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Phytochemicals at the Plant-Insect Interface

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Opportunities for genetic engineering of natural products are increasing, while discovery and development of synthetic insecticides and developmental regulators are declining. However, discovery and potential applications of natural compounds are constrained by present ecological knowledge and theory. Biochemistry offers additional perspective to chemical interaction across the interface between plant and herbivore. Phytochemical effects on an insect herbivore may be determined by physical, chemical, and biotic characteristics of the microenvironment during phytochemical transfer between plant and insect. The midgut lumen is often overlooked as part of this microenvironment. It initially determines rates of metabolism and uptake of phytochemicals into hemolymph, and ultimately the quantity of a compound seen by affected tissues. Additive processes such as absorption, binding, and transport by proteins in hemolymph may ultimately prove more crucial to toxication than subtractive processes such as metabolism and excretion. Uptake and transport of coumarins in hemolymph are being studied in larvae of the citrus root weevil *Diaprepes abbreviatus*. Studies with synthetic 7-amino-3-phenyl coumarin (coumarin-10) have preceded studies with natural coumarins. The fluorescence properties of coumarin-10 have enabled determination of absorption and binding to hemolymph proteins.

Key words: chemical ecology, digestive absorption, natural products, citrus root weevil, pyranocoumarins, protein binding

INTRODUCTION

During the last two decades, natural products from plants have yielded a number of insecticides and insect growth regulators. Discovery and development of natural products into insecticides and bioregulators have reflected changing scientific, environmental, and economic opportunities. The agrochemical industry has benefitted from natural products such as the pyrethrins, rotenones, azadirachtins, avermectins, certain alkaloids, juvenile hormones, and precocenes. These and other compounds have often provided models for chemical syntheses and targets for large-scale screening based on mode of action or taxonomic origin. However, the same social and economic pressures that

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have promoted these developments are also increasing the economic risk and limiting the potential benefits of new product development. This works against ventures into new discoveries and commercial development.

Simultaneously, efforts to genetically engineer plant defenses require the availability of effective primary and secondary gene products. Phytochemicals constitute one of the most diverse biological reserves of such gene products, due to the diversity of defenses that have evolved in plants against insects. In studying those defenses and their mechanisms, biologists recognize many potential chemical activities. However, it is a significant challenge to develop assays and isolate, characterize, and finally synthesize (or biosynthesize) the relevant compounds.

Considering the many potential activities and compounds to be explored, detailed chemical study of plant-insect interactions is often limited to the most accessible, apparent, or dramatic examples. The discovery and use of active phytochemicals may be better guided by perspective in two areas: 1) the chemical ecology of interactions between insect and plant, and 2) the biochemistry of those interactions. Principles derived from these areas of study may highlight potential targets, including biochemical lesions and bioassays, and then indicate potential problems and opportunities in application.

This article will present some concepts of chemical ecology relevant to allelochemical activity, transfer from plant to insect, and final effect upon the insect. It will introduce the concept of an environmental interface between the plant and insect, at which many factors can alter the efficacy of plant allelochemicals. Finally, some biochemical determinants of the final crucial step across the interface—absorption and internal transport of phytochemicals in an insect—will be discussed in light of one exemplary plant-insect system.

HERBIVORE-PLANT RELATIONSHIPS AND CHEMICAL ECOLOGY

A chemical line of defense by a plant against one or more species of herbivore is often composed of multiple compounds. Despite this, employment or genetic incorporation of single active compounds or their analogs may lead to successful crop defenses. However, maintaining those defenses could be another matter: lack of ecological perspective favors debacles such as development of catastrophic resistance to defenses in a pest [1]. Theories of chemical ecology, which oversimplify the nature of chemical influence between plant and insect, nonetheless can aid in the discovery and use of natural plant products.

The plant apparency theory [2,3] defined plant defenses according to conspicuousness of a plant to herbivores. If a plant was not apparent to herbivores, it would contain qualitative defenses, i.e., chemicals that exhibit toxicity in an acute all-or-none fashion, exhibit rapid effects, and are effective at low concentrations. If a plant was apparent, it would contain quantitative defenses, i.e., less toxic chemicals that are effective in a chronic, slow-acting (e.g., growth-inhibiting), dose-dependent manner.

