Phenylalkanoic acids also have antimicrobial properties (*14*), suggesting yet another, albeit remote, functional possibility for the turtle secretion.

Integumental glands occur in all four orders of reptiles, but their products have so far been identified in only few species, including a crocodilian, which produces citronellol (*15*), a leptotyphlopid snake, which discharges a mixture of glycoprotein and long-chain aliphatic acids (*16*), and a gopher tortoise, which also secretes aliphatic acids. In the tortoise, the glands are well developed in the male only, and the secretion has a proven pheromonal role (*17*). For biologists and chemists working in concert, reptilian glands could provide a fruitful subject of research.

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Herbivore-Plant Interactions: Mixed-Function Oxidases and Secondary Plant Substances

Abstract. *The mixed-function oxidases of a polyphagous insect larva (the southern armyworm,* Spodoptera eridania*) were found to be induced by a diversity of secondary plant substances. The induction proceeds rapidly and in response to a small quantity of secondary substance. Following induction, the larva is less susceptible to dietary poisoning. It is argued that mixed-function oxidases play a major role in protecting herbivores against chemical stress from secondary plant substances.*

The so-called mixed-function oxidases (MFO) are a group of enzymes widely distributed in organisms and are best known for their well-established role in the primary degradation of drugs, pesticides, and other synthetic compounds. These enzymes are attached to the endoplasmic reticulum of cells in association with an electron transport pathway that delivers reducing equivalents from NADPH (reduced nicotinamide adenine dinucleotide phosphate) to the terminal oxidase, cytochrome P-450. Here, as a consequence of binding and subsequent reaction with activated oxygen, the compounds undergo any of several types of oxidative transformations (*1*).

Of the large number of foreign compounds to which organisms are exposed, the lipophilic ones are frequently more hazardous than others since they are often difficult to excrete and tend to accumulate in body tissues. The primary general function of MFO enzymes is to convert such lipophilic compounds into more polar hydrophilic metabolites that are more readily excreted (*2*). The fact that these enzymes metabolize the many lipophilic synthetic organic chemicals represented by modern drugs and insecticides should be viewed as a consequence of this basic capacity. The decrease in toxicity (detoxification) usually associated with the MFO system is secondary to the primary effect of increasing hydrophilicity.

The MFO system is ideally suited for its role as a general clearinghouse for lipophilic compounds. It is nonspecific in accepting a large variety of compounds as substrates, and effects the lipophile-hydrophile conversion by means of numerous reactions, including aromatic and aliphatic hydroxylations and epoxidations, *N*- and *O*-dealkylations, and nitrogen and thioether oxidations (*1*). In addition, its ability to be induced by many chemicals (*3*) provides the system with the proper flexibility for responding to conditions of increasing environmental chemical stress.

An unsettled question concerns the evolutionary "what for?" of MFO enzymes. Given their proven defensive action against man-made chemicals, and the fact that they are involved in the metabolic degradation of many naturally occurring compounds, including toxicants such as nicotine, rotenone, and the pyrethrins (*4*), it was proposed that the MFO enzymes play a major role in the feeding strategies of herbivores (*5*). Demonstration of a correlation between polyphagy and high MFO activity led to the conclusion (*6*) that the evolution (or at least evolutionary refinement) of the MFO system in herbivorous animals might have been forced by exposure to the so-called secondary substances of plants—compounds such as phenolics, quinones, terpenoids, and alkaloids, which are widely distributed in plants and frequently repellent or toxic to animals, and hence in themselves defensive (*7*). The critical questions are (i) whether the MFO enzymes of an animal are induced by secondary plant substances in its food, and (ii) whether the induction proceeds with enough speed and to sufficient levels to provide the animal with increased protection against these potentially offensive dietary factors. We now provide evidence that, for a broadly polyphagous insect, the larva of the southern armyworm moth *Spodoptera eridania*, the answers to these questions are indeed in the affirmative.

Our experiments were done with sixth (last) instar larvae that had been raised through the fifth instar on kidney bean plants (*Phaseolus vulgaris*) and shifted after the last molt to a semidefined artificial diet to which various known secondary plant substances were added. Control larvae were fed the artificial diet without the compounds. All larvae used in any given test were of closely matched age, having undergone the previous molt within a 3- to 4-hour period. MFO activity was routinely assayed by *p*-chloro *N*-

methylaniline *N*-demethylase activity measurements in crude midgut homogenates (*8*, *9*).

