Natural Pest Control Agents

A symposium sponsored by the Pesticides Subdivision of the Division of Agricultural and Food Chemistry at the 149th Meeting of the American Chemical Society, Detroit, Mich., April 8, 1965.

Donald G. Crosby, *Symposium Chairman*

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FOREWORD

ADVANCES IN CHEMISTRY SERIES was founded in 1949 by the American Chemical Society as an outlet for symposia and collections of data in special areas of topical interest that could not be accommodated in the Society's journals. It provides a medium for symposia that would otherwise be fragmented, their papers distributed among several journals or not published at all. Papers are refereed critically according to ACS editorial standards and receive the careful attention and processing characteristic of ACS publications. Papers published in ADVANCES IN CHEMISTRY SERIES are original contributions not published elsewhere in whole or major part and include reports of research as well as reviews since symposia may embrace both types of presentation.

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Natural Pest Control Agents

DONALD G. CROSBY

Agricultural Toxicology and Residue Research Laboratory,
University of California, Davis, Calif.

Man has made extensive use of chemicals in his continual battle against insects, weeds, pathogenic fungi, and the many other "pests" which compete for his food and fiber. However, he is not original in this use—other life forms have long employed a variety of compounds in attempts to control their own competitors. Insects, plants, and fungi all are known to affect one another chemically, and knowledge about such substances and their biological significance can lead to important new methods and agents for our benefit.

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"Pesticide" has become a household word. Through the combined but not always parallel efforts of certain federal agencies, local newspapers, popular writers, and others, this important class of agricultural chemicals is the subject of increasingly widespread discussion, research, and use. The manufacture of pesticides has become a billion-dollar industry.

The American College Dictionary defines the word as follows: "Pesticide. A chemical substance for destroying pests such as mosquitos, flies, etc." This implied restriction of the term to chemical insecticides was the accepted practice for many years—"pesticide" and "insecticide" were used interchangeably and, to some extent, still are so used today. However, many other types of living things long have been recognized and fought as enemies of man's health, food supply, clothing, and general welfare, and broader definitions now have come into general use.

Webster's Third International Dictionary refers to a pesticide as "an agent (as a chemical) used to destroy a pest: Economic poison." The *Federal Register* for March 27, 1964, further defines

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economic poisons to include "all preparations intended for use as insecticides, rodenticides, nematocides, fungicides, herbicides, amphibian and reptile poisons or repellents, bird poisons or repellents, fish poisons or repellents, mammal poisons or repellents, invertebrate animal poisons or repellents, plant regulators, plant defoliants, and plant desiccants." The *Federal Register* also offers further official definitions for many of these kinds of pesticides. For example, the term "fungicide" includes "all preparations intended for preventing, destroying, repelling, or mitigating any fungi or viruses (other than those on or in living man or other animals)," including but not limited to "plant fungicides, seed fungicides, fungicidal wood preservatives, and mildew and mold preservatives, and disinfectants, sanitizers, and sterilizers, except those for use only on or in living man or other animals." And so on.

Complete as the above definitions appear to be, neither "pesticide" nor "economic poison" seems to convey clearly the major purpose in man's use of such chemicals—that is, the control over the undesired presence and activities of other living species. Now, man is not alone in this purpose although his meager success in some aspects of the control of his pests with chemicals often has led him to congratulate himself on his inventiveness and intelligence. It is apparent that most if not all other kinds of living things also are in constant competition with their own pests, and many have developed chemical methods of defense and aggression which are remarkedly effective. It is with these agents—the natural pest control chemicals used by both man and other organisms—that this volume is concerned.

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Control of Insects

With the present major emphasis on the preparation and testing of a seemingly endless number of synthetic organic compounds for pesticidal activity and the clamor over their possible adverse effects on public health and our general environment, comparatively few people realize that certain natural insecticides have been used by man for centuries. The ancient Romans apparently utilized false hellebore as a rodenticide and insecticide (*13*), and preparations of *Tripterygium wilfordii* (thunder-god vine) and *Derris* species have been employed by the Chinese for hundreds of years as insecticides (*16*). The insecticidal properties of sabadilla (from *Schoenocaulon* species) were known in the sixteenth century; tobacco was in use as

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an insecticide in France prior to 1690; the manufacture of pyrethrum was begun in Europe about 1828; and rotenone was used against nutmeg insects in Singapore before 1848.

All together, thousands of higher plant species have been examined for insecticidal properties, and more than 2000 have been indicated to possess some activity. In many instances, the effectiveness of the constituents is equivalent to that of commercial botanical compounds such as rotenone, and frequently the plant or its extractives have a history as folk remedies for combating insect pests. Several of the most interesting examples are discussed briefly by Feinstein; *Nicandra physaloides* is a case in point. This species, which is known also as the "Peruvian ground cherry" and the "shoo-fly plant," repels insects and is used as an insecticide in India. "Stories told about it are many: The plant distributed around a room repels flies; in a greenhouse, it causes the whitefly to disappear; a few hundred planted near a barn apparently keep the animals from being bothered by flies" (*13*). Although these properties have been known and used for decades, it is only very recently that work has been reported on the chemistry of the active principle—a still unidentified ketone for which the name "nicandrenone" was coined (*23*).

Unfortunately, nicandrenone is typical of many instances in the field of natural pesticides where elucidation of chemical structure has not previously received the attention it deserves. However, with the increased availability of powerful instrumental techniques, considerable progress has been made during the past decade. Since the last general review (*14*), active principles from several more of the time-honored insecticidal plants have yielded to structure elucidation (Table I).

Table I. Recently Identified Natural Insecticides

Species and Common Name	*Compound*	*Structure*	*Ref.*
Mammea americana (Mamey)	Mammein	I	(*10*)
Mundulea sericea	Mundulone	II	(*15*)
Pachyrrhizus erosus (Yam bean)	Pachyrrhizin	III	(*29*)
	Erosnin	IV	(*11*)
Quassia amara (Quassia)	Quassin	V	(*6*)
Schoenocaulon officinale (Sabadilla)	Cevine	VI	(*18*)
	Veracevine	VII	(*18*)

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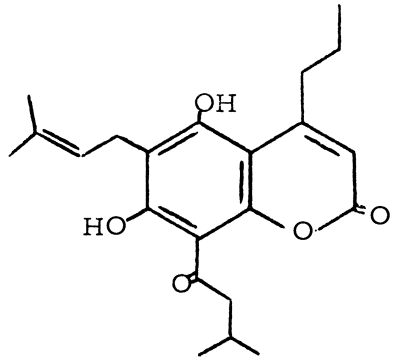

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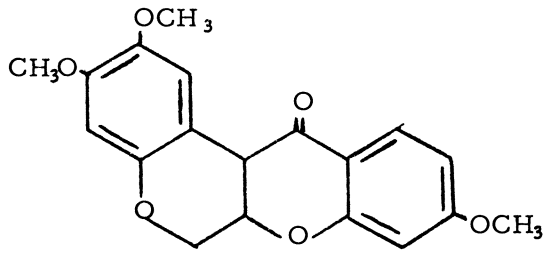

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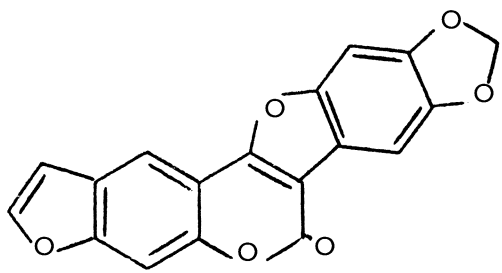

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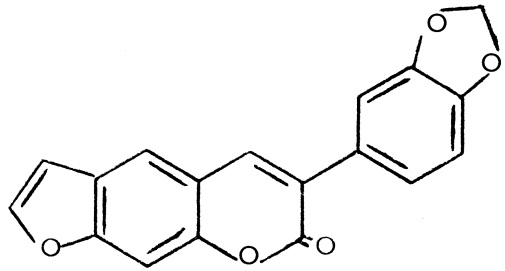

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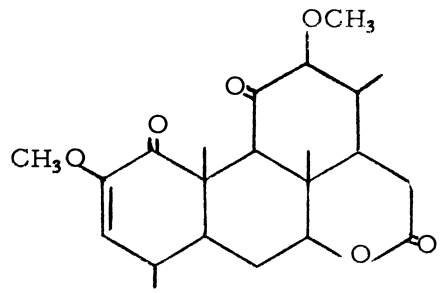

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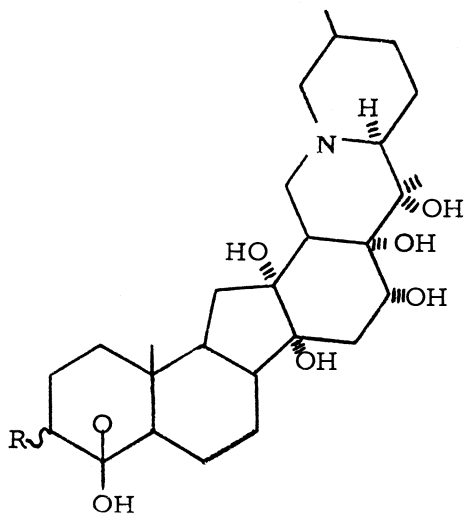

VI: R = α — OH VII: R = β — OH

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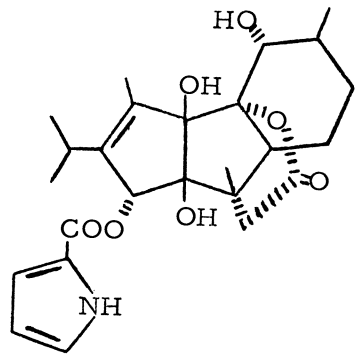
Despite the recent efforts of Beroza (*3*), the insecticidal alkaloids of the thunder-god vine (*Tripterygium wilfordii*) remain only partially characterized. A structure has been proposed for anhydroryanodine (VIII), a major degradation product of the active principle (ryanodine) of the commercial insecticide ryania (*34*), and a structure for the parent compound will be proposed soon by Rapoport and his coworkers (*27*). This again illustrates the importance of collaboration between physical and biological scientists and the intriguing research which each can reveal to the other.



VIII

It would seem obvious that efforts should be concentrated on following up the many leading observations which have been reported and determining the structure of the substances already isolated. However, re-examination of the biology and chemistry of species which have yielded insecticidal compounds in the past certainly should not be overlooked. Dr. Beckman will show that careful investigation of such a well-known commercial preparation as pyrethrum extract can reveal the presence of unsuspected and active new constituents; Dr. Moore will indicate how some such observations may be turned to profit through synthesis of simple analogs.

Despite the extensive screening of plants for insecticidal properties, only a vanishingly small proportion of the world's species have been examined. Apparently, even some very common kinds of plants have been overlooked: Dr. Lichtenstein will discuss his research on natural insecticides in turnips, rutabaga, and other garden vegetables.

Lower plants appear to have been virtually ignored in the search for natural insecticides, although Wilcoxon, Hartzell, and Wilcoxon

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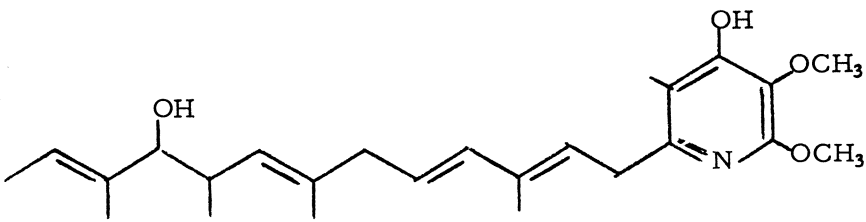
(*35*) described the isolation of potent compounds from extracts of the fern *Aspidium filix-mas*, and recent Japanese work on "piericidin" (IX) (*32*) indicates that common fungi such as *Streptomyces* species also may produce new types of insecticidal substances. The large volume of microbial cultures under examination for antifungal and antibacterial activity may represent a wealth of new leads for natural pesticides if the concept of "antibiotics" could be extended to include larger, but still unwanted, forms of life. It is probable that many cultures which have been discarded because of insufficient antimicrobial activity actually contain natural pesticides which either could be used commercially themselves or serve as models for synthesis of more simple, active substances.

A possible example of this thesis is the crystalline insect toxin found in *Bacillus thuringiensis* spores and discussed here by Dr. Anderson. Although neither the bacillus nor its spores exhibit useful "antibiotic activity" against other microorganisms, the very specific toxicity to insects has become of major commercial interest. The enormous number and variety of fungal species available for further examination must lead inevitably to one or more which produces pesticidal metabolites.



