10, 1976), pp. 39–48, and other editions]. Height measurements were also compared to the HSPH norms. The Composite International and Interracial Graphs compiled by G. Nellhaus [*Pediatrics* **41**, 106 (1968)] were used for head circumference. These references were chosen because they are widely utilized in pediatric growth measurements [see, for example, R. R. Puffer and C. V. Serrano, *Pan. Am. Health Organ. Sci. Publ. No. 262* (1973)]. They differ only slightly from other standards. See M. Behar [in *Malnutrition, Learning, and Behavior*, N. S. Scrimshaw and J. E. Gordon, Eds. (MIT Press, Cambridge, Mass., 1968), p. 83] for discussion of reference standards for physical growth.

12. Information concerning birthweight and maturity at birth, although available on all the subjects, was judged to be of doubtful reliability in some cases.
13. To determine the ratio of weight to age and height to age, the patient's height and weight are compared to the normal reference standards to ascertain the age at which the patient's measure would fall on the normal mean. The patient's height and weight ages were always below their chronological ages.
14. The methods and procedures described in Barnet *et al.* (*5*) were followed as closely as possible. Electrode placements were C_z, C_3, C_4, O_z, O_1, and O_2, referred to joined mastoids [H. H. Jasper, *Electroencephalogr. Clin. Neurophysiol.* **10**, 371 (1958)]. The EEG was amplified by means of an Elema Schonander EEG machine with half-amplitude bandpass of 0.7 to 70 Hz.
15. All but 11 of the evoked potentials were recorded during non-rapid eye movement sleep. The results were similar whether or not the 11 REM evoked potentials were included in the analyses; see also (*16*).
16. We found that sleep stages in the marasmic subjects could be scored by conventional criteria [T. Anders, R. Emde, A. Parmelee, Eds., *A Manual of Standardized Terminology, Techniques and Criteria for Scoring of States of Sleep and Wakefulness in Newborn Infants* (Neurological Information Network, National Institute of Neurological Diseases and Stroke, Los Angeles, 1971); A. Rechtschaffen and A. Kales, Eds. *A Manual of Standardized Terminology, Techniques, and Scoring System for Sleep States in Human Subjects* (No. 204, U.S. Public Health Service, Washington, D.C., 1968)]. Background EEG's often showed abnormalities including slowing, reduced amplitude, and decreased sleep spindles; or very-high-amplitude fast activity. (Patients whose EEG's showed paroxysmal abnormalities were excluded from the study.) Background EEG and sleep stage interacted with evoked potential characteristics (M. Shkurovich *et al.*, in preparation), but did not fully account for the observed EPI abnormalities. For example, the extremely large N_1P_2 and P_2N_2 components characteristic of the evoked potentials of many marasmic subjects at follow-up were found in all sleep stages. Evoked potentials with high EPI's were often found in "typical" stage 2 or 3 sleep.
17. Norms were based on Barnet *et al.* (*5*). These norms were validated in a subsequent study of 254 evoked potentials [Ohlrich *et al.* (*6*)]. The rules followed for identifying the peaks are given in Barnet *et al.* (*5*). In instances where abnormal evoked potential characteristics made identification of peaks uncertain, the peaks chosen were the ones which minimized the EPI, that is, the scoring method tended to underemphasize deviance.
18. In two previous, similarly conducted evoked potential studies of normal infants [see references in (*17*)] no sex differences were found.
19. We have no information on the heights of the parents of the control subjects from which estimates of genetic growth potential could be made. See Cravioto *et al.* (*2*) and Scrimshaw and Gordon [(*3*), part 2, pp. 16–90] for discussion of height-and-weight-for-age as indicators of nutritional status during infancy and early childhood in developing countries.
20. The evoked potentials were recorded during a study of infant malnutrition being conducted by the Nutrition Service of the Hospital de Niño. Interactions of EPI's with sex and other cofactors will be reexamined later (J. Cravioto *et al.*, in preparation).
21. We thank the personnel of the Servicio de Nutricion, R. Arrieta, chief, for their cooperation. We thank M. Campos for assistance in data collection and J. Auñon, B. Shanks, and B. Barnet for assistance and advice. The research was supported in part by the W. T. Grant Foundation and Public Health Service grants and awards HDO2296 and K2MH45472.