Initial tests were designed to determine the relative inducing capacities of various known secondary plant substances. The compounds were added to the diet at low concentrations (0.05 to 0.26 percent) comparable to those that, at least in some cases, occur in the succulent parts of plants (*10*). The results (Table 1) show that, with the exception of benzaldehyde, all compounds tested had an inducing effect on MFO activity. Levels of induction were moderate with some compounds, but with several they were high (200 to more than 300 percent of control activity) and comparable to the higher levels of induction observed in insects and vertebrates in response to synthetic compounds (*11*). The most potent inducers were (+)-α-pinene and myrcene.

Subsequent tests, designed for finer analysis of the dose dependency and time course of the inductive process, were carried out with three secondary substances, all proven inducers: sinigrin (allyl glucosinolate), a mustard oil glycoside from crucifers; (+)-α-pinene, a bicyclic monoterpene from conifers and other plants; and *trans*-2-hexenal, the widely distributed "leaf aldehyde" of plants (*10*).

Dose dependency was established for each compound by feeding larvae diets containing different concentrations of the compound. Assays were carried out after a 24-hour feeding period. The results (Fig. 1) show that the MFO system is responsive to even the lowest concentrations of the compounds tested, and that it responds in a graded rather than "all or none" fashion to increasing concentrations of the compounds. With synthetic substances such graded responsiveness had already been demonstrated (*9*). The decreased MFO activity at high concentrations might have been due simply to reduced intake by the larvae of diets containing the larger quantities of the compounds. *trans*-2-Hexenal and α-pinene, at appropriate levels, can act as repellents or topical irritants to insects (*12*), and sinigrin, probably by virtue of its metabolite allyl isothiocyanate, can act as a toxicant (*13*).

For resolution of the time course of the induction process, larvae were fed on diets containing fixed concentrations of one of the three compounds; groups were killed for assay at intervals (30 minutes to 24 hours) after initiation of feeding. With sinigrin and (+)-α-pinene the rise in MFO activity was immediate and proceeded rapidly over much of its course within the first few hours (Fig. 2). The results with *trans*-2-hexenal were essentially similar except for an initial depression of activity (*14*). Comparable rapid increases in MFO activity have been shown in insects exposed to pentamethylbenzene and other synthetic compounds (*9*, *15*).

The preceding suggested that even a brief exposure to a small quantity of secondary substance might suffice to trigger a measurable increase in MFO activity. That this is so was shown in tests with larvae that were fed a single, measured quantity of diet containing either (+)-α-pinene or sinigrin (which they consumed in a few minutes) and then sampled for assay at intervals of 2, 30, and 60 min-

Table 1. Effect of secondary plant substances on *N*-demethylase activity in the midgut of the armyworm. Newly molted sixth instar larvae were given free access to semidefined artificial diets containing the test compound for 24 hours. Larvae of the same age feeding on diets without any additions served as controls. Enzyme activity in midguts from control larvae was 0.70 ± 0.06 nmole per milligram of protein per minute, which is considered 100 percent. Assays were as described (*8*); S.E., standard error.

Compound	Percent in diet	Specific activity* (mean ± S.E.)	Percent of control activity
Aldehydes			
Benzaldehyde	0.10	0.83 ± 0.03	119
	0.20	0.64 ± 0.04	92
trans-2-Hexenal	0.10	0.91 ± 0.10	130
	0.20	1.02 ± 0.05	145
Terpenoids			
(+)-α-Pinene	0.09	1.50 ± 0.08	214
	0.26	2.18 ± 0.08	312
(−)-α-Pinene	0.05	1.28 ± 0.10	183
	0.10	1.42 ± 0.10	203
β-Pinene	0.07	1.49 ± 0.10	213
	0.14	1.51 ± 0.12	216
(+)-Limonene	0.10	0.70 ± 0.08	100
	0.20	1.30 ± 0.12	186
Camphene	0.10	1.18 ± 0.06	168
	0.20	1.48 ± 0.17	211
Myrcene	0.10	1.48 ± 0.12	211
	0.20	2.70 ± 0.20	386
β-Carotene	0.10	1.11 ± 0.13	159
	0.20	1.13 ± 0.07	161
Cadinene	0.10	1.19 ± 0.08	170
Steroids			
Stigmasterol	0.10	1.16 ± 0.05	165
	0.20	1.00 ± 0.10	143
Sitosterol	0.10	0.86 ± 0.08	124
	0.20	1.94 ± 0.09	277
N-Heterocyclics			
Quinoline	0.10	1.89 ± 0.15	270
Quinazoline	0.10	0.90 ± 0.07	129
	0.20	1.09 ± 0.12	156
Glycoside			
Sinigrin	0.10	1.26 ± 0.12	180
	0.20	1.39 ± 0.06	198