The dichotomy of plant apparency has encountered contradictions and complications. Net defensive effects may be due to mixtures of compounds showing both qualitative and quantitative effects, and it is sometimes difficult to maintain distinctions between qualitatively and quantitatively effective com-

pounds [4]. The theory rests heavily on distinctions between plants defending against general herbivores vs. those defending against specialized herbivores. This aspect of the theory has been poorly supported by relative abundance of those two types of herbivores on apparent vs. unapparent plants and by relative efficacies of qualitative and quantitative defenses vs. generalists and specialists [5]. Finally, organisms of trophic levels higher than herbivore and host must be considered, whether represented by symbiotic microorganisms or parasites and predators of an herbivore, since such organisms often have substantial impact on herbivore population levels [4].

Coley et al. [5] provided an updated alternative to the plant apparency theory. They suggested that plant defenses directly correlate with resource availability, and that resource availability is in fact the major evolutionary determinant of the quality and quantity of plant defenses. By this theory, abundant resources favor plants that exhibit rapid growth rates, and such plants overcome herbivore damage through high foliar turnover rates without investment in specialized defenses. Limited resources favor slow growth rates and relatively high levels of specialized defense to protect foliage. References to both theories of plant defense generally specify foliage and ignore root system defenses, although subterranean herbivores often have substantial impact on roots. Difficulty of observation complicates collection of data on root herbivory, and presumably accounts for absence of theories on root herbivory. Classifications concerning growth and tissue turnover rates, apparency, and types of defense presumably (though not necessarily) extend to root systems.

Regardless of theory, the ecological complexity of phytochemical defenses is evident. Natural defenses often involve multiple secondary products, and attempts to chemically engineer crop defenses with only one, or perhaps even several, compounds may meet natural limitations on efficacy [1]. Isolation and characterization of phytochemicals are therefore necessary but not sufficient to substantiate plant defense theories or to engineer crop defenses. Rather, comprehension and management of plant-herbivore interactions must include an area somewhere between chemistry and population ecology. Biochemical mechanisms of interaction contribute both to ecological theory and to direction of effort in chemical isolation and assay.

AT THE INTERFACE: BIOCHEMICAL AND BIOPHYSICAL COMPONENTS OF INTERACTION

Chemical transfer between a plant and its insect herbivore occurs across an interface, or common boundary. Chemicals and their effects are subject to modulation by physical, chemical, and biotic factors when crossing over the interface through a stylet, mandibles, cuticular contact, or the atmosphere (Fig. 1). Physical factors such as light and temperature can alter the chemical structure of compounds, their rates of transfer from plant to insect, or their reactivities in the environment or in the insect [6]. Rates of diffusion and thus the transfer of compounds vary with changes in temperature, density, pressure, and viscosity of a medium. Chemical characteristics such as the charge and polarity of both the compound and its environment profoundly affect rates of interchange, especially across membranes and cuticle. Redox potentials and pH

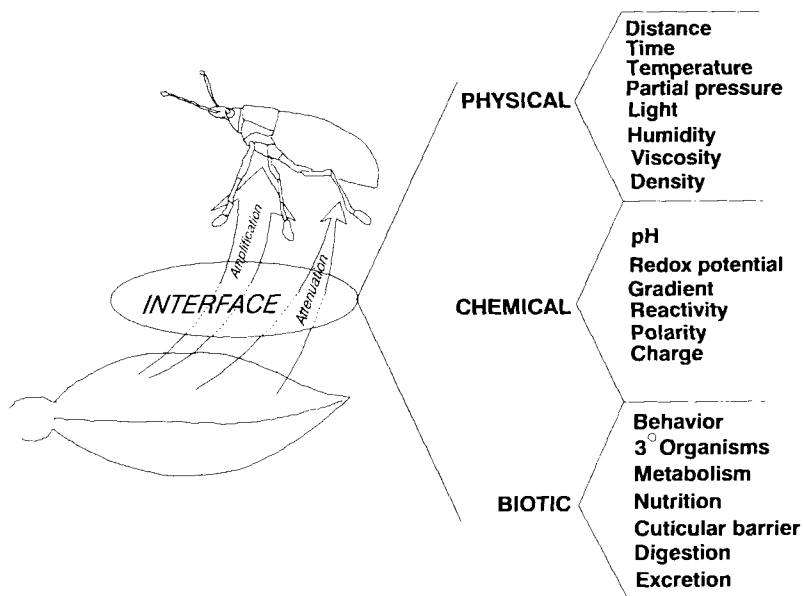


Fig. 1. Physical, chemical, and biotic factors active in phytochemical transfer from plant to insect across the external interface. Passage through the interface often results in modulation (amplification or attenuation) of allelochemical effect. Kinetics of transfer, exposure to degrading or activating environmental components (including other organisms), characteristics of receptive tissues, and attributes of the phytochemicals themselves all contribute to modulation.

can alter the rates of chemical reactions. Finally, the behavior, metabolism, and nutritional state of the host and herbivore [7], and interaction with tertiary organisms such as parasites and symbiotes [8], will profoundly alter rates and quality of chemical transfer.