IX

Thus, far, this discussion has been concerned with plant products which kill insects through toxic action. Other means also should be considered important to the solution of insect pest problems, and among the most useful may be attractants and repellents. Many of these substances show a high degree of species specificity in their activity and so might offer the hope of pest control without harm to the useful insects inhabiting the same area. Dr. Jacobson has provided an excellent review of current work in this broad field. Insect sex attractants are among the most selective agents and are receiving considerable scientific attention, but other mechanisms for attraction

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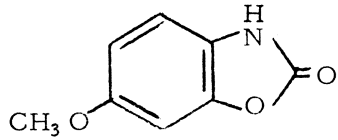
undoubtedly are of major significance. For instance, the strong attraction of fruit flies to certain fruit-borne yeasts, the biological basis for the attractiveness of commercially used protein hydrolyzates, and the agents responsible for the ability of certain plants to lure specific insects to them still require scientific investigation and explanation.

Until rather recently, our choice among chemicals repellant to insects was very limited (*9*), and some of the available remedies seem to have been almost equally repellent to their human users. The most familiar of them undoubtedly is the classical "oil of citronella," a mixture of plant terpenes which consists principally of geraniol, citronellol, and citronellal. It is a remarkable coincidence that at least one insect species, an ant discussed by Dr. Happ, also makes use of some of these same terpenes as repellents against other insects. It biosynthesizes them *de novo* rather than simply taking them from plant sources. Many other examples of insect repellency have been observed (*9*), and Roth and Eisner (*28*) list over 30 compounds which have been identified as defense substances of anthropods.

The insect's choice of food may be governed to a considerable extent, as ours is, by "attractants" and "repellents." In many instances, the actual insecticidal action of plant extractives may be due primarily to an artificially high level of application, while, in fact, the parent plants are only repellent in the field. This repellency may appear to be resistance on the part of the plant, and the chemistry of such "resistance factors" has begun to receive much-needed attention. For example, Smissman and his coworkers have examined the chemical basis for the inherited resistance of some strains of corn to attack by the European corn borer. 6-Methoxybenzoxazolinone (X) was isolated (*2, 30*) and shown to be one of the principal resistance factors, and a number of synthetic analogs were found to



X

possess as great or greater insecticidal activity. With information such as this, it should become possible for plant breeders to aim directly toward a high level of resistance against specific insects by means of a rapid and objective chemical analysis. Such investigations

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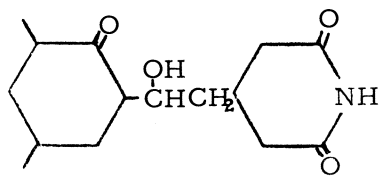
also can lead to the discovery and development of new types of insecticidal compounds and new mechanisms of insecticidal action.

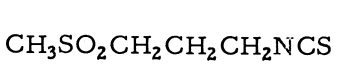
Control of Plant Disease

Research on natural fungicides and bactericides has become of major worldwide importance. The U. S. antibiotics industry, based entirely on this field of research, now produces over 1000 tons of these substances annually, valued at more than $250,000,000. The names are becoming common words–streptomycin, penicillin, bacitracin, Terramycin.

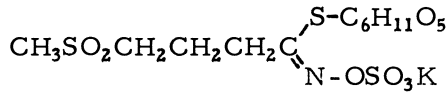
By far the greatest emphasis in the research and application of microbial antibiotics has been concerned with animal and human health. Despite the extent of the problems relating to plant disease, the practical use of antimicrobial antibiotics in plant agriculture has been limited to a few crops and compounds: Only streptomycin and actidione (XI) at present enjoy commercial importance. This may be due in part to the wider applicability of the less expensive synthetic fungicides and in part to the difficulty of arousing much public sympathy for a sick plant.



XI



XII



XIII

A few antibiotics have been isolated from higher plants, but none have been used commercially in practice. Such substances might be found in any plant part: Seeds of the wallflower (*Cheiranthus cheiri*) have yielded cheirolin (XII) and its parent thioglucoside (XIII) (*20*); pinosylvin (XIV) and several thujaplicins (XV = α — thujaplicin) are found in conifer heartwoods and equal sodium pentachlorophenate in their fungicidal effectiveness (*21*); and allicin (XVI) is an active constituent of garlic, the bulb of which actually has provided extracts used in practice as an antibiotic spray!

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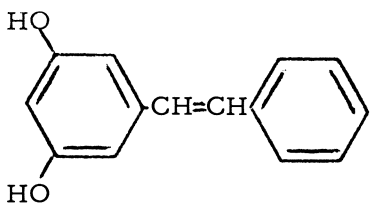

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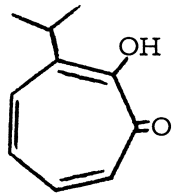
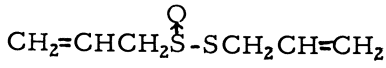
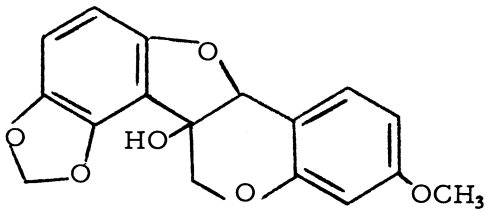


XV



XVI



XVII

It is apparent that we are surrounded by natural fungicides which must be very effective indeed. All together, several thousand higher plant species have been tested for antifungal activity (*31*). However, most plants seem to be remarkably resistant to the wide variety of pathogenic fungi and bacteria to which they must be almost continually exposed. Although a certain proportion of this resistance is mechanical or structural, recent investigations have already shown that many species can produce chemical agents which actually control invading microorganisms (7). Pisatin (XVII), recently isolated from the garden pea *Pisum sativum*, is one example. Dr. Stoessl describes his own current research on another example: an interesting antifungal substance in barley which appears to be responsible for the resistance of certain varieties to *Helminthosporium* infection. Some natural fungicides appear to be normal constituents of higher plants while the formation of others is induced by fungal attack. The chemistry, biochemistry, and eventual practical application of these findings offer an exciting realm of possibilities.

Plant viruses present a related and economically very serious agricultural problem. Although there appears to be little established

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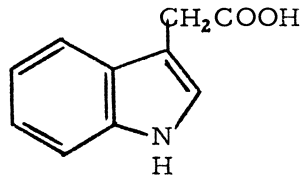
information on the reasons for host-virus specificity in plant species or varieties, Dr. Cheo provides some original and intriguing thoughts which give hope for future chemical means of virus control.

Control of Plant Processes

Present knowledge about the variety of natural chemical compounds which regulate the living processes in higher plants—processes including germination, shoot and root growth, flowering, and senescence—has been reviewed recently by Nitsch (*24*). Chemical research in this field is surprisingly scant, and, of the few regulators



XVIII

whose structures have been proved, only the growth substance mixture known as "gibberellic acid" has achieved commercial use at present. Indole-3-acetic acid (XVIII) also has seen limited application, and the recent advent of a very economical process for its synthetic production (*19*) may offer more encouragement.

One of the economically most important areas in the control of plant processes is defoliation—the intentional removal of leaves. Large quantities of several chemicals currently are employed for this purpose, including magnesium chlorate and DEF (*S,S,S*-tributyl phosphorotrithioate), and the principal commercial value is for defoliation of cotton. Several synthetics such as 4-chlorophenoxyacetic acid and naphthalene-1-acetic acid are used to provide the opposite but related effect of retarding or limiting fruit drop in apples, stone fruits, and grapes.

The phenomenon of leaf and fruit drop is known as abscission and has been suspected for some time to be under hormonal control. Dr. Addicott's description of the structure and properties of "Abscisin II," the abscission hormone isolated from cotton, climaxes over 12 years of his own investigations on the subject and represents perhaps the greatest advance in plant hormone research in the past decade. It also provides an unusual and unsuspected structural model to guide

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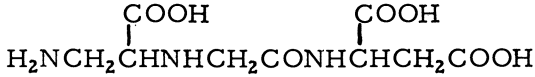
rational synthesis of new, more effective, and less hazardous means for the intentional control of abscission.

Natural herbicides produced by microorganisms have had a profound influence on world agriculture. Many species of higher plants have survived the ages because of their ability to detoxify or avoid the toxic effects of these compounds, and extensive effort has gone into the commercial breeding of other species to develop these abilities. Still, the structural variety of natural herbicides appears to be so great that their effects are still of major concern in food production.

I refer, of course, to the toxins produced by phytopathogenic fungi and bacteria. Considering the widespread damage which they cause, it is remarkable that so little is known about the chemistry of these substances. Perhaps the first "toxin" to receive detailed chemical examination was lycomarasmin (XIX) (*36*), an isolate from



XIX

culture media of *Fusarium lycopersici* which could bring about the typical wilting in treated tomato plants. Although both the structure and the importance of lycomarasmin in "fusarium wilt" disease are in question (*21*, *25*), its identification nonetheless represents one of the first recognitions of even the existence of fungal toxins.

Alternaric acid (XX) from the "early blight" fungus *Alternaria solani* (*1*), diaporthin from the sweet chestnut blight organism *Endothia parasitica* (*4*), and fusaric acid (XXI) (*33*) from *Fusarium lycopersici* and *Gibberella fujikuroi* represent other phytotoxic compounds thought to be associated with fungal diseases of higher plants, and a structure (XXII) has been proposed for the bacterial toxin responsible for "wildfire" disease (*37*). Current work has been reviewed recently (*26*). In view of the relative simplicity of these structures and the comparative ease with which fungi may be cultured in quantity, it is to be hoped that this promising field of investigation will receive greatly increased attention in the future.

As an example of the fascinating current progress, Dr. Spencer will describe a beautiful piece of research on the isolation and structure proof of the toxin from *Helminthosporium sativum*, an important

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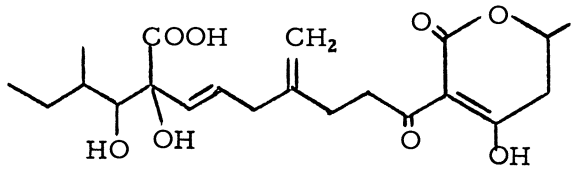

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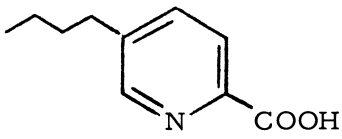

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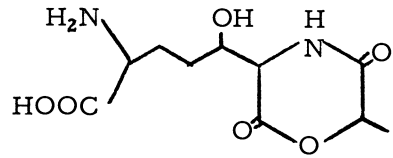
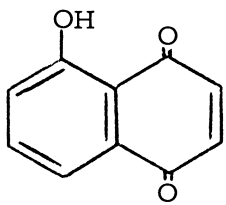


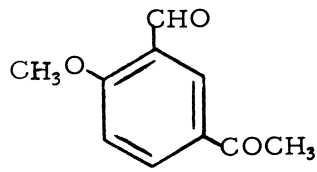
XXII

pathogen of barley, wheat, corn, and many other grasses. It not only represents an unusual type of terpene not previously reported to occur in microorganisms, but it also offers a structural type of systemic herbicide which probably would never have been suspected to possess this biological activity otherwise.

Higher plants, too, have been shown to produce phytotoxins. "Allelopathy," as the chemical effect of one higher plant on another is called, has been recognized for many years (*21, 22*), but again, chemical investigation has lagged far behind the biological observations. Several natural systemic herbicides have been identified, however: 5-hydroxy-1,4-naphthoquinone (XXIII) from the black walnut (*Juglans nigra*) (*8*), 3-acetyl-6-methoxybenzaldehyde (XXIV) from



XXIII



XXIV

Encelia farinosa (*17*), and *trans*-cinnamic acid from guayule (*Parthenium argentatum*) (*5*). Some chemical and biological properties of natural herbicides from *Thamnosma montana* are described in this

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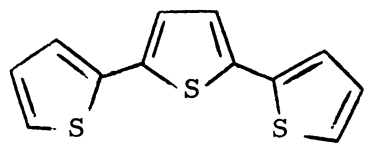
volume, and, like them, extractives from many other higher plants have been shown to inhibit seed germination (*12*). It appears that the concept of neither pre-emergent nor postemergent herbicidal action is anything new in nature. However, Dr. Moreland discusses not only the general subject of herbicides from higher plants but also the reverse of pre-emergent herbicidal action—germination stimulation as a means of weed control. He also presents some interesting observations on the use of such substances as models for a commercially interesting and novel application.