*Nanomoles per minute per milligram of protein; $N = 2$ or 3.

Table 2. Effect of (+)-α-pinene and sinigrin on armyworm midgut *N*-demethylase activity. Sixth instar larvae that had been feeding on control diet for 20 hours, and then starved for 4 hours, were fed previously weighed disks of control diet, or comparable disks containing (+)-α-pinene (98 ± 3.8 μg per gram of larva) or sinigrin (106 ± 5.6 μg per gram of larva) and killed for assay, at the times indicated. Consumption times were roughly similar for the different disks: 4.6 minutes for control disks; 6.5 and 6.6 minutes for disks containing (+)-α-pinene and sinigrin. Assays were as described (*8*); $N = 6$, except with 60-minute data, where $N = 5$.

Time after consumption (min)	Specific activity* (mean ± S.E.)		
	Control	(+)-α-Pinene	Sinigrin
2	0.866 ± 0.036	0.958 ± 0.078	0.866 ± 0.080
30	0.826 ± 0.045	1.000 ± 0.063†	1.032 ± 0.019†
60	0.798 ± 0.017	0.953 ± 0.049†	0.988 ± 0.105†

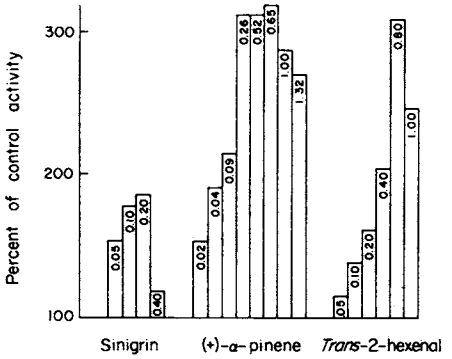
*Nanomoles per milligram of protein per minute. †Indicates $P < .1$ relative to corresponding control (Student's *t*-test).

utes after termination of the meal. The dosage consumed of either compound—about 100 μg per gram of body weight of larva—elicited a significant increase in MFO activity at 30 minutes (Table 2). Such a dosage of secondary substance could easily be ingested by a late instar larva in a few minutes of feeding on an actual leaf (*16*) and could therefore suffice to set in motion the induction process under natural conditions. Interestingly, armyworms do not feed continuously, but pause regularly and for a comparatively long time (16 to 34 minutes) between periods of feeding (22 to 36 minutes) (*17*). Such habits could obviously benefit a polyphagous herbivore whose MFO system may require adjustment to the changing chemical challenges imposed by its diet.

Larvae with an induced MFO system are better protected against a toxic secondary substance than uninduced controls. Induction was effected by feeding larvae for 24 hours on diets containing 0.05 or 0.1 percent (+)-α-pinene. Nicotine (*18*) was given orally to both induced and uninduced larvae, either with or without piperonyl butoxide, an insecticide synergist known to be a potent inhibitor of the MFO system (*19*). Prior feeding of the larvae on (+)-α-pinene resulted in lowered susceptibility to nicotine poisoning as compared to the corresponding controls (Table 3); this effect, as expected, was abolished by piperonyl butoxide. Moreover, the LD_{50} (lethal dose for 50 percent of the animals) for each of the two piperonyl butoxide–treated groups was lower than that for the corresponding controls, indicating that even in these "uninduced" larvae the MFO system was operating at a level that provided some protection.