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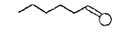
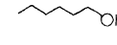
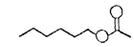
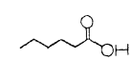
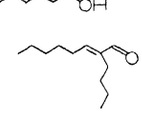
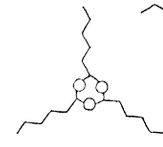
Proteins in a Nonvenomous Defensive Secretion: Biosynthetic Significance

Abstract. *In common with many arthropods, the true bug,* Leptoglossus phyllopus, *when disturbed, emits a two-phase secretion that consists of an organic phase and an aqueous phase. The organic phase is a mixture of highly reactive low-molecular-weight compounds, analogous to those produced by other arthropods, and is deterrent to many kinds of predators. The aqueous phase, heretofore ignored in most analyses of arthropod defensive secretions, contains proteins. Even though the secretion is not injected, the proteins enzymatically catalyze the derivation of the most reactive components within the impermeable cuticular storage reservoir and, thus, constitute part of the defensive system that appears to be commonly used by arthropods producing irritating chemicals.*

Arthropods are conspicuous for emitting chemical irritants for defense against would-be predators (*1*). Natural product chemists have identified such reactive compounds as hydrogen cyanide, quinones, α,β-unsaturated aldehydes, and carboxylic acids in the defensive secretions of arthropods, ranging from millipedes, centipedes, opilionids, and whip scorpions to the phylogenetically advanced cockroaches, walkingsticks, stinkbugs, and beetles (*1–6*). Yet, despite the fact that practically all of the defensive secretions examined closely have been reported to contain an immiscible aqueous phase (*3, 4, 7*), in addition to the reactive organic phase, the chemical analysis of the aqueous phase has been almost totally ignored (*3*). We have found that the defensive secretion of the coreid bug *Leptoglossus phyllopus* contains a mixture of compounds not unlike those identified from other so-called stinkbugs or true bugs in the order Hemiptera (*4–6*). In addition, the aqueous phase contains at least four proteins. The secretion is not injected as are the venoms of some bees, wasps, spiders, snakes, and various marine organisms. We now report that the protein fraction of the secretion catalyzes the production of the most irritating constituents of the defensive blend from a relatively nontoxic precursor within the impermeable cuticular reservoir of the gland.

Adults of *L. phyllopus* were reared in the laboratory, and the volatile components of the defensive secretion from the capacious metathoracic gland were analyzed immediately (Table 1) (*8*). The metathoracic gland in terrestrial Hemiptera (Georcorisae) consists of a large nonglandular storage reservoir lined with cuticle; two pairs of accessory glands, the primary and secondary accessory glands, empty into the reservoir (Fig. 1) (*5, 7*). The secretion released from the reservoir by 1-week-old bugs contained equivalent amounts of hexyl acetate and hexanal (Table 1). In 10-week-old bugs, the amount of hexyl acetate had declined by almost 45 percent, with a corresponding increase in the proportion of hexanal. Comparison of an extract of the primary

Table 1. Composition of the metathoracic gland secretion from 1- and 10-week-old *Leptoglossus phyllopus*.

Compound	Structure	1 week old (%)	10 weeks old (%)
Acetic acid		N.D.*	N.D.*
Hexanal		42.1 ± 8.0	87.8 ± 3.7
1-Hexanol		6.6 ± 1.8	1.2 ± 0.6
Hexyl acetate		49.4 ± 7.7	5.1 ± 3.7
Hexanoic acid		Trace	4.0 ± 4.7
2-*n*-Butyloct-2-enal		N.D.*	N.D.*
Hexanal trimer†		0.2 ± 0.1	2.0 ± 0.6

*Not determined. †Both *cis* and *trans* isomers.