Conclusion

This brief preface and the following pages offer examples of the chemical substances with which insects influence other insects, fungi influence plants (and *vice versa*), plants influence other plants, and many other agents regulate the complex interactions of living things. Still other intriguing substances include natural nematocides such as terthienyl (XXV), natural rodenticides and repellents such as the



XXV

Scylla glycosides and actidione (XI), the furocoumarin and rotenoid fish poisons, and many other toxic, repellent, or attractive natural pest control chemicals. Most of these compounds undoubtedly offer survival value for their living manufacturers and permit them to compete more successfully in their own particular environments than could their less-endowed relatives. A wide variety of chemical types is represented in these natural products although terpenoid heterocycles appear with unusual frequency, and perhaps at least a few may provide useful models toward either more simple synthetic agents or new modes of regulatory action. There are important lessons to be learned from observation of the attempts of other species to control with chemicals the competing living forms which jeopardize their welfare. In fact, our observations generally have only begun to yield insight in this field, and the most interesting and useful work undoubtedly remains to be done. Compared to many of our living co-inhabitants

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of this planet, man appears to be strictly a neophyte in the pest-control field.

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2

Natural Insect Attractants and Repellents, New Tools in Pest Control

MARTIN JACOBSON

Entomology Research Division, Agricultural Research Service,
U. S. Department of Agriculture, Beltsville, Md.

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Chemical attractants and repellents produced by plants and by insects themselves may be the answer to the public's demand for more potent insect-control agents and the demand of health agencies for less toxic residues. The most active attractants, which may be effective in infinitesimal concentration at a distance of ¼ mile or more, are the sex attractants; they are highly specific and therefore excellent tools for detecting and estimating insect populations. These and other natural attractants and repellents are potentially useful in several ways for controlling harmful insects. Developments in present investigations as well as those conducted during the past five years are reviewed.

One of the most important aspects of pest control now being investigated by the Entomology Research Division is the use of insect attractants and, to a lesser but nonetheless increasing extent, of insect repellents. Plagued by the growing problem of insect resistance to insecticides, insecticide-residue problems, and the need for more selective and effective control measures, scientists have been investigating insect attractants and repellents as a means of combating the insect hordes that take heavy toll of our crops, livestock, timber, household products, and even our health. These investigations have turned up powerful materials of both synthetic and natural origin and amply demonstrated that, when properly used, they can greatly increase the efficiency of control or eradication measures.

What is the new concept in this field? Simply this. Research within only the past two or three years has made clear that substances

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produced by insects themselves as a requisite to their reproduction and defense, substances which have enabled certain species to survive or thrive in a hostile environment, can now be used by man to cause these species to promote their own destruction and that of other harmful species. In addition, numerous plants produce chemical substances which they use to defend themselves from insect attack. Many compounds isolated from natural sources are free of the toxic effects to humans shown by many common synthetics.

This is not a complete review of the literature of naturally occurring substances that are being or may be used in pest control. Excellent reviews cover well the older literature of natural insect attractants (*9*, *32*, *37*, *40*, *41*, *43*, *44*, *71*) and repellents (*7*, *17*, *22*, *25*, *54*, *63*, *64*). Only the more important recent developments in this field are discussed here. I hope that this report will stimulate the increased interest and investigation on the part of both chemists and entomologists, working together, that is needed to make the concept a reality.

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Attractants

The natural attractants, especially those which elicit a sexual response in an insect, hold the greatest potential for use in insect control, either alone or as supplements to other control agents. The combined potency and specificity of the sex attractants have made them invaluable for the detection and estimation of insect populations. The insect, responding to a lure-baited trap, is caught and thereby signals the presence of its species. Thus, a good attractant can provide the earliest detection of an infestation before it can enlarge or spread. Control measures need be applied only to areas where the insect is found and only as long as it continues to be present. In this manner, considerable economies may be effected and needless broadcasting of toxic materials be avoided. If the goal is eradication, a good lure can help by accurately delineating an infestation and locating the last few hard-to-find insects. Excellent examples of such use involve the survey programs for the gypsy moth [*Porthetria dispar* (L.)] in New England (*35*, *37*) and the pink bollworm moth [*Pectinophora gossypiella* (Saunders)] in the cotton-growing areas of the United States and Mexico (*2*, *8*, *55*). In the case of bollworm moth, traps baited with a crude methylene chloride extract of the female bodies are being successfully used for survey in Texas, Ari-

zona, and northern Mexico. At Beltsville, we have just isolated and identified the powerful sex attractant of the pink bollworm moth and are attempting to synthesize it.

The sex attractants will lure large numbers of one sex into field traps, where they may be destroyed, with or without the use of a toxicant, or exposed to a chemical sterilant. Certain chemosterilants considered too hazardous to expose in the field could be exposed along with the attractants in dispensers that allow access only to the insect. Insects responding to the attractant could thus be brought in contact with the chemosterilant, or possibly be induced to feed on it by admixing it with a food lure. An excellent example of the tremendous potential for such annihilation or mass-sterilization procedures is the sex attractant of the introduced pine sawfly [*Diprion similis* (Hartig)] (*15*, *19*). Wooden traps utilized in a field program consisted of a board with a 2.5-inch screened opening in the center, suspended from trees in infested areas. A virgin female was placed in the screened portion and Tanglefoot was spread over the wooden portion. Traps baited with one virgin female attracted an average of 1000 males each; large numbers of uncounted males also fell to the ground. One trap with a virgin female exposed from 11 A.M. to 4 P.M. attracted more than 7000 males during this period; she continued attracting males at approximately 1000 per day until she died on the fifth day, after which small numbers were caught for the next 3 days. Males could be lured 200 feet out of the forest over an open field. Field control of the sawfly based on luring the males to an insecticide-attractant mixture seems unlikely, but chemosterilant-attractant mixtures should be more effective in reducing or eradicating a field population of this insect. Much greater damage to the insect population can be effected by rendering insects infertile by a chemosterilant than by killing them outright.

Once a sex attractant has been identified and prepared synthetically so that it is available in quantity, it might possibly be used to confuse males seeking females, making the use of a toxicant unnecessary. Wright (*73*, *75*) has theorized that insects can be controlled, at least in principle, by permeating the atmosphere with a sex attractant chemical so that the small additional quantity emanating from a female is imperceptible. He has coined the term "Metarchon" (*74*) for an external stimulus artificially introduced into the environment of an organism for the purpose of modifying its behavior by eliciting an inappropriate response or inhibiting an appropriate one.

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It has very recently been reported that insect sex attractants used in conjunction with black light traps will increase the catch of such traps severalfold. For example, Hoffman (*34*) has found that the presence of virgin female tobacco hornworm, tobacco budworm, or corn earworm moths in or near black light traps increased sixfold the number of male hornworms trapped. Shorey and Gaston (*66*) observed increased male cabbage looper responses to a visible light source during studies with sex pheromone extracts from female cabbage looper moths. Field and laboratory studies conducted by Henneberry and Howland (*33*) showed that black light traps baited with 50 virgin female cabbage looper moths caught approximately 20 to 30 times as many males as light traps alone; the number of females caught was not influenced by the presence of virgin females.

To date, the females of more than 150 species of insects have been reported to produce a sex attractant or excitant for the male, and males of more than 50 species have been shown to produce such materials to attract or sexually excite the females (*39*). Table I lists insect pests of the order Lepidoptera in which the occurrence of sex attractants has been reported since 1960. In addition, investigators

Table I. Lepidopterous Insect Pests in Which Sex Attractants Have Been Demonstrated Since 1960

Scientific Name	*Common Name*	*Ref.*
Agathymus baueri (Stallings and Turner)		(*59*)
Agathymus polingi (Skinner)		(*59*)
Autographa californica (Speyer)	Alfalfa looper	(*28*)
Carpocapsa pomonella (L.)	Codling moth	(*57*)
Diatraea saccharalis (F.)	Sugarcane borer	(*55*)
Galleria mellonella (L.)[a]	Greater wax moth	(*58*)
Grapholitha molesta (Busck)	Oriental fruit moth	(*23*)
Heliothis virescens (F.)	Tobacco budworm	(*29*)
Heliothis zea (Boddie)	Bollworm, corn earworm	(*28*)
Pectinophora gossypiella (Saunders)	Pink bollworm moth	(*53*)
Prodenia eridania (Cramer)	Southern armyworm	(*14*)
Prodenia ornithogalli (Guenée)	Yellow-striped armyworm	(*28*)
Protoparce sexta (Johannson)	Tobacco hornworm	(*3*)
Rhyacionia buoliana (Schiffermüller)	Pine shoot moth	(*56*)
Rhyacionia frustrana (Comstock)	Nantucket pine tip moth	(*72*)
Spodoptera exigua (Hübner)	Beet armyworm	(*25*)
Synanthedon pictipes (Grote and Robinson)	Lesser peach tree borer	(*18*)
Trichoplusia ni (Hübner)	Cabbage looper	(*36*)

[a] Sex attractant produced by the male.

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have reported during this same period the presence of female-produced sex attractants in eight species of cockroaches (*4, 6, 67*), the banded cucumber beetle [*Diabrotica balteata* (LeConte)] (*20*), introduced pine sawfly [*Diprion similis* (Hartig)], (*19*), Virginia-pine sawfly [*Neodiprion pratti pratti* (Dyar)] (*12*), mosquito [*Culiseta inornata* (Williston)] (*47*), housefly [*Musca domestica* (L.)] (*60*), *Hemicrepidius morio* (LeConte) (*16*), and *Praon pallitans* (Muesebeck) (*65*), and male-produced sex attractants in three species of cockroaches (*4, 5, 62, 67*), the boll weevil [*Anthonomus grandis* (Boheman)] (*46*), Mediterranean fruit fly [*Ceratitis capitata* (Wied.)] (*27*), and scorpion flies [*Harpobittacus australis* (Klug) and *H. nigriceps* (Selys)] (*13*).

Insects have demonstrated a remarkable ability to become resistant to insecticides. The use of an attractant upon which a species depends for survival, such as a sex lure emitted by the female, will probably be a permanent tool whether or not it is used with a toxicant since the insect's ability to reproduce will be seriously impaired if the male becomes less responsive to the scent that helps it find a mate (*12*).

Aside from the sex attractants, other powerful attractants are produced by insect host plants, and it is readily conceivable that the active substances, once they are known and available, may be placed with a toxicant at field locations to lure destructive insects away from crops and to their deaths. According to Yamamoto and Fraenkel (*76*), the same, as yet unidentified, glycosides are responsible for the attraction of host plants to larvae of the tobacco hornworm [*Protoparce sexta* (Johansson)] and Colorado potato beetle [*Leptinotarsa decemlineata* (Say)]. Powerful unidentified feeding stimulants have recently been reported for adults of the large milkweed bug [*Oncopeltus fasciatus* (Dall.)] (*26*), European elm bark beetle [*Scolytus multistriatus* (Marsh.)] (*48*), and corn rootworm [*Diabrotica longicornis* (Say) and *D. virgifera* (LeConte)] (*21*) in extracts of milkweed seed coats, elm bark, and corn kernels, silks, leaves, and roots, respectively, and for the desert locust [*Schistocerca gregaria* (Forsk.)] (*30*) in various extracts of a number of host plants.

In 1963, Keller and coworkers (*45*) and Neff and Vanderzant (*52*) reported the presence of a boll weevil attractant in cotton squares and seedlings. In the same year, an arrestant (a substance

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found accidentally by an insect, which then stops to feed thereon) and feeding stimulant for this insect was reported by Maxwell, *et al.* (*50*) in water extracts of all parts of the cotton plant, and by Jenkins *et al.* (*42*) in aqueous extracts of fresh cotton, okra, green bean, and cucumber.

The attraction of the bark of red pine (*Pinus densiflora*) to pine beetles, *Cryphalus fulvus* (Niijima) and *Blastophagus* (*Myelophilus*) *piniperda* (L.), has been found to be due to benzoic acid (*78*). This finding prompted Yasunaga *et al.* (77) to test for attractancy a number of esters of this and related acids. The most active compounds were *n*-propyl and isoamyl benzoates, *o*- and *p*-toluic acids, linolenic acid, and methyl linolenate. Adlung (*1*) had previously determined that a mixture of linseed oil methyl esters (especially methyl linoleate and methyl linolenate) in field traps was highly attractive to spruce bark beetles, *Ips typographus* (L.) and *Hylurgops glabratus* (Zetterstedt), and the products obtained by autoxidation of these esters showed increased attractiveness.