The process of phytochemical transfer across the interface into an insect is especially relevant to acutely toxic compounds characteristic of qualitative defenses. The processes of uptake, distribution within an insect, and sequestration or effect of such a phytochemical in the insect are best understood as kinetic phenomena [9,10]; the rates and routes of absorption and metabolism of a compound, from immediate environment to target tissue, will determine whether it reaches that tissue at intoxicating concentrations.

Contact between phytochemicals and an insect occurs at both the external (cuticular) and internal (digestive) surfaces of the insect. Although external contact has been of primary interest in considering insecticide transfer and effect [11], internal contact is paramount in phytochemical transfer, since the most intimate contact between plant and insect herbivore is between ingested plant material and the midgut epithelium. Effects of active phytochemicals can be attenuated at either the cuticular or digestive surface through sequestration and/or enzymatic activity, but enzymatic mechanisms of detoxification have been considered foremost. The mixed function oxidases are perhaps the best studied enzymes with respect to absorptive detoxification at both cuticular and midgut epithelial surfaces. Typically, mixed function oxidases will oxidize a high percentage of administered phytotoxin prior to excretion (e.g.,

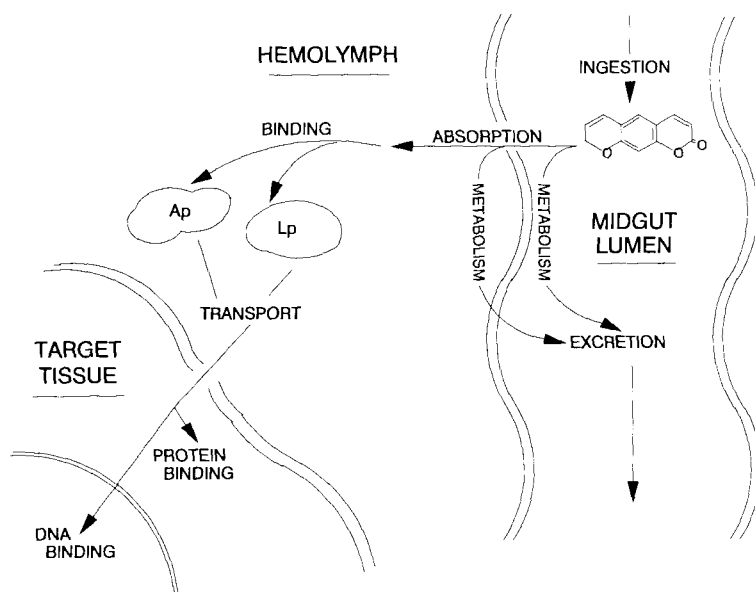


Fig. 2. Additive and subtractive processes determining phytochemical passage across the internal interface between ingested phytochemicals and target tissues. Hemolymph proteins such as lipophorins (Lp) and arylphorins (Ap) may be key components of additive processes, as shown, or may act subtractively by sequestering phytochemicals. (Reprinted by permission of Westview Press from *Advances in Insect Rearing*, edited by Thomas E. Anderson. Published by Westview Press, 1991, Boulder, Colorado.)

[12]). However, the high profile of that route of detoxification has perhaps overshadowed the roles of other detoxicative and intoxicative factors [13,14]. Alternative mechanisms for avoiding toxification may be critical, even when most of a compound is metabolized, because only a small proportion of an applied compound needs to bind to target sites for effect [15].

Additive processes, or those that contribute to absorption, distribution, and arrival of a compound at the active site, are counteracted by subtractive effects of a biological system on phytochemicals, i.e., processes that result in removal of an active substance from the system. Some selected additive and subtractive processes are diagrammed in Figure 2. To reach internal active sites, an allelochemical first penetrates into the insect from the plant surface or ingested plant tissue at the insect cuticle or epithelium, respectively. Next, the allelochemical or toxic metabolites diffuse or are transported to target tissues at a rate and concentration sufficient to be toxic. The quantity of a compound that finally reaches and binds to a target tissue or receptor, and not the quantity ingested or absorbed, determines the degree of intoxication. Only a small proportion of a compound that is highly active at the target site need reach that site, while a much larger proportion of compound with low activity must reach its target site. Therefore, quantification of subtractive processes such as metabolism and excretion may only be relevant relative to ultimate potency of a compound at its active site.