Additional experiments with microsomal preparations confirmed that the aforementioned effects are indeed associated with induced MFO activity. Midgut microsomes, isolated from larvae fed on diets containing either secondary substances or synthetic inducing agents, were assayed for *N*-demethylase, aniline hydroxylase, and aldrin epoxidase activities, as well as for cytochrome P-450 titer (*20*). The values obtained were in all cases higher than those of the corresponding controls (Table 4), showing that secondary substances can be potent inducers. The inducing capacity of (+)-α-pinene, for example, was comparable to that of phenobarbital and pentamethylbenzene, two synthetic compounds known to be (*20*) potent inducers of the armyworm MFO system.

The MFO system of armyworms has the attributes of an effective biochemical defense. Through the process of induction it responds in a rapid and graded fashion to potentially hazardous dietary factors and, once induced, provides increased protection against chemical stress. To the extent that they are known, MFO systems appear to be basically similar in animals of diverse types (*21*). What has been demonstrated about the system's role vis à vis secondary plant substances in armyworms might therefore apply to herbivores as a whole. This is not to say that MFO systems evolved specifically in herbivores for biochemical protection against the chemical weaponry of plants, or that they



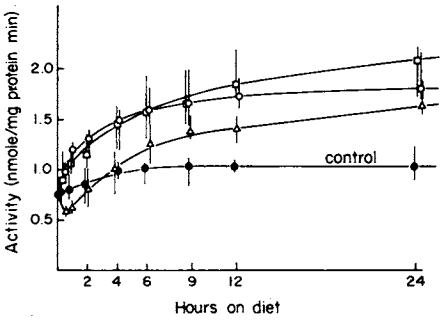


Fig. 1 (left). *N*-Demethylase activity in the midgut of the armyworm as a function of concentration of sinigrin, (+)-α-pinene, and *trans*-2-hexenal in diet. Newly molted sixth instar armyworms were fed freely for 24 hours on diets containing the given concentrations of the compound. Larvae fed on the control diet were used for measuring 100 percent activity (0.72 ± 0.08 nmole per milligram of protein per minute). Assays were as previously described (*8*). Fig. 2 (right). *N*-Demethylase activity in the midgut of the armyworm as a function of time of feeding on various diets. Sixth instar larvae that were first fed for 24 hours on control diet were transferred to diets containing 0.04 percent (+)-α-pinene (□), 0.1 percent sinigrin (○), or 0.2 percent *trans*-2-hexenal (△), and killed for assay after having been given free access to these diets for periods of up to 24 hours. Controls (●) were continued on the control diet. Assays were as described (*8*). Data points are given as means and ranges (N = 2 or 3).

Table 3. In vivo toxicity of nicotine to armyworms. Sixth instar larvae were fed for 24 hours (pretreatment) on the control diet, or on the control diet with (+)-α-pinene at the concentrations indicated. They were then individually confined and fed small pieces of bean leaf to which the nicotine (with or without 5 parts per weight of piperonyl butoxide) was applied. Larvae were tested in samples of 12, and mortality was checked after 24 hours (22°C). Values of LD_{50} ± S.E. are means obtained from three separate log dosage–probit mortality lines (fitted by eye) for each treatment. Each line was based on the mortalities at four to six dosage levels. Midgut *N*-demethylase activity (NCH_3) was determined (in larvae killed at end of pretreatment period) by the assay previously described (*8*).

Prior treatment (diet)	NCH_3 (percent of control)	Test substance	LD_{50} ± S.E. (mg/kg)
Control	100	Nicotine	2670 ± 430
0.05 percent (+)-α-pinene	207	Nicotine	4870 ± 580
0.10 percent (+)-α-pinene	227	Nicotine	4600 ± 210
Control	100	Nicotine plus piperonyl butoxide	770 ± 120
0.10 percent (+)-α-pinene	220	Nicotine plus piperonyl butoxide	530 ± 40

Table 4. Effect of (+)-α-pinene, sinigrin, *trans*-2-hexenal, pentamethylbenzene and phenobarbital on microsomal mixed-function oxidation in the midgut tissues of the armyworm. *N*-Demethylation of *p*-chloro *N*-methylaniline (NCH_3), *p*-hydroxylation of aniline (AOH), and epoxidation of aldrin (AE) were measured in in vitro incubations by means of microsomal preparations; activities are given as nanomoles per milligram of protein per minute. Cytochrome P-450 (P-450) levels are given as change in absorbancy from 490 nm to that at 450 nm per milligram of protein. Numbers are means (N = 2 or 3; S.E. 10 to 20 percent); numbers in parentheses are percent of control values. Sixth instar armyworms were fed freely for 3 days on diets containing 0.12 percent of the various compounds.