A good example of the usefulness of attractants produced by plants for controlling insects is that of methyl eugenol, the most powerful attractant yet found for the male Oriental fruit fly [*Dacus dorsalis* (Hendel)]. This compound, now available synthetically, is effective for one-half mile downwind; it is sought out and so greedily devoured by the males that they will, if permitted, engorge themselves until they die. Cane-fiber squares saturated with a mixture of *O*-methyleugenol and the toxicant naled (Dibrom) were dropped from planes onto the Pacific Island of Rota, located 37 miles north of Guam. Within 6 months, the insect was eradicated completely from the Island (*69*).

Repellents

An excellent review by Roth and Eisner (*63*) summarized the chemical defense substances found in arthropods up to 1962. These authors listed 31 defense substances of known structure: one anhydride, three carboxylic acids, nine aldehydes, one furan, three hydrocarbons, two ketones, one lactone, eight quinones, and three inorganic compounds. Many of these same compounds (unsaturated aldehydes and quinones) have been found in other arthropods since 1962 (*38*). The compounds are discharged when the animal is disturbed by predators, and there can be no doubt that the action of most of them

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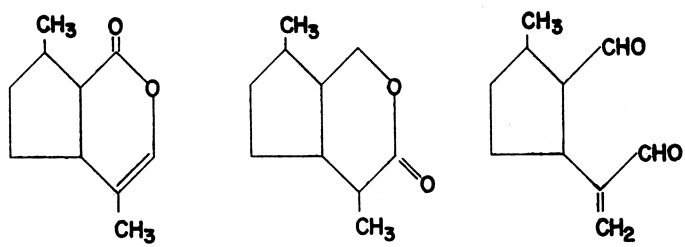
is repellent. It is therefore possible that a number of them can be used to protect forests, crops, man, and animals from insect attack. Some arthropods produce more than one volatile substance and in some cases each of these substances appears to have a specific function and to be considerably less effective for defense or repulsion when employed as a single compound. For example, acetic and formic acids are effective defense agents, if they are able to penetrate the protective armor and waxy coating of insects. The superior solvent properties of caprylic acid appear to aid penetration of the epicuticle by more toxic substances of lower molecular weight (*51*). Dramatic examples of the direct repellent effect of the unsaturated aldehydes (especially *trans*-2-hexenal) on ants and other insects have been cited by Bevan *et al.* (*10*), Blum (*11*), and Weber (*70*). The diplopods, such as millipedes, produce mainly quinones for defense, whereas the insects produce mainly aldehydes and acids.

In 1964, Eisner (*24*) reported on his investigations of catnip (nepetalactone) (I) as a possible defensive substance protecting the plant (*Nepeta cataria*) that produces it against phytophagous insects. The response of 17 species of insects to the vapors emanating from the tip of a capillary tube filled with liquid nepetalactone was distinct avoidance for most of them. Only relatively few species remained undisturbed by it. Insects that responded to the vapors also reacted characteristically to direct contact with the liquid. Two species of ants foraging along trails stopped abruptly before a droplet of nepetalactone placed on the trail. A freshly killed cockroach with a droplet of it on its abdomen repelled attacking ants. In view of the proved repellency of I and of the related iridomyrmecin (II) (*61*) and anisomorphal (III) (*68*), the cyclopentanoid monoterpenes might be profitably investigated for potential practical use as insect repellents.



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Although the boll weevil, a very serious cotton pest in the South, feeds voraciously on cotton squares and bolls in response to an attractant or attractants contained therein, these same cotton parts (*49*) contain also a boll weevil repellent that shows its effect once the attractant has volatilized completely. Department of Agriculture scientists are attempting to isolate and identify this repellent, which presumably could be applied to ward off weevil attack. Cotton seedlings painted with an aqueous emulsion of the material effectively repelled 100% of the weevils for 5 hours and only medium damage was evident after 12 hours, whereas control seedlings were completely destroyed after only 2 hours.

Repellency appears to be associated with the highly pungent odor of the material since physical contact is unnecessary. The oily repellent is stable to heat and is not phytotoxic to the seedlings when proper aeration is provided.

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Biosynthesis of Monoterpenes in an Ant *(Acanthomyops claviger)*

G. M. Happ[1] and J. Meinwald

Department of Chemistry and Division of Biology, Cornell University, Ithaca, N. Y.

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Terpenoid substances are of broad distribution and diverse function in insects. One set, elaborated by the mandibular glands of Acanthomyops claviger, *acts both as a defensive secretion and as an alarm releaser. When fed* C^{14}*-labeled acetate or mevalonate, laboratory colonies of these ants produce radioactive citronellal and citral, providing unambiguous evidence for* de novo *synthesis of these terpenes by the ant. The incorporations of these precursors implicate the mevalonic acid pathway as the likely biosynthetic route.*

The defensive glands of arthropods produce a variety of chemical substances, among which are formic acid, *p*-benzoquinones, and aliphatic aldehydes (*14, 16*). Often the particular molecular species employed as toxins are representatives of chemical classes of wide biological distribution. Although Brower and Brower (*2*) have suggested that many natural insect toxicants are derived from secondary plant substances, at present it is far from clear whether most must come from such dietary sources or whether the majority can be synthesized *de novo* by insects. As defensive secretions can be isolated relatively easily from their capacious integumentary reservoirs, they offer especially favorable material for biosynthetic studies. Aside from the work of Waterhouse and coworkers (*10*) which demonstrates the incorporation of C^{14}-labeled acetate, propionate, caproate, or decanoate into all major aliphatic constituents of the

[1] Present address: Department of Biology, Catholic University of America, Washington, D. C.

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defensive secretion of a pentatomid bug, little data is available on their biogenesis.

The monoterpenes, which include citronellal and citral identified in the mandibular gland secretion of the ant *Acanthomyops claviger* (Roger) (*3*), comprise one of the more interesting classes of defensive substances. These and related isoprenoid molecules serve as physiological or behavioral messengers in a variety of insect groups (*19*, *23*, *24*). In spite of a few exceptional cases (*4*, *6*, *15*), as a rule insects do not manufacture steroids (*5*, *11*); thus ecdysone, the molting hormone, appears to be derived from ingested cholesterol (*12*). In contrast, Schmialek (*17*) has shown that after the injection of 2-C^{14}-mevalonic acid into silkworm caterpillars, radioactive farnesol can be recovered. In the present study, we are concerned with the biogenesis of the monoterpene aldehydes which serve as alarm releasers and defensive substances for *Acanthomyops* (*9*).

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Table I. Experimental Results

	Sodium Acetate		*Mevalonic Acid Lactone*	
	1-C^{14}	*2*-C^{14}	*1*-C^{14}	*2*-C^{14}
Compound fed				
Amount, mg.	0.18	0.048	6.2	2.32
Activity, d.p.m.	2×10^7	3×10^7	1×10^7	1×10^7
Derivatives isolated				
Citronellal				
Total activity, d.p.m.	2.5×10^4	2.5×10^4	10	1×10^4
% incorporation	0.1	0.07	–	0.1
Specific activity, d.p.m./mmole	3.3×10^6	3.3×10^6	–	1.4×10^6
Citral				
Total activity, d.p.m.	2.5×10^2	1.5×10^3	0	6.0×10^2
% incorporation	0.001	0.005	–	0.006
Specific activity, d.p.m./mmole	3.3×10^5	2.7×10^6	–	1×10^6

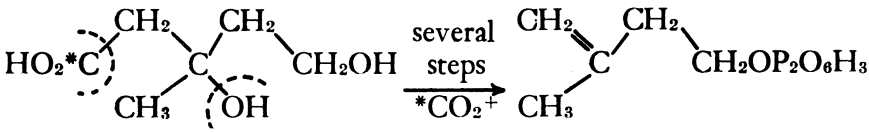
Laboratory colonies of worker ants were fed sugar-water containing 1-C^{14}-acetate, 2-C^{14}-acetate, or in a carefully controlled simultaneous feeding, 1-C^{14}- or 2-C^{14}-mevalonate. After an appropriate period, the ants were frozen and extracted with methylene chloride and the terpene aldehydes (citronellal and citral) isolated by thin-layer chromatography. These were then converted into their dini-

trophenylhydrazones, which were further purified to constant radioactivity.

Results and Discussion

Acetate, labeled at either the methyl or carboxyl position, was significantly incorporated into both citronellal and citral. 2-C^{14}-Mevalonate was similarly well incorporated, but, in contrast, 1-C^{14}-mevalonate produced only very slight radioactive labeling in the terpenes. The results of these experiments are summarized in Table I.

It is immediately clear that *Acanthomyops* need not rely on dietary sources of terpenes but can synthesize citronellal and citral from either acetate or mevalonate. The higher total activity of the citronellal as compared with the citral probably reflects the natural preponderance of citronellal (*ca.* 90%) in the ant secretion. As the specific activities show, these results are consistent with a common biogenetic origin of both terpenes. In the mevalonic acid pathway as described from other organisms (*13*), the radioactive carbon of 1-C^{14}-mevalonate is lost upon formation of isopentenyl pyrophosphate.



In *Acanthomyops*, the strikingly different incorporations following the two mevalonate feedings indicate that mevalonate is not degraded before being built into terpenes but rather is decarboxylated, as in the classical mevalonic acid pathway.

The presence of the mevalonic acid pathway for terpene biosynthesis suggested that these ants might also manufacture steroids. To investigate this possibility, a new sample of ants from the 1-C^{14}-acetate experiment were examined for the presence of radioactive cholesterol or other β-hydroxysteroids. The ants were extracted with ethanol and ether, this lipid extract was saponified, and the nonsaponifiable material (plus a few milligrams of nonradioactive carrier cholesterol) chromatographed on a deactivated alumina column (*18*). Upon elution with a solvent series of increasing polarity (*see* Figure 1) the cholesterol was recovered in fractions 96 to 104 (benzene). It was further purified by thin-layer chromatography and by preparation

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of its digitonide. To make certain that other β-hydroxysteroids of *Acanthomyops* would not be missed, the remaining fractions were pooled under their respective solvents and the digitonides of each were prepared. When assayed for C^{14}-incorporation, none of the

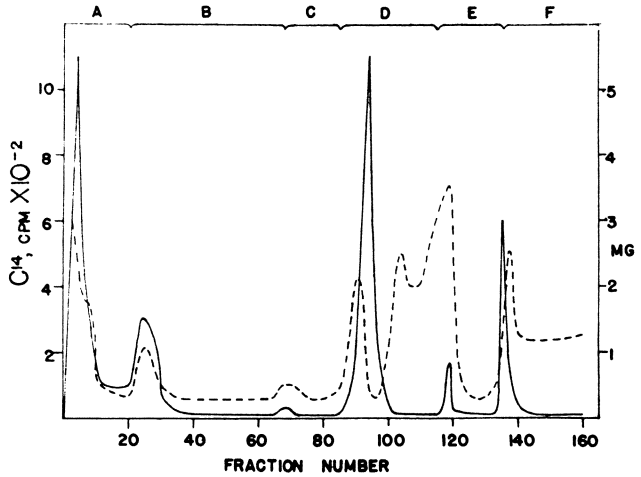

Figure 1. Chromatography of nonsaponifiable lipids from A. claviger *after feeding of 1-C^{14}-acetate*

Absorbent: 20 grams of deactivated Merck alumina eluted with:

A. Petroleum ether
B. Petroleum ether-benzene (10 to 1)
C. Petroleum ether-benzene (4 to 1)
D. Benzene
E. Diethyl ether
F. Methanol
____ *Weight recovered*
---- *C.p.m. recovered*

digitonin-precipitable material was radioactive. Apparently the mevalonic acid pathway is employed by *Acanthomyops* for the synthesis of monoterpenes but not for the formation of steroids.

Experimental

Assay of Radioactive Compounds. The radioactive samples were counted on steel planchets in a Nuclear Chicago Model D-47 low-background gas-flow counting chamber with an absolute counting efficiency (estimated by comparison with a standard) of about 20%.

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Purification of Carrier Compounds. Citronellal (b.p. 99–99.5°/25 mm.) and citral (b.p. 92–93°/4.2 mm.) were purified by distillation, and the purity was checked by vapor-phase chromatography. Cholesterol (m.p. 148.5–49.5°) was purified via its dibromide (*8*).