THE ROLE OF HEMOLYMPH IN PHYTOCHEMICAL TRANSPORT ACROSS THE INTERFACE

The most apparent route for distribution of a compound from the environmental interface at either cuticle or gut to affected tissue is through hemolymph [15]. An alternative, though less likely, route is through cuticle into the tracheal system, directly to target tissues [16,17]. Though the roles of hemolymph in toxicological processes are not yet clear, they are bound to be significant [9,10] and merit continued investigation. The topic is relevant not only physiologically and toxicologically, but ecologically as well, since hemolymph is the primary body fluid contacting an insect's parasites, parasitoids, and predators [4]. Allelochemicals in hemolymph could therefore affect not only the herbivore, but any of its antagonists.

However, many active phytochemicals are hydrophobic, and therefore of limited solubility in aqueous solution [9]. For significant transport into and through hemolymph, these compounds would have to dissolve through contact with amphiphilic molecules (molecules compatible with both aqueous and organic phases). The primary amphiphiles in hemolymph are glycoproteins and lipoproteins, and since both forms of protein are often present at very high concentrations, they offer excellent opportunity for dissolution of compounds in the process of assimilation. Many hemolymph proteins have only been isolated and characterized within the last two decades, and specific functions for some are yet unknown.

The potential importance of hemolymph as an amphiphilic matrix of cells, macromolecules (proteins), and chemical compounds is indicated by comparing experiments of Haunerland and Bowers [18] with those of Shah and Guthrie [19] and Shah et al. [20]. During studies *in situ* [19,20], isolated midguts were incubated in buffer and appreciable metabolism of insecticides was observed as they passed from lumen through epithelium and into the buffer. Studies on binding of insecticides to hemolymph proteins *in vitro* [18] later demonstrated binding to two proteins, lipophorin and arylphorin, based on insecticide polarity. If we assume that the protein content of hemolymph enhances its binding affinity and capacity, incubating the midguts in hemolymph instead of buffer might have enhanced the flux of insecticide through epithelium, decreasing the period of contact with epithelium and attenuating the rates of metabolism.

The lipophorins are the best-known hemolymph proteins, both in specific function and transport of hydrophobic compounds. These large (approximately 500,000 M_r) lipoproteins were first known for their ability to shuttle high concentrations of diacylglycerol through hemolymph for use in flight metabolism [21,22]. Other potential roles were soon discovered. Absorption of sterols and fatty acids by lipophorin has been well demonstrated [22], and absorption of carotenoids from diet is evident in the distinct yellow color of lipophorin when isolated from hemolymph [22,23]. Prior to the description of lipophorins as a common class of insect lipoproteins [24], several observations indicated that they help transport apolar insecticides such as DDT in hemolymph [25,26]. Various other insecticides were later shown to bind *in vitro* to lipophorin and

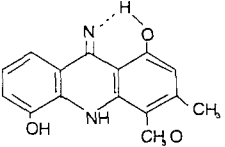
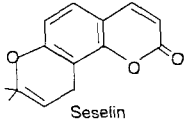
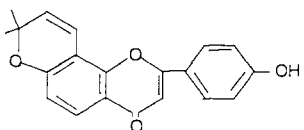
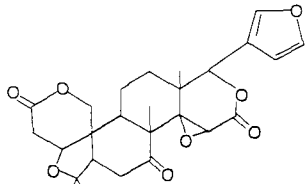
COMPOUND CLASS (subclass shown)	REPRESENTATIVE STRUCTURE	No. COMPOUNDS IDENTIFIED
ALKALOIDS (Acridone Alkaloid)	 Citrusinine-I	27
COUMARINS (Pyranocoumarin)	 Seselin	60
FLAVONOIDS (Flavone)	 Limonianin	3
LIMONOIDS	 Limonin	6

Fig. 3. Phytochemical classes and representative structures identified in roots of several citrus species. The number of characterized compounds in each class is from an informal literature survey of work published during the last decade.

arylphorin of *Heliothis zea*, prompting the hypothesis that xenobiotics partition between arylphorin and lipophorin in a ratio dependent on polarity of the xenobiotic [18].

While these concepts in internal chemical transport have yet to be tested or applied relative to phytochemicals, advances in hemolymph protein characterization during the past decade have increased testability. We have recently completed studies on hemolymph binding and transport using a synthetic coumarin (coumarin-10, or 7-amino-3-phenyl coumarin; Fig. 3) meant to serve as a model phytochemical. Our subject was the curculionid *Diaprepes abbreviatus*, a pest of citrus in central Florida. The weevil-citrus system exemplifies several points discussed above.