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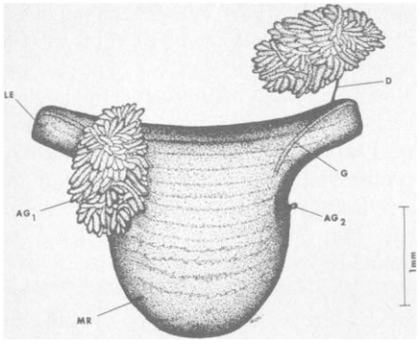

Fig. 1. The metathoracic gland of *Leptoglossus phyllopus*. Abbreviations: AG_1, primary accessory gland; AG_2, secondary accessory gland; *D*, duct; *G*, chitinous groove; *LE*, lateral extension; and *MR*, median reservoir.

accessory glands from several bugs to an extract of the metathoracic gland reservoir from the same bugs showed that, in the gland, hexyl acetate was the major component whereas, in the reservoir, hexanal and hexyl acetate were present in equivalent amounts, in agreement with investigations of other Geocorisae (*9*). Not only did the amount of the ester drastically decline in bugs several weeks old, but four compounds eluting after hexyl acetate significantly increased in amount (Table 1). The first two of these compounds were identified by low-resolution mass spectrometry as hexanoic acid and the aldol condensation product of hexanal, 2-*n*-butyloct-2-enal (*6*, *8*). The remaining two compounds eluted within 1 minute of each other. They had nearly identical mass spectra resembling the spectrum of hexanal (M = m/e 100) (m-e, mass to charge), but with characteristic ions at m/e 201, 229, and 299, suggesting a molecular weight about three times that of hexanal. Saturated aldehydes form stable trimers which exist as *cis* (all three side chains on the same side of the ring) and *trans* (two side chains above the ring and one below) isomers (*10*). High-resolution mass spectrometry (*11*) and comparison of the gas-liquid chromatography (GLC) retention times of synthetic hexanal trimers with those of the volatiles from the bug demonstrated that the latter were indeed the trimerization products of hexanal, not previously detected in arthropod defensive exudates.

By first chilling and then anesthetizing laboratory-reared *L. phyllopus* adults, it was possible to remove the adjacent tissue by dissection under saline solution, and to expose the metathoracic gland without the insects ejecting their secretions. The reservoir wall of a replete gland was transparent, so that the two-phase fluid could be viewed within. The secretion in the reservoir appeared as a "bubble within a bubble." Upon rupturing the wall of the reservoir the outer fluid layer, which varied greatly in volume, appeared to dissolve in the surrounding saline solution, in agreement with the observations of Carayon (*7*) for the metathoracic gland secretions of other Hemiptera. The fluid forming the inner bubble of the two-phase secretion was insoluble in saline solution and, when squeezed from a ruptured gland, floated to the surface. This phase had the characteristic odor of the bug. Separate analyses of each phase collected either directly from the reservoir in microcapillaries or by centrifugation of secretion collected from the peritreme surrounding the external openings of the gland confirmed that the internal fluid phase was the organic phase, and that the external phase contained only traces of scent volatiles.

Electrophoresis of the aqueous phase of the metathoracic gland secretion on polyacrylamide gels (*12*) revealed four proteins (Fig. 2), three of which showed strong enzymatic activity. A strong esterase band was detected between the two lower bands, with α-naphthyl acetate as the substrate, and the two lower bands stained positively for dehydrogenase activity when 1-hexanol was used as the substrate. Therefore, it appears that the major aldehydic constituent in the defensive secretion of *L. phyllopus*, and probably other Geocorisae as well, is produced by enzymatic hydrolysis of the ester and oxidation of the alcoholic product. Since neither of the lower two bands stained positively for dehydrogenase activity when hexanal was the substrate, hexanoic acid is apparently produced by the nonenzymatic oxidation of hexanal, as has been suggested (*6*). The relatively small increases in concentration with time of hexanoic acid, 2-*n*-butyloct-2-enal, and trimerized hexanal apparently occur spontaneously.