Administration of Tracers. Workers of *Acanthomyops claviger* (Roger) were freshly collected near Ithaca, N. Y., for each experiment. Over the course of each feeding, 1000 to 1500 ants were maintained in a two-chamber Lucite Wilson nest. One chamber was filled with moist earth and shielded from light while the other served as a foraging area. C^{14}-labeled acetate and mevalonate were fed in glucose solution and were distributed throughout the colony by regurgitative feeding (*22, 24*). In the experiments with 1-C^{14}- and 2-C^{14}-mevalonate, all workers were collected from a single natural colony and were individually sorted into one or the other laboratory colonies to avoid any possible bias due to physiological caste differences analogous to those reported in other ant species (7).

Isolation of Citronellal and Citral. At the close of each experiment (7 to 10 days), the nests were frozen intact. Groups of 200 workers were placed in a micro-Soxhlet apparatus and extracted for 8 hours with methylene chloride. A few milligrams of carrier citronellal and citral were added and the mixture was applied to a thin-layer chromatoplate (silica gel G) which was developed with hexane–ethyl acetate (92 to 8) to separate citronellal and citral (*3*). The aldehydes were detected by spraying with a solution of 2, 4-dinitrophenylhydrazine in tetrahydrofuran (*20*) and the citronellal and citral peaks were scraped off and allowed to react with excess dinitrophenylhydrazine reagent for a further 12 hours.

The dinitrophenylhydrazones were separated from the reaction mixture by thin-layer chromatography (silica gel G developed with benzene) and further purified by thin-layer chromatography on aluminum oxide G (petroleum ether–diethyl ether (96 to 4), silica gel G (chloroform), and silica gel G (diethyl ether)). In all cases, the specific activities of the dinitrophenylhydrazones remained constant over the course of the last two purifications.

Collection of Nonsaponifiable Lipids. Two hundred ants from the 2-C^{14}-acetate feeding were ground with sand and the resulting brei refluxed for 4 hours in 25 ml. of ethanol, ethanol-diethyl ether (3 to 1), and diethyl ether (twice). The extracts were pooled, the solvents were evaporated, and the residue was saponified by refluxing for 1 hour with 20 ml. of methanolic potassium hydroxide (10% potassium hydroxide in 60% aqueous methanol). An equal quantity of water was added and the aqueous solution extracted three

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times with diethyl ether (100 ml. total) to isolate the nonsaponifiable fraction. The ether extract was then shaken with 100 ml. of 1% aqueous potassium hydroxide to remove any free fatty acids. The activity of the total extract was about 8.0×10^6 d.p.m. and that of the nonsaponifiable material 3.0×10^5 d.p.m.

Chromatography of Nonsaponifiable Lipids. The nonsaponifiable residue plus 4.5 mg. of carrier cholesterol was applied to the top of a 7.5×1.7 cm. column containing 20 grams of Merck alumina (suitable for chromatographic adsorption) which had been previously deactivated by mixing with 7% aqueous acetic acid (10% glacial acetic acid in distilled water) (*18*). The column was packed in petroleum ether (redistilled, b.p. 60-70° C.) and 10 ml. fractions were collected. The eluting solvents are shown in Table II.

Table II. Eluting Solvents

Fraction	*Solvent*
1–20	Petroleum ether
21–65	Petroleum ether–benzene (10:1)
66–85	Petroleum ether–benzene (4:1)
86–115	Benzene
116–135	Diethyl ether
136–160	Methanol

The only white solid recovered was in fractions 97 to 104. Thin-layer chromatography on silica gel G impregnated with Rhodamine 6G showed that only this fraction contained cholesterol (*1*). After a second chromatography using chloroform as the solvent, 3.25 mg. of white crystalline material with an activity of 2×10^2 d.p.m. was recovered. This cholesterol was further purified by preparation of its digitonide (*21*). All other fractions were pooled under their respective solvents, 1 mg. of carrier cholesterol was added to each, and the digitonides were prepared. No activity above background was detected in any of the digitonin precipitates.

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4

Insecticides Occurring Naturally in Crops

E. P. LICHTENSTEIN

Department of Entomology, University of Wisconsin, Madison, Wis.

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The edible parts of parsnips contain a chemical of insecticidal and strong synergistic nature. This chemical, present in a concentration of about 200 p.p.m., was isolated and identified as 5-allyl-1-methoxy-2, 3-methylenedioxybenzene or myristicin. Its toxicity to various insects (fruit flies, etc.) was established and compared with pyrethrum and aldrin. A second chemical, identified as 2-phenylethyl isothiocyanate, was found in the edible parts of turnips and rutabaga, which also have been consumed for centuries by humans without obvious harm. In the turnip, the concentration was 63 p.p.m. Toxic action against various insects was established. Two cabbage varieties, cauliflower, brussels sprouts, broccoli, kale, mustard, and kohlrabi also contain 2-phenylethyl isothiocyanate as evidenced by gas liquid chromatography and bioassay. Only root tissues had insecticidal activity.

One of the most difficult aspects of the search for useful insect control chemicals is the attempt to evaluate the potential hazard of the compounds on prolonged ingestion by man. It is therefore intriguing to seek insecticidal components in the edible parts of plants long consumed by man and animals without causing any obvious harm. Such chemicals might be of further importance in naturally protecting the edible portions of certain crops from insect attack, thus reducing the need for applied methods of insect control.

Since 1960, experiments have been conducted at the University of Wisconsin with edible parts of various crops grown on soil that was free of any pesticidal residues. In these tests, two different

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chemicals of strong insecticidal and, in one case, also of synergistic properties were discovered in some crops.

The edible parts of parsnips (*Pastinaca sativa* L.), which have been consumed for centuries by humans without causing any obvious harm, were found to contain a chemical of insecticidal and strong synergistic nature (*1*). The insecticidal constituent, present at about 200 p.p.m., was isolated and identified as 5-allyl-1-methoxy-2, 3-methylenedioxybenzene or myristicin. Its toxicity to various insects [vinegar flies, houseflies, Mediterranean fruit flies, mosquito larvae, Mexican bean beetles, and pea aphids] was established and compared with pyrethrum and aldrin (Tables I and II). The knockdown effect, although definite, was not as great as that of pyrethrum. In tests

Table I. Toxicity of Myristicin and Pyrethrum[a] (0.5% w./v.)

Insect	*Exposure, Hr.*	*Per Cent Mortality*	
		Myristicin (Nat.)	*Pyrethrum*
Mexican bean beetle	48	100	100
Mosquito larvae	24	100	100
Pea aphids			
Contact	48	60	100
Systemic (72+)	48	50	20
Houseflies	24	6	98
Spider mites	120	28	100

[a] From (*1*, p. 413).

Table II. Toxicity to Vinegar Flies, LD_{50}, μg.

	Exposure Time	
	1 hr.	*24 hr.*[a]
Myristicin	430	300
Pyrethrum	62	26
Aldrin	35	1

[a] Plus corn oil.

with vinegar flies (*Drosophila melanogaster* Meig.), it acted as a repellant and also killed through fumigant action, characteristics not evident with pyrethrum (Table III). Comparison of the synergistic activity of myristicin and piperonyl butoxide with houseflies showed that piperonyl butoxide was better with the pyrethroids, and myristicin with the carbamates tested (Table IV). With vinegar flies, the synergistic activity of myristicin was superior with Sevin, similar

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with *p,p'*-DDT and allethrin, and inferior with pyrethrum as compared with piperonyl butoxide (Table V).

The second chemical, identified as 2-phenylethyl isothiocyanate (*3*) was found in the edible parts of turnips (*Brassica campestris* var.

Table III. Fumigant Toxicity to Vinegar Flies

Dose, μg.		*% Knockdown, 24 Hr.*	*% Mortality, 24 Hr.*	*% Revived*
Myristicin				
	300	34	19	44
	400	79	67	15
	800	100	96	4
Pyrethrum	1500	0	0	—

Table IV. Toxicity to Houseflies
LD_{50}, μg./g., 24 hours

Insecticide	*Flies*[a]	*Synergists, 1:1,W./W.* None	*Myristicin*	*Piperonyl butoxide*
None			700	2000
Sevin	C.S.	>3500	9	39
Dimetilan	C.S.	4	2	4
p,p'-DDT	C.S.	5	5	5
p,p-DDT	Resist.	3250	325	715
Pyrethrum	C.S.	26	15	5
Allethrin	C.S.	9	7	6

[a] C. S. = C.S.M.A. strain–DDT-susceptible flies; Resist. = Fowler strain–DDT-resistant flies.

Table V. Effect of Synergist on Toxicity to Vinegar Flies

Insecticide, μg.		*Synergist* μg.	*Ratio, 1:S*[a]	*None*	*Myristicin*	*Piperonyl butoxide*
None		100			0	0
Sevin	100			10		
	100	20	1:0.2		100	40
p,p'-DDT	25			22		
	25	100	1:4		75	57
Pyrethrum	10			0		
	10	50	1:5		13	51
Allethrin	25			3		
	25	100	1:4		95	96

[a] Insecticide: Synergist (W./W.)

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Table VI. Toxicity of 2-Phenylethyl Isothiocyanate and Lethane 334[a]

Insect	*Expt. Time, Hr.*	*% Mortality, 0.5% W./V.*	
		2-Phenylethylisothiocyanate	*Lethane 384*
Houseflies	0.3	90	100
	24	96	28
Pea aphids	48	100	100
Mexican been beetle	48	0	0
German cockroach	48	0	0
Spider mites	120	100	87

[a] *From* (*3*, p. 32).

rapa) and rutabaga (*Brassica napus* var. napobrassica), which also have been consumed for centuries by humans without causing any obvious harm. In the turnip variety investigated (Purple Top Strap Leaf), the concentration amounted to 63 p.p.m., as determined by bioassay. The toxic action of 2-phenylethyl isothiocyanate against various insects was established (Table VI). It has a definite knockdown effect which is not as great as that of Lethane 384; however, its killing effect is superior to that of Lethane 384. Sixty and 47% of the totally recovered 2-phenylethyl isothiocyanate from roots of turnip and rutabaga were located within their peelings, respectively. Boiling of the edible parts of these crops in water resulted in a rapid loss of the insecticide by steam distillation.

Table VII. Distribution of 2-Phenylethyl Isothiocyanate in Cruciferous Crops[a]

	Cabbage	*Cauli-flower*	*Brussels Sprouts*	*Broc-coli*	*Kohlrabi*	*Cress*	*Radish*
Edible portion							
% mortality/[b] min.	0	0	0	0	0	100/210	50/120
P.p.m.[c]	0.18	0.13	0.21	0.21	0	0	0
Roots							
% mortality/ min.	50/15	50/200	50/10	50/47	50/16		
P.p.m.	279	83	568	181	165		

[a] From (*2*, p. 160).
[b] Extract. 15 grams root, 100 grams greens.
[c] Gas-liquid chromatography.

The wide distribution of this insecticide in other cruciferous crops was investigated later, and the stability and occurrence of this compound were studied. Two cabbage varieties, cauliflower, brussels sprouts, broccoli, kale, mustard, and kohlrabi contained 2-phenylethyl isothiocyanate in various amounts as evidenced by gas-liquid chromatography and bioassay (houseflies and vinegar flies) (Table VII). Insecticidal activities were encountered only with root tissues. Compounds of insecticidal activity, not attributable to 2-phenylethyl isothiocyanate, were found in the edible parts of radish and leaves of turnip, rutabaga, and garden cress.

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5

Chemistry and Biochemistry of Pyrethrins

JOSEPH B. MOORE

McLaughlin-Gormley-King Co., Minneapolis, Minn.

A review of the chemistry and biochemistry of pyrethrum is presented based mainly on work done subsequent to 1945. The greatest advances in the chemistry of pyrethrum have occurred since the disclosure of the heterogeneity of pyrethroids. On the biological side, the most recent work has been made possible by the use of chromatographic column separation and gas chromatography separation of the four active components of pyrethrins.

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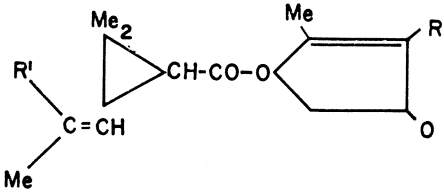
The publications of LaForge and Barthel (*24*), Harper (*5*, 7, *11-17*), Inouye (*20*), and Crombie (*6*) some fifteen to twenty years ago focused attention on the then current knowledge of the chemistry of pyrethrum.