The relationship between *D. abbreviatus* and citrus is an example of a generalist herbivore feeding on an "apparent" slow-growing perennial that seems to defend itself with a diversity of acute toxins (i.e., qualitative defenses [2]). The larval weevil feeds on root bark and rootlets of the citrus tree, and on foliage as an adult. However, both larvae and adults will feed on a wide vari-

ety of monocots as well as dicots. As a slow-growing perennial, citrus contains a wide range of potential allelochemicals. Numerous furanocoumarins, terpenoids, limonoids, flavonoids, etc., are abundant in foliage and fruit [27–29], while roots contain acridone alkaloids, pyranocoumarins, and some flavonoids and limonoids [30–34]. Figure 3 gives an indication of the abundance of phytochemicals in roots alone. Tests have not been reported for potential allelochemical activity in most of these compounds. Because a citrus plant is composed of a scion grafted onto a rootstock, independent breeding and genetic engineering of the scion and rootstock are necessary. Phytochemical defenses can be exclusively developed against either subterranean or aerial pests. The division of labor also offers an excellent opportunity to incorporate into rootstocks otherwise absent allelochemicals or to enhance those already present at low levels without fear of doing the same to edible portions of the plant.

The internal processes of ingestion, absorption, and binding to hemolymph proteins were studied in larval *D. abbreviatus* by feeding them coumarin-10 introduced ad libidum in diet or in oil by forced-feeding. In both circumstances, absorption and binding to hemolymph proteins were demonstrated. Two hours after forced-feeding, 95% of the coumarin-10 in hemolymph was bound to proteins, and the remainder was bound to lipophorin [35,36]. The primary binding entities were large glycoproteins that bound coumarin-10 at a K_d of approximately $1.5 \mu\text{M}$ [36]. As a highly fluorescent tracer, coumarin-10 proved useful in directly demonstrating binding to proteins through transfer of fluorescence energy. In this experiment, spectrofluorometric excitation of coumarin-10 resulted in fluorescence emission by tryptophan residues of hemolymph proteins (Fig. 4), demonstrating close proximity of coumarin-10 to tryptophan residues. Naturally fluorescent phytochemicals such as the furano- and pyranocoumarins may offer opportunity for similar experiments.

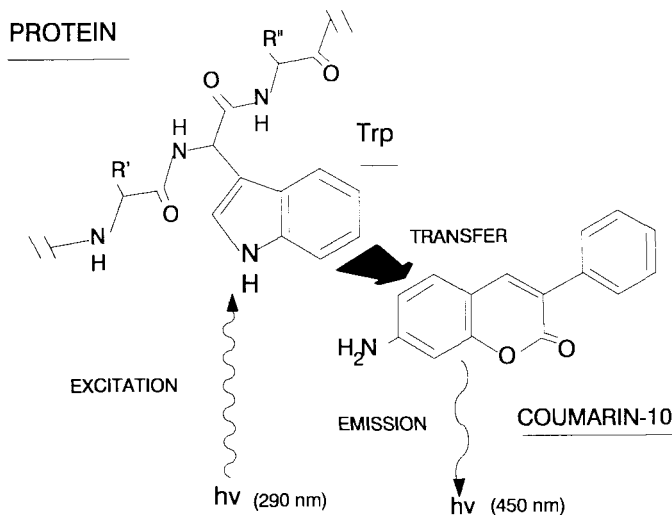


Fig. 4. Fluorescent energy transfer between bound coumarin-10 and tryptophan residues in binding proteins. Energy is absorbed by tryptophan residues at 290 nm and a portion is emitted at 455 nm after transfer to bound coumarin-10; the natural peak of emission from tryptophan is at 340 nm.

CONCLUSIONS

To date, the processes of phytochemical transfer from plant to insect and distribution within the insect have been considered in limited senses only. Both insecticide and phytochemical studies have stressed enzymatic detoxification and elimination (subtractive flux), neglecting processes of additive flux into the insect to target sites. This neglect is due partly to lack of knowledge about uptake, transport, and distribution in insects of xenobiotics such as insecticides, and partly to conflicting ideas about those processes [16,17]. Advances in biochemical understanding, especially of hemolymph protein chemistry, will contribute to concepts and methods for studying mechanisms of additive flux.

In a sense, hemolymph serves as the final buffer between external environment and internal tissues, and is in contact with that environment only through cuticular or gut epithelia. Its prime relevance to plant-insect interaction lies in its role in the disposition of acutely toxic defensive compounds (i.e., in qualitative plant defenses).

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