In arthropods, the biosynthesis of reactive defensive molecules does not occur in the glandular cells which produce the less toxic precursors (*1*). Rather, this is almost universally achieved by the inclusion of a cuticular "end-apparatus" within each secretory cell where the later stages of synthesis occur (*13*). At the opposite extreme, there are some species in which the production of particularly noxious chemicals is triggered at the moment of discharge by admixture of precursors with appropriate catalysts (*2*). A less spectacular, but probably more common, autodefensive adaptation of arthropods that secrete highly reactive compounds is the enzymatic derivation

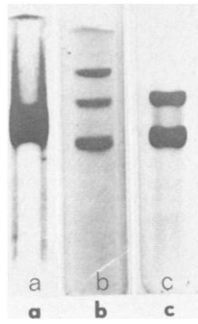

Fig. 2. Gel electrophoresis of the metathoracic gland secretion from *Leptoglossus phyllopus*: (a) stained for esterase with α-naphthyl acetate as the substrate, (b) stained for proteins, and (c) stained for dehydrogenase with 1-hexanol as the substrate.

of the ultimate toxins inside the storage reservoir long before discharge, as occurs in the metathoracic gland of *Leptoglossus* (*14*). The enzymes in the defensive secretion of *L. phyllopus* are apparently synthesized in the secondary accessory glands (*9*, *15*), whereas the ester precursor is produced in the primary accessory glands. In contrast, the adult defensive glands of the primitive aquatic Hemiptera and the defensive glands of all immature Hemiptera lack primary and secondary accessory glands; the reservoir is covered by a layer of histologically identical secretory cells (*7*). Thus, in the true bugs there has been both a phylogenetic and an ontogenetic evolutionary tendency toward the separation of catalyst synthesis from precursor synthesis.

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8. The metathoracic gland was dissected from each bug and extracted in *n*-pentane. The composition of the secretion, excluding acetic acid and 2-*n*-butyloct-2-enal, was determined with a gas-liquid chromatograph (GLC) (Tracor 222) with a flame ionization detector coupled to a chromatography data system (Varian CDS-111) peak area integrator on a 3 percent OV-1 column programmed from 50° to 215°C at 10°C per minute. Four adult bugs were analyzed separately for each age class. The mean percentage is reported for each compound, followed by the 95 percent confidence interval. Mass spectra were obtained for each compound with a GLC and mass spectrometer (LKB-9000 GLC-MS) and a 1 percent SP-1000 column programmed from 50° to 225°C at 10°C per minute.
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11. High-resolution mass spectra for both of these GLC peaks gave diagnostic ions at *m/e* 229.1797 ($C_{13}H_{25}O_3$), 201.1841 ($C_{12}H_{25}O_2$), 101.0972 ($C_6H_{13}O$), and 83.1857 (C_6H_{11}), plus metastable ions at *m/e* 50.75, 68.20, and 36.45.
12. The bugs were chilled, their metathoracic glands were removed intact, and the secretion was mixed with 100 μl of cold 0.1*M* tris-HCl buffer (*p*H 8.2). Each sample (30 glands) was divided into three equal portions and placed on the gels. Electrophoresis was performed on 7.5 percent polyacrylamide gels with spacer gels, at 1.5 mA per gel for 4 hours; the gels were then stained differentially for esterases, proteins, and alcohol dehydrogenase. When assayed for 1-hexanol or hexanal dehydrogenases, the gels were washed in 0.1*M* tris-HCl buffer (*p*H 7.4) for 5 minutes and then incubated at 37°C in 50 ml of 0.1*M* tris-HCl buffer containing 1 ml of either 1-hexanol or hexanal (20 percent in acetone), 25 μmole of tetrazolium, 30 μmole of nicotinamide adenine dinucleotide phosphate, and 15 μmole of phenazine methosulfate for 10 minutes.
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14. Few nonvenomous secretions have been analyzed for proteins. We electrophoretically analyzed the defensive exudates of some opilionids (*Leiobunum* spp.) and found an array of low-molecular-weight proteins.
15. The secondary accessory glands of whole excised metathoracic glands from *L. phyllopus* stained positively for esterases using α-naphthyl acetate as a substrate.