The great advance in the field of instrumentation, coupled with the discovery of the heterogeneity of the pyrethrolone radical, has advanced the knowledge of pyrethrum chemistry considerably beyond that known in 1945. LaForge and Barthel (*24*, *25*) have shown the structure of the active ingredients of pyrethrum, known collectively as pyrethrins, to be esters as represented by the structure shown in Table I.

Recently a new constituent of pyrethrum extract was described by Godin *et al.* (*9*): jasmolin II, the *cis*-pent-2-enylrethronyl ester of pyrethric acid. Jasmolin II differs from pyrethrin II in that the terminal double bond of the alcoholic side chain is saturated. This constituent forms about 3% of the total pyrethrins. Jasmolin II is less toxic to the insects tested than a similar concentration of pyrethrins. The pyrethrum extract was 16 to 17 times as toxic as jasmolin II to *Aedes aegypti* and *Phaedon cochlearia* adults, less than 17 times

In Natural Pest Control Agents; Crosby, D.;
Advances in Chemistry; American Chemical Society: Washington, DC, 1966.

Table I. Active Ingredients of Pyrethrum



	R	R'
Pyrethrin I	$-CH_2-CH=CH-CH=CH_2$	Me
Cinerin I	$-CH_2-CH=CH-Me$	Me
Pyrethrin II	$-CH_2-CH=CH-CH=CH_2$	COOMe
Cinerin II	$-CH_2-CH=CH-Me$	COOMe
Jasmolin II	$-CH_2-CH=CH-CH_2-CH$	COOMe

to *Tenebrio molitor* adults, and at least 100 times to *Tribolium castaneum* adults.

Jasmolin II in pure form was nearly equal to cinerin II in toxicity to houseflies but much less when synergized. The synergistic factor of the synergized compounds is: pyrethrum extract 9.6; jasmolin II, 7.0; cinerin II, 12.0.

Chemistry of Pyrethrins

Pyrethrins are esters and therefore may be discussed in terms of the chrysanthemumic or pyrethric moiety and the keto alcohol moiety.

Chrysanthemumic acid may exist in four stereoisomers, because of the two asymmetric carbon atoms in the cyclopropane ring. The natural acid has the D-*trans* configuration and this has been shown to be more insecticidally active than any of the other isomers or the racemic form. Harper *et al.* (*4*, *18*) have synthesized, separated, and optically resolved all of the isomers of this acid.

Julia *et al.* (*21*) worked out a synthesis of (DL) *trans*-chrysanthemumic acid which comprised treating the 4-methyl-3-isobutenyl-γ-valerolactone with hydrogen chloride or hydrogen bromide in methanol or ethanol to open the lactone ring, cyclizing the resulting alkyl-5-methyl-3(1-halogenoisopropyl) hex-4-enoate into a (DL) *trans*-alkyl chrysanthemumate by treating with an alkali metal such as *tert*-butylate or *tert*-amylate, alkali metal amides, and alkali metal anhydrides, and converting the ester group of the chrysanthemumate into a carboxylic acid group by saponification.

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Godin *et al.* (*10*), working on the effect of insecticidal activity of pyrethrum flowers from fresh and dried flowers, showed that extracts from fresh flowers had little or no greater effect on pyrethroid content and insecticidal activity than extracts from dried flowers with drying temperatures up to 80°C. However, some of all the pyrethrin was lost at 120°C. The principal loss was in pyrethin I and not pyrethin II.

Chrysanthemum dicarboxylic acid or pyrethric acid may exist in eight stereoisomers, owing to the trans or cis configuration on the side chain of the double bond as well as that of the cyclopropane. The natural acid has been shown to be the trans-trans acid. As in the case of the chrysanthemum monocarboxylic acid, the naturally occurring configuration is more insecticidally active than the racemic form or any of the three isomers synthesized.

Pyrethrolone and cinerolone make up the keto alcohol moiety of the pyrethrins. Both of these keto alcohols have one asymmetric carbon at the 4-position and a double bond in the side chain which is capable of cis-trans isomerism in the 2-position. It is possible, therefore, to have four stereoisomers for each keto alcohol. Katsuda *et al.* (*22*) show that only the (+) form occurs in the natural esters. Elliott (*8*) has shown recently, by a new procedure developed to obtain pure (+) pyrethrolone, that the hitherto unidentified pryethrolone C is in reality pyrethrolone contaminated with thermally isomerized material. (+) Pyrethrolone forms a crystalline monohydrate from which the pure alcohol is obtained. The natural configurations of the keto alcohols in the esters are insecticidally more active, as is the case with the acid moiety.

Goldberg *et al.* (*11*) recently reported on work with distillation of pyrethrum extract in a wiped-wall, falling-film, short-path still, showing the separation of pyrethrins I and II and the determination of their relative biological activities. They confirmed the work of Sawicki *et al.* (*33*) on the relative effectiveness of pyrethrins I and II on various insects. They were able to use this process in decolorizing pyrethrins without forming isopyrethrins, with a consequent loss in biological activity normally observed with thermal isomerization of pyrethrins.

They indicated that this method might be advantageous for the partial separation of pyrethrins I and II on an industrial scale, thus tailoring pyrethrin extracts for specific uses depending upon the insect to be controlled.

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Sawicki *et al.* (*33*) prepared by reconstitution pure samples of pyrethrins I and II and cinerins I and II and compared the toxicity of these esters with the regular pyrethrum extract on houseflies 5 to 6 days old.

Table II. Relative Toxicity of Pyrethrum Constituents at LD_{25}, LD_{50}, and LD_{75} Levels

	Experiment A		*Experiment B*	
Substance	LD_{50}	LD_{25}	LD_{50}	LD_{75}
Pyrethrum extract	100	100	100	100
Pyrethrin I	95	95	105	93
Pyrethrin II	147	147	127	126
Cinerin I	40	40	65	42
Cinerin II	57	57	69	52

Pyrethrins I and II are, respectively, more toxic per unit weight than cinerins I and II against *Musca domestica* and *Phaedon cochlearia;* however, pyrethrin II (pyrethrin II + cinerin II) is more toxic to houseflies, and pyrethrin I (pyrethrin I + cinerin I) is more toxic to mustard beetles. The reason for this species specificity is unknown.

Sawicki and Elliott (*31*) re-examined the insecticidal activity of pyrethrin extracts and its four insecticidal constituents against four strains of houseflies, and checked the relative toxicity of pyrethrins I and II. This work confirmed the earlier results, showing that pyrethrin II was 1.3 to 1.6 times more toxic than pyrethrin I, but that the relative toxicities of pyrethrins I and II against the four strains of flies differed little. Resistance to knockdown but not to killing was associated in these strains with resistance to organophosphorus and chlorinated insecticides.

They found that the methods of immobilizing flies during treatment had no effect on the relative toxicity of the pyrethrins. Strong chilling possibly increased the susceptibility of the flies to the pyrethrins, at least during the first 24 hours after treatment, but did not alter the relative toxicities.

More detailed information on the chemistry of pyrethrins is given by Zechmeister (*35*). The chapter on the "Chemistry of the Natural Pyrethrins" by Crombie and Elliott is an excellent and complete review of this field.

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Synergists

A number of synergists are used with pyrethrum to increase its toxicity. Most of the better ones, as far as flying insects are concerned, are of the methylene dioxyphenyl type. The best known of this type is piperonyl butoxide.

Piperonyl butoxide increases the absolute toxicity of the four constituents of pyrethrum.

Table III. Relative Toxicity of Four Constituents of Pyrethrum at Six Ratios of Synergist to Active Ingredient

Ratio of Active Ingredients to Synergist	*Pyrethrum Extract*	*Pyrethrin I*	*Pyrethrin II*	*Cinerin I*	*Cinerin II*
10:1	1.00	1.10	1.14	1.04	0.62
2:1	1.00	1.14	1.27	1.11	0.60
1:2	1.00	1.12	1.03	1.23	0.63
1:4	1.00	1.24	1.04	1.30	0.66
1:8	1.00	1.31	0.90	1.28	0.58
1:20	1.00	1.23	0.82	1.17	0.62
No synergist	1.00	0.85	1.37	0.53	0.49

The largest increase is in cinerin I, the cinerolone ester of chrysanthemumic acid; the smallest, with pyrethrin II. Pyrethrin I and cinerin II have similar synergistic factors up to a ratio of 1 to 4, but at higher ratios pyrethrin I is better synergized than cinerin II. The increase in toxicity in the presence of synergist depends on the constituents; so it follows that the order of toxicity relative to the standard extract is different in the presence of the synergists from the order of relative toxicity of the constituents alone. Sawicki *et al.* (*32*) have shown that at a ratio of 1 to 8, cinerin I and pyrethrin I are more toxic than pyrethrin II and cinerin II. However, when alone, pyrethrin II is more toxic than pyrethrin I, cinerin I or cinerin II.

Bates, Hewlett, and Lloyd (*1*) found that both piperonyl butoxide and SKF 525A, the ester of 2-diethylaminoethyl 2,2-diphenyl-*n*-pentanoate, synergized the action of pyrethrins on insects of species of the lesser mealworm beetles and houseflies but both antagonized the action of malathion. SKF 525A is known to increase the effects on mammals of drugs of various types and has been shown to synergize pyrethrins.

Bates, Hewlett, and Lloyd (*2*) therefore studied the synergistic effects of some compounds related to 2-diethylaminoethyl 2,2-diphenyl-*n*-pentanoate on the insecticidal activity of pyrethrins. They found active compounds when a 2-diethylamino moiety was joined to the diphenylmethyl moiety through an ester, ketone, or ether linkage. However, none of the compounds investigated approached piperonyl butoxide in synergistic activity with pyrethrins.

Why there should be wide differences in the extent to which very closely related chemical compounds are affected by piperonyl butoxide is not known. Studies on the mode of action of pyrethrins and synergists indicate the possibility that decreased detoxification is the mechanism of synergism. According to Veldstra (*34*), synergistic action such as that discussed here is caused by blocking the sites of loss (mechanisms by which an active substance can be lost before it reaches the effective receptor). If it is assumed that there are differences in the activity of the four constituents at the site of action, differences in the relative toxicity of the pure and the synergized constituents could be explained as follows: if two insecticides, similar in chemical composition and action, differ in their toxicity at the site of action, this difference will not necessarily be reflected in their relative toxicities unless they are both equally affected by the sites of loss. The substance with a lower toxicity at the site of action may show a greater toxicity if less is lost before it reaches the site of action. Blocking sites of loss by synergists will allow insecticides to reach the site of action and show their true relative toxicities. If it is assumed that the esters of chrysanthemumic acid are more toxic at the site of action and are more affected by sites of loss than esters of pyrethric acid, and if cinerins show these properties more than corresponding pyrethrins, the order of increase in toxicity with the addition of synergists will be cinerin I, pyrethrin I, cinerin II, and pyrethrin II.

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Mode of Action of Pyrethrum

The biochemistry or mode of action of pyrethrum is not as well known as its chemistry. There are several theories of the toxic action of pyrethrum. Lauger *et al.* (*26*) consider that a highly effective contact insecticide must possess a toxic component (toxaphore) and must have groups attached which absolutely insure pronounced lipid solubility. They consider in the case of pyrethrins that in the cyclopro-

pane ring, the methyl, dimethylethylene, and allene groups are responsible for the lipid solubility of the molecule.

Hurst (*19*) discusses the similarity in action of the pyrethrins and of DDT as indicated by a dispersant action on the lipids of insect cuticle and internal tissue. He has developed an elaborate theory of contact insecticidal action but provides no experimental data. Hurst believes that the susceptibility to insecticides depends partially on the cuticular permeability, but more fundamentally on the effects on internal tissue "receptors" which control oxidative metabolism or oxidative enzyme systems. The access of pyrethrins to insects, for example, is facilitated by adsorption and storage in the lipophilic layers of the epicuticle. The epicuticle is to be regarded as a lipoprotein mosaic consisting of alternating patches of lipid and protein receptors which are sites of oxidase activity. Such a condition exists in both the hydrophilic type of cuticle found in larvae of *Calliphora* and *Phormia* and in the waxy cuticle of *Tenebrio* larvae. Hurst explains pyrethrinization as a preliminary narcosis or knockdown phase in which oxidase action is blocked by adsorption of the insecticide on the lipoprotein tissue components, followed by death when further dispersant action of the insecticide results in an irreversible increase in the phenoloxidase activity as a result of the displacement of protective lipids. This increase in phenoloxidase activity is accompanied by the accumulation of toxic quinoid metabolites in the blood and tissues—for example, *O*-quinones which would block substrate access to normal enzyme systems. The varying degrees of susceptibility shown by different insect species to an insecticide may be explainable not only in terms of differences in cuticle make-up but also as internal factors associated with the stability of oxidase systems.

Blum and Kearns (*3*) showed in the American cockroach that topically applied pyrethrum shows a negative temperature coefficient of action between 15° and 35°C. The 24-hour LD_{50} for pyrethrum is about 1 μg. at 15°C. and about 6 μg. at 35°C.

By employing C^{14}-labeled pyrethrum, it has been determined that at 35°C. the rate of penetration of pyrethrum is more than twice that at 15°C. Cockroaches prostrate at 15°C. can be returned to normal by transferring them to 35°C., a process which can be repeated for several hours. Since cockroaches transferred from 35° to 15°C. became prostrate more rapidly than those held continuously at 15°C., the insecticide (or some metabolic toxin) was probably in the vicinity of its site of action at 35°C. but was ineffective.

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The detection of microgram quantities of pyrethrins, cinerins, keto alcohols, and chrysanthemum acids by paper chromatography and by application of these techniques to a study of possible metabolites enabled certain tentative conclusions that imply hydrolysis in insects of a large portion of the radioactive pyrethrins and synergists to corresponding keto alcohols and chrysanthemum acids.

The characteristic paralytic effect of pyrethrins and their rapid action are due to action on the insect's central nervous system. Roy *et al.* (*30*), showed that pyrethrins injected into the abdomen or into a spiracle produced a progressive weakening or pseudoparalysis in the legs, starting with the leg innervated by the ganglion nearest the point of injection, passing to its opposite leg, and so on, either cephalically or caudally, depending on the point of injection.

Page *et al.* (*28*) studied the activation threshold of pyrethrins for certain insects. This threshold point of toxicant in the insect occurred when the natural activity was replaced by forced activity caused by the action of the pyrethrins on the peripheral nervous system.

In the case of *Aedes aegypti* the activation threshold is about 0.25 μg. per gram, compared to the knockdown threshold of 1.25 μg. per gram. The approximate ratio is 1.65 for the log of knockdown threshold over the log of activation threshold for *Aedes aegypti*, *Culix pipiens*, *Coccinella septempunctata*, *Rhagonycha fulva*, *Musca domestica*, *Vespula germanica*, and *Vespula vulgaris*.

When the pyrethrin concentration in a spray dosage was increased, the mean time for paralysis was reduced until it reached a minimum of 2.5 minutes and was then independent of concentration. This time was considered to represent the period required for diffusion through the cuticle to the site of action.

Lowenstein (*27*) found an approximate correlation between the nerve action potential produced by 1.6% pyrethrins applied externally and 0.3% pyrethrins applied directly to the nerve cord.

Synthetic Pyrethroids

The work of LaForge and Barthel (*24*, *25*) was the prelude to intensive work on synthesis of pyrethroid materials. Some of the synthetic pyrethroids developed are:

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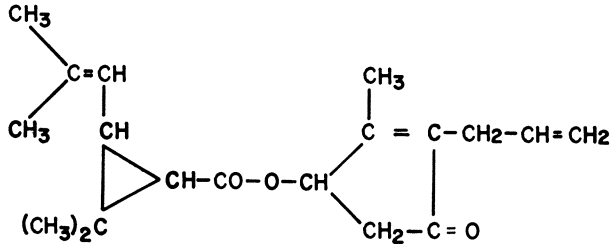
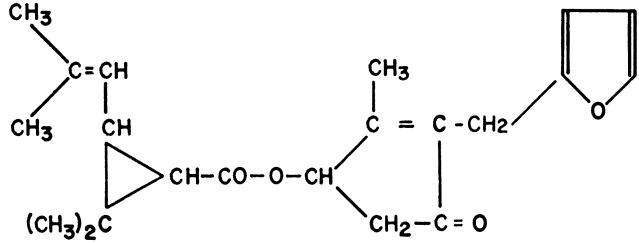
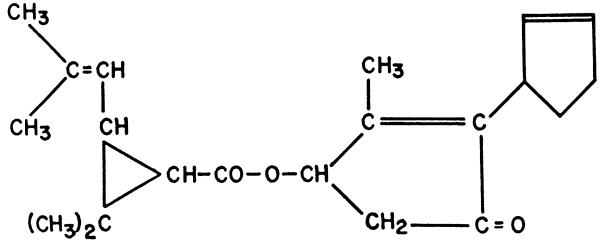
Allethrin, DL-2-allyl-3-methyl cyclopenten-1-one ester of DL-*cis-trans*-2, 2-dimethyl-3 (-2-methylpropenyl) cyclopropanecarboxylic acid



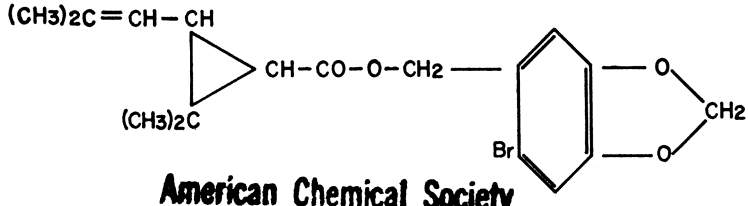
Furethrin, furfurylrethronyl ester of chrysanthemum monocarboxylic acid



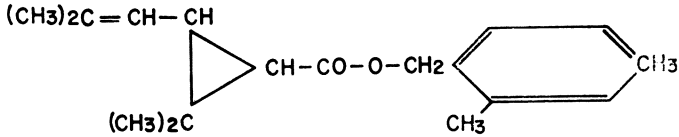
Cyclethrin, 3-(2-cyclopentanyl)-2-methyl-4-oxo-2-cyclopentenyl chrysanthemum monocarboxylate



Barthrin, 6-bromopiperonyl ester of chrysanthemum monocarboxylic acid

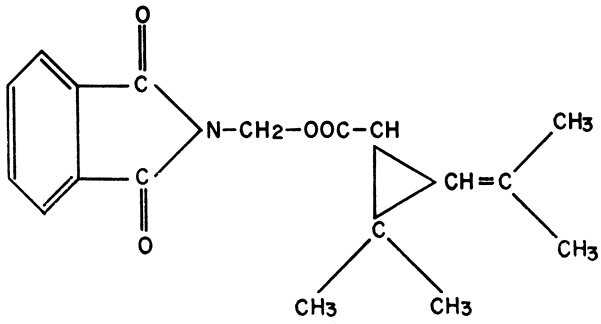


Dimethrin, 2,4-dimethyl benzyl ester of chrysanthemum monocarboxylic acid



and most recently, developed in Japan (*23*),

Super Pynamin, 1-(phthalimidomethoxycarbonyl)-2-2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropane



Clyde Kearns (personal communication) suggests that the first step in the possible detoxification of pyrethrins, including the cinerins as well as the pyrethrins, is the epoxidation of the double bond.

This is interesting when one considers the effect of synergists on the synthetics. All of the synthetics mentioned above are based on chrysanthemum monocarboxylic acid and in the case of allethrin, cyclethrin, and furethrin on the alcohol moiety there is only one double bond. When checked against the standard synergists, these synthetics do not show the degree of synergism shown by pyrethrins and this may be because of the fact that there is only one double bond for epoxidation, compared with two in the pyrethrolone radical, and therefore the synergist would not block this epoxidation step as effectively.

Philleo *et al.* (*29*) recently confirmed the inhibitory action of methylene dioxyphenyl groups on biological oxidations in insects, including epoxidation and hydroxylation of certain compounds.

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Conclusions

The analytical chemistry of pyrethrins leaves much to be desired as far as current methods are concerned. A great deal of effort is being expended to get more accurate methods of assay including ultraviolet absorption, infrared, and gas-liquid chromatography.

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6

Analysis of Pyrethrins

HERMAN BECKMAN and PAUL T. ALLEN[1]

Agricultural Toxicology and Residue Research Laboratory,
University of California, Davis, Calif.

The development and adaptation of procedures for the separation, isolation, purification, identification, and analysis of the components of the pyrethrum mixture have been studied and evaluated. Results of studies to determine the molar extinction coefficient of pyrethrin I as well as a gas chromatographic procedure for the determination of pyrethrins are reported. The use of chromatographic separation procedures (including partition, adsorption, gas, and thin-layer chromatography), colorimetry, and infrared spectrophotometry are discussed.

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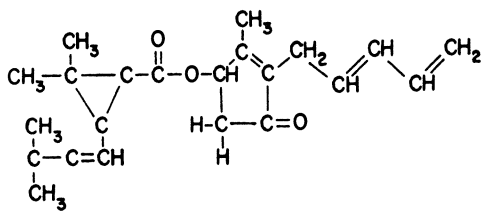
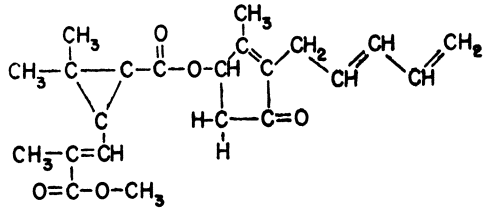
For several years this laboratory has taken an active interest in the analysis of pyrethrum preparations and improved procedures (*1*, *2*). The aim of the present investigation was to develop a procedure, based on gas chromatography, that would quantitatively measure the four principal constituents of the pyrethrum mixture. Progress has been made toward this goal. At this point in our study we have many facts and the first step toward the complete method.



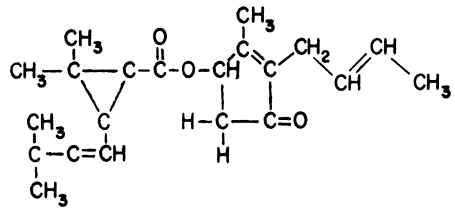
PYRETHRIN I

[1] Present address, Mobil Chemical Co., Beaumont, Tex.

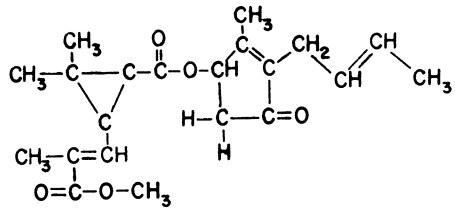
In Natural Pest Control Agents; Crosby, D.;
Advances in Chemistry; American Chemical Society: Washington, DC, 1966.



PYRETHRIN II



CINERIN I



CINERIN II

It is apparent from the literature on pyrethrum analysis that there is little agreement on what is needed or wanted in new methodology. As soon as it is possible to obtain complete information on all the significant components of the pyrethrum mixture, a better understanding of the methodology needed will be available.

The purpose of this paper, then, is to summarize the present condition of some of the work done in this and other laboratories and present information on the separation, isolation, and identification of some of the components of pyrethrum. Work of others has, in part, been confirmed and some new evidence is presented on compounds isolated from the pyrethrum mixture.

Separation Procedures

Column Chromatography. Liquid-liquid partitioning has been used by several investigators for several pesticides, including pyrethrum (*1, 2, 3, 5, 10, 13*). The most useful procedure allows the

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separation of the bulk of the nonpyrethroid extractives from the active constitutents of the pyrethrins. Probably the two most common solvent pairs are nitromethane with hexane and acetonitrile with hexane. Dimethylsulfoxide and dimethylformamide have been experimented with on a small scale as the immiscible phase with hexane. No conclusive information has been developed as to their potential.

Partition column chromatography for separating several of the primary constituents of the pyrethrum extract has been reported. The elution pattern for some of the constituents of the pyrethrum mixture recovered from the partition column is shown in Table I.

Table I. Elution Order of Pyrethrum Constituents from Gas Chromatograph as Compared with That from Partition Column

Identifications in terms of gas chromatographic responses, infrared data, and colorimetric tests

Compound	*B.P., °C. (9)*	*Elution Order*	
		Gas chromatograph	*Partition column*
Cinerin I	136–38°/8×10^{-3}mm.	1	1
Cinerin I type		2	2
Pyrethrin I	146–48°/2×10^{-3}mm.	3	3
Cinerin II type		4	5
Cinerin II	182–84°/1×10^{-3}mm.	5	4
Pyrethrin II	196–98°/7×10^{-3}mm.	6	6

The elution pattern from the gas chromatograph is included for comparison, as well as the boiling points for the known compounds as given by Elliott (7). Figure 1 identifies the gas chromatographic peaks. In general, a silicic acid column is used as the supporting medium for acetonitrile or nitromethane, with hexane as the mobile phase. Some work using Celite in place of silicic acid has been done to fractionate the pyrethrum components (Figures 5 and 6). The nitromethane-hexane solvent mixture was used with a silicic acid column as the basis for separations in a colorimetric analysis for pyrethrins (*1*). In this procedure pyrethrin I was recovered in quantity and used to relate to the total amount present. The colorimetric procedure will detect compounds containing conjugated unsaturation in the side chain. The side chain must also terminate in a double bond as with pyrethrins I and II. Thus, no color is observed with

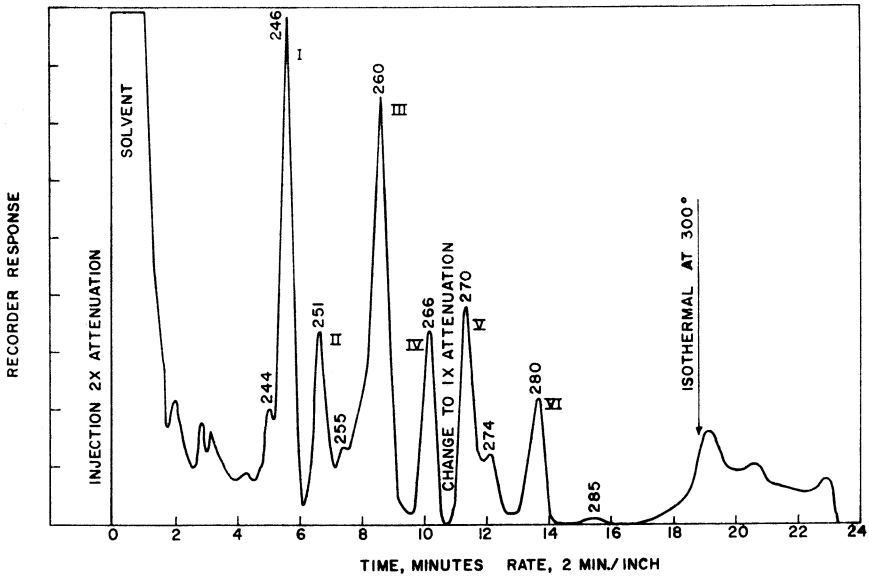

Figure 1. Gas chromatographic responses from a pyrethrum concentrate
Identity of numbered peaks given in text

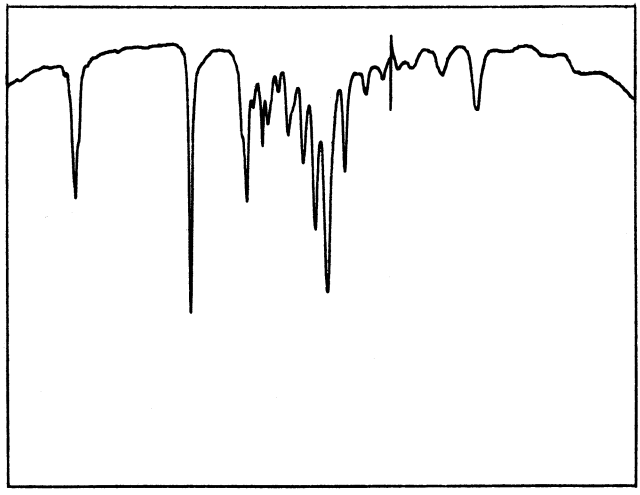

*Figure 2. Infrared spectrum of methyl-*trans*-chrysanthemate Prepared from DL-*trans*-chrysanthemum acid and collected as pure ester from gas chromatograph*

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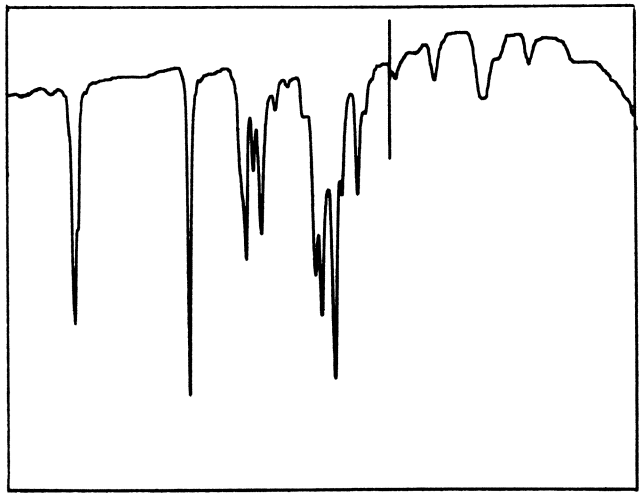

Figure 3. Infrared spectrum of methyl-cis-chrysanthemate Prepared from DL-cis-chrysanthemum acid and collected as pure ester from gas chromatograph

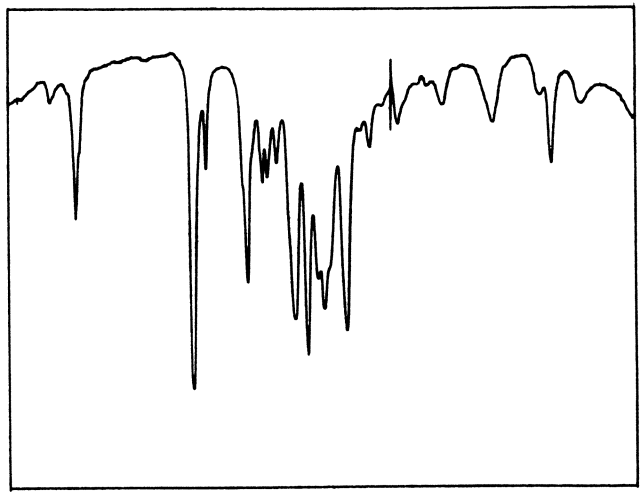

Figure 4. Infrared spectrum of methyl-trans-pyrethrate Prepared from pyrethric acid isolated from hydrolysis mixture of pyrethrum concentrate. Pure ester isolated by gas chromatography

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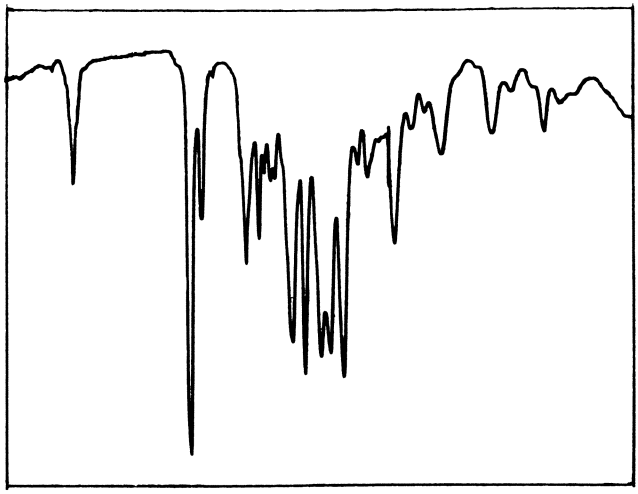

Figure 5. Infrared spectrum of pyrethrin II Fraction isolated from silicic acid–acetonitrile-hexane partition column

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cinerin I or II or related compounds such as allethrin. The recently reported jasmolin I (7) will not respond to the color test.

Adsorption column chromatography has been employed to separate the constituents of pyrethrum. Florisil and aluminum oxide have been used as adsorption columns to retain much of the pigmented materials. The pyrethroids may be caused to elute with several solvents. In our experience mixtures of hexane with ethyl acetate, methanol, ethyl ether, dichloromethane, or acetone have provided different elution patterns.

Gas Chromatography. In recent work we have followed the progress of a partition column using a combination of thermal conductivity gas chromatography and the colorimetric test. This has shown that some of the unidentified peaks originally observed by gas chromatography and not identified are related to the general pyrethrum structure. These compounds are present as part of the original mixture as they have been separated by the column partition chromatography and give the same infrared spectra before and after gas chromatography. Their retention times correspond to peaks found in the mixture and are judged not to be thermally isomerized products as has been suggested (*3*, *4*, *6*, 7).

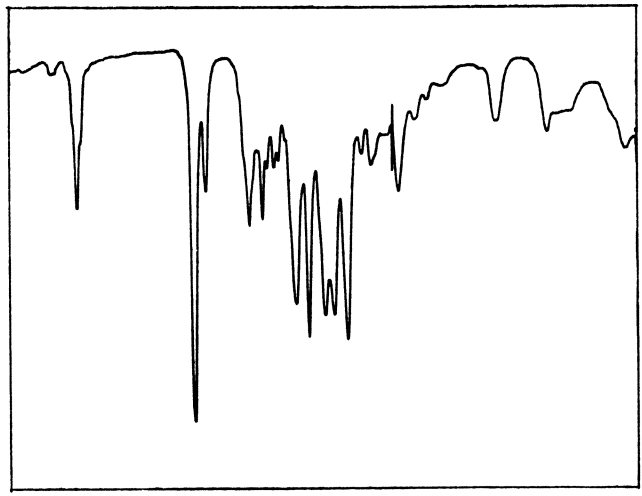

Figure 6. Infrared spectrum of cinerin II Collected from gas chromatograph after isolation from silicic acid–acetonitrile-hexane partition column

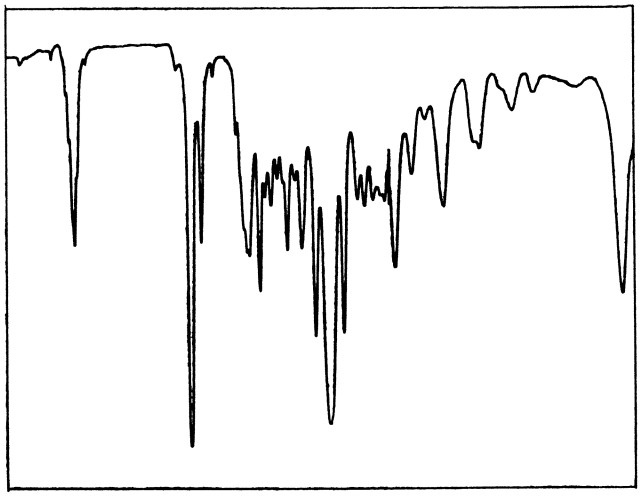

Figure 7. Infrared spectrum of pyrethrin I Isolated after dual partition chromatography First partition column. Celite-acetonitrile-hexane Second partition column. Silicic acid–nitromethane-hexane (with 5% acetone). Corresponds to peak 3 of gas chromatographic separation of pyrethrum mixture

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Colorimetric Analysis

The colorimetric procedure has been applied to each of the fractions isolated from the partition column. The response to the color test has allowed an accurate prediction of the general type of infrared spectra ultimately found. The color test has also been applied to fractions collected from the gas chromatograph. Of the major responses observed when the pyrethrum mixture is passed through the chromatograph, three of the components respond to the color test. At least two other pyrethrin-like compounds of long retention and small quantity also give the color test. No infrared data are available on these.

While the colorimetric procedure has not found wide application as a quantitative method, it has proved its worth as a diagnostic tool.

The separations allowed by the partition column provided a rather pure sample of pyrethrin I, demonstrated by the gas chromatograph and by comparison with known infrared spectra. The purified pyrethrin I was weighed quantitatively and a color test performed to determine the extinction coefficient. The figure obtained from ten runs is 1120, calculated from the formula:

$$a = \frac{A}{bc}$$

where A is the absorbance measured,

b is the path length of the cell in centimeters, and

c is the molar concentration of the compound.

Under the conditions of operation we observed the following:

$$a = \frac{0.25}{1 \times (0.232 \times 10^{-4})}$$

$$a = 1120$$

This is expressed as molar absorptivity at 550 mμ.

Thin-Layer Chromatography. A study using thin-layer chromatography both for separating components of a pyrethrum extract and for purifying single components has been made. Other reports have mentioned the use of thin-layer chromatography in various connections related to pyrethrin analysis. Stahl (*14*) reported on pyrethrins I and II and evidence for presence of other compounds from

pyrethrum concentrate. Part of the additional compounds found were the result of ultraviolet irradiation of the pyrethrum mixture.

Thin-layer plates were made with silica gel–calcium sulfate and each contained a mixture of zinc silicate and zinc cadmium sulfide as phosphors. Separated components are generally visible under ultraviolet light by fluorescence quenching. This was true, in part, for the pyrethrins, except that some of the separated components possessed a natural fluorescence under the ultraviolet lamps.

