, one ozonolysis fragment would be detected from any monounsaturated 14-carbon acetate isomer.
21. Reconstructed mass chromatograms for any *m/e* can be recalled by the data system from stored total mass spectra. These can be plotted in the same format as mass fragmentograms. However, mass fragmentography analyzes and stores only a few ions, disregarding the remainder of the mass range. For a more complete description of mass fragmentography, see (*1*) and (*6*).
22. The retention times (spectrum numbers) for the *m/e* 241 and 269 leaf extract components, as well as standard tetradecenyl acetates, are different for CI and EI mass spectra in Fig. 3 because of differences in chromatographic conditions on these instruments.
23. The only GC-MS ionization mode available in this laboratory during the original identification of pheromones in plants (*1*) was EI.
24. In CI spectra the *m/e* 241 and 269 leaf components give certain fragment ions similar to diagnostic tetradecenyl acetate ions. For example, the 269 component has a minor *m/e* 61, a strong *m/e* 195 fragment, and a strong signal at *m/e* 253 which produces a trace isotope pattern extending to *m/e* 255.
25. Saturated tetradecyl acetate does appear to be a true leaf extract component when analyzed before the DEGS collection which is timed to remove most of it.
26. The carrier gas used for collection from QF-1 was He (30 ml/min at 160°C, isothermal).
27. The mass spectrum of this leaf extract component shows a prominent $(M + 1 - 32)^+$ characteristic of fatty acid methyl esters, and would be consistent with a methyl pentadecenoate ($C_{14}H_{27}COOCH_3$). Other plant extract components may be due to a homologous series of fatty esters.
28. No leaf tetradecenyl acetates were detected by GC-MS analysis of 50 g of plant material. One nanogram was an approximate lower sensitivity limit for adequate full CI mass spectra including $(M + 1)^+$, $(M + 29)^+$, and $(M + 41)^+$. However, trace amounts below 100 pg of tetradecenyl acetates would be indicated by reconstructed mass chromatograms of prominent fragments such as *m/e* 195 and 61.
29. T. Eisner, J. S. Johnessee, J. Carrel, L. B. Hendry, J. Meinwald, *Science* **184**, 996 (1974); L. P. Brower, *Sci. Am.* **220** (12), 22 (1969); T. Reichstein, J. Von Euw, J. A. Parsons, M. Rothschild, *Science* **161**, 861 (1968); L. P. Brower, P. B. McEvoy, K. L. Williamson, M. A. Flannery, *ibid.* **177**, 426 (1972); G. M. Happ and J. Meinwald, *J. Am. Chem. Soc.* **87**, 2507 (1965).
30. For helpful discussions we thank R. D. Minard, M. Shamma, and other members of the Department of Chemistry and R. O. Mumma of the Department of Entomology and Pesticide Research Laboratory, Pennsylvania State University. We also thank K. W. Dillan for assistance in the purification of leaf extracts.
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21 May 1976; 24 August 1976

The Stomach as a Site for Rapid Nutrient Reinforcement Sensors

Abstract. *Rats with inflated cuffs placed around the pyloric sphincter were given a choice between two nonnutritive solutions. Ingestion of one solution was paired with nutritive intragastric injections, and ingestion of the other was paired with saline injections. The preference of rats for the nutrient-paired flavors indicates that the stomach alone can rapidly detect the arrival of nutritive substances.*

When predigested milk is injected into the stomachs of rats that are choosing between two samples of flavored water, the rats will choose the flavor paired with the nutrient (*1*). It is unlikely that this result is due to stomach distention, because the effect occurs when an intragastric saline load of equal volume is injected into the stomach when the other sample is chosen. Nor is the rewarding effect due to absorption. Each daily choice session lasts only 10 minutes, and most rats sample both flavors in each session (Table 1). Since the effect does not occur when glucose is substituted for the milk, the rats probably do not regurgitate and thus taste the flavor. Although the predigested milk is injected directly into the stomach, the site of action could actually be in the duodenum, as a considerable volume immediately enters the duodenum at this time (*2*). We have attempted to determine whether nutrient can be detected by the stomach or whether nutrient is detected by the duodenum alone; we now show, for what we believe to be the first time, that such detection does occur in the stomach. In order to distinguish between these alternatives, an inflatable cuff was implanted and placed around the pyloric sphincter of rats. The cuff was inflated so as to close off completely the entrance to the duodenum while rats were allowed for 10 minutes to choose between two flavored nonnutritive solutions. When the rat chose and drank one solution, an equal volume of predigested milk was injected into the stomach. When the rat drank the other flavored solution, an equal volume of saline was also injected into the stomach.

Table 1. Mean intake [together with standard error of the mean (S.E.M.)] of flavor paired with predigested milk injection and of flavor paired with saline injection, and the number of rats (out of eight) sampling both flavors.

Day	Intake				Rats (No.)
	Nutrient-paired flavor (ml)		Saline-paired flavor (ml)		
	Mean	S.E.M.	Mean	S.E.M.	
1	4.29	0.95	2.31	0.98	7
2	4.15	1.01	2.22	0.81	8
3	5.40	0.65	1.62	0.57	8
4	5.97	0.52	0.95	0.37	8
5	6.32	0.35	0.67	0.27	6

The subjects were 15 naive male rats (Sprague-Dawley) weighing between 350 and 450 g at the time of surgery. Ten rats served as experimental animals and five as donors. In the experimental rats, two stomach tubes were implanted [according to the method of Deutsch and Hardy (*3*)] and an inflatable cuff. In the donors, one stomach tube was implanted. Animals were allowed to recover for 1 week after surgery. During this period, milk (Carnation evaporated) and water were freely available.

After recovery, donor rats were put on a 22-hour food and water deprivation schedule. They were allowed access to milk for 2 hours every day.

At the same time, experimental rats were also put on a food and water deprivation schedule. Every morning two burettes were filled with water and their drinking spouts were inserted into each cage for 10 minutes. One hour later, subjects were given 8 g solid food. After daily watering and feeding, each rat's intragastric fistulae were cleared with 1 ml water. At this time the cuff was temporarily inflated in order to accustom animals to the experimental procedure. No apparent physical discomfort was exhibited by the animals. After rats had shown a stable water intake for at least 3 days and had sampled from both spouts, preliminary training ended.

Once the experimental conditions began, the daily routine was as follows: First, donors were allowed to drink milk. When they had stopped drinking for 5 minutes, milk was pumped out through the intragastric fistulae and cooled to room temperature. Second, the cuffs of the experimental animals were inflated. These rats were then given a choice between two flavored solutions: banana (0.5 percent banana flavoring, Schilling) and almond (0.5 percent almond flavoring, Schilling). When half the rats drank the banana flavor, predigested milk was injected into their stomachs at the rate at which they drank. When these rats drank the almond flavor, physiological saline (Tis-u-sol) was injected. For the remaining rats, the pairings were reversed. There were five 10-minute sessions, one each day for 5 days.

After this phase, each of the ten experimental rats was anesthetized as before, a laparotomy was performed, and the infla-