Compound	NCH_3	AOH	AE	P-450
Control	2.71 (100)	0.46 (100)	0.51 (100)	0.015 (100)
(+)-α-Pinene	6.37 (235)	2.19 (479)	1.32 (258)	0.026 (176)
Sinigrin	4.88 (180)	0.88 (191)	0.66 (129)	0.020 (138)
trans-2-Hexenal	3.52 (130)	0.85 (185)	0.67 (130)	0.020 (136)
Pentamethylbenzene	6.23 (230)	2.41 (527)	1.28 (250)	0.035 (240)
Phenobarbital	5.83 (215)	1.44 (315)	1.05 (206)	0.026 (176)

serve only for metabolic conversion of exogenous chemicals. On the contrary, MFO enzymes are also involved in important endogenous functions (*22*), and it may be some of these that are more truly indicative of the ancestral activities of the system. But secondary plant substances are ubiquitous and of broad potential offensiveness, and, in the world of today, they may be the major group of hazardous substances against which the MFO enzymes of herbivores must operate.

A point of further evolutionary interest is that the MFO systems of animals are not "foolproof." Being programmed primarily to effect lipophile-hydrophile conversions rather than detoxifications per se, MFO enzymes sometimes transform substances that are initially relatively innocuous into ones that are actually toxic. Substances thus "bioactivated" include not only certain synthetic compounds (*23*), but also some familiar natural products (*24*). Among the latter are the so-called pyrrolizidine alkaloids, a group of widely distributed plant substances converted by mammalian MFO activity into hepatotoxic pyrroles (*25*). Plants that produce such bioactivatable compounds may, in effect, have succeeded in overcoming the biochemical defenses of their herbivorous enemies.

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Asymptomatic Gonorrhea in Men: Caused by Gonococci with Unique Nutritional Requirements

Abstract. *In a retrospective case-control study, gonococci with nutritional requirements for arginine, hypoxanthine, and uracil were recovered from 24 of 25 men with asymptomatic gonorrhea and 10 of 25 men with symptomatic gonorrhea (P = .0001). These strains represent a smaller proportion of gonococcal isolates from blacks than from whites. Asymptomatic urethral infection is important in the epidemiology of gonorrhea, particularly among whites.*

The proportion of heterosexual men with gonorrhea who lack signs or symptoms of urethritis is low among men who voluntarily seek care in venereal disease clinics (*1*), but is high among infected men brought to treatment because they were named as sex contacts of women with gonorrhea. No signs or symptoms were noted in 44 percent of culture-positive sex partners of women with acute pelvic inflammatory disease (*2*), in 57 percent of infected sex partners of women with disseminated gonococcal infection (DGI) (*3*), and 39 to 56 percent of infected sex partners of infected women detected by routine screening in a family planning clinic (*4*). Thus men with asymptomatic infection are important as transmitters of gonorrhea.

It has not been determined whether it is the characteristic of the host or the organism, or a combination of both, which determine whether urethral gonococcal infection produces urethritis. However, it is known that many men with DGI have asymptomatic urethral infection as the apparent primary focus from which their bacteremia arises (*5*), and most gonococci which cause DGI are uniquely susceptible to penicillin G (*6*), and resistant to the complement-dependent bactericidal action of normal human serum (*7*). In some areas of the United States, isolates from patients with DGI also have unique nutritional requirements for arginine, hypoxanthine, and uracil on chemically defined media. For example, in Seattle, gonococci whose nutritional requirements include arginine, hypoxanthine, and uracil ($Arg^-Hyx^-Ura^-$) were recovered from 89 percent of patients with DGI, and only 38 percent of patients with uncomplicated gonococcal urethritis (*8*).

Our case-control study was undertaken to determine whether Arg^-Hyx^-

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