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Biosynthesis and Accumulation of Formic Acid in the Poison Gland of the Carpenter Ant *Camponotus pennsylvanicus*

Abstract. *Formic acid synthesis in the poison gland of* Camponotus pennsylvanicus *is closely related to the C-1 metabolism of the glandular cells. Serine, glycine, and histidine are potential C-1 donors to formic acid by several tetrahydrofolate intermediates. Formic acid is accumulated by its transfer to an insulated reservoir, so that the ant avoids the acid's cytotoxicity. This combination of biochemical and morphological features provides an autodefensive mechanism. Possible factors that regulate the biosynthesis of formic acid in the poison gland apparatus are discussed.*

Formic acid, identified from a distillate of formicine ants (Formicidae: Hymenoptera) more than 300 years ago, was the first natural product isolated from insects (*1*). Later it was identified in the defensive secretions of moth larvae (Notodontidae:Lepidoptera) (*2*), and as a pygidial gland product of ground beetles (Carabidae:Coleoptera) (*3*). In the notodontids and carabids, formic acid is utilized as a defensive compound, but in some species of formicine ants it also functions as an alarm pheromone (*4*). In view of the high reactivity of formic acid, we were interested in determining how insects biosynthesize and accumulate large amounts of this cytotoxic acid. In order to understand this mechanism, we have studied its in vitro synthesis and storage of formic acid in the poison glands of the carpenter ant, *Camponotus pennsylvanicus*.

We assumed that (i) formic acid was derived from one or more of the free amino acids in the glandular tissue, and (ii) that its biosynthesis was closely related to the C-1 metabolism in the gland and probably involved tetrahydrofolic acid (H_4folate). All incubations were conducted with the 20 intact poison glands dissected from worker ants belonging to the same colony (*5*). Time-course studies, in which poison glands were incubated with uniformly labeled serine, in vitro, indicated that this amino acid serves as a precursor for formic acid production. Formic acid was synthesized at a rate of 40 nmole per hour for the first 3 hours of incubation. Incubation of poison glands with [U-^{14}C]serine, [3-^{14}C]serine, or [1-^{14}C]serine, suggests that serine contributes both its α and β carbons to formic acid but not its carboxylic carbon, since the incorporation of [U-^{14}C]serine was twice that of [3-^{14}C]serine (Table 1). The α carbon of glycine was also converted to formic acid by the poison glands, suggesting that both serine and glycine serve as precursors of formic acid. Histidine also contributes a carbon fragment to the C-1 pool in cells (*6*), and in fact contributes its C-2′ (in the imidazole ring) to formic acid (Table 1). These results suggest that the production of formic acid by the poison gland is linked to the C-1 metabolism in its glandular cells.

To test our second hypothesis, that H_4folate is involved in the transfer of the β carbon of serine to formic acid, we studied the incorporation of labeled H_4folate derivatives, that could be precursors. An H_4folate-free extract of poison glands (desalted on a Sephadex G-25 column) synthesized formic acid when incubated with 5,10-[^{14}C]methylene H_4folate—which is formed spontaneously when [^{14}C]formaldehyde and H_4folate are mixed in solution (*7*)—whereas a similar extract supplemented only with [^{14}C]formaldehyde failed to do so (Table 2). But when [^{14}C]formaldehyde was incubated with intact poison glands, it was readily converted to formic acid. This was probably due to its intracellular combination with H_4folate, forming 5,10[^{14}C]methylene H_4folate. A second H_4folate derivative, 5,10[^{14}C]methenyl H_4folate, had an even higher degree of incorporation when incubated with intact poison glands, suggesting that both H_4folate derivatives are intermediates in formic acid biosynthesis.

Table 1. Incorporation of labeled amino acids into formic acid in the poison glands of *C. pennsylvanicus*.

Precursor	Formic acid produced (dpm/nmole)	Total formic acid (nmole)	Percent incorporation
L [U-^{14}C]serine	983	56 ± 5	1.67
DL [3-^{14}C]serine	574	56 ± 5	0.98
L [1-^{14}C]serine	21.4	56 ± 5	0.036
[2-^{14}C]glycine	605	47 ± 3	0.87
L-[2′-^{14}C]-histidine	347	42.5 ± 3	0.45

Table 2. Incorporation of labeled formaldehyde and H_4folate derivatives into formic acid by intact poison glands, or poison glands extracts, of *C. pennsylvanicus*.

Precursor	Tissue	Formic acid produced (dpm/nmole)	Percent incorporation
5,10[^{14}C]Methylene H_4folate	Gland extracts	924	1.41
[^{14}C]Formaldehyde	Gland extracts	12	None
	Intact glands	832	1.27
5,10[^{14}C]Methenyl H_4folate	Intact glands	1180	1.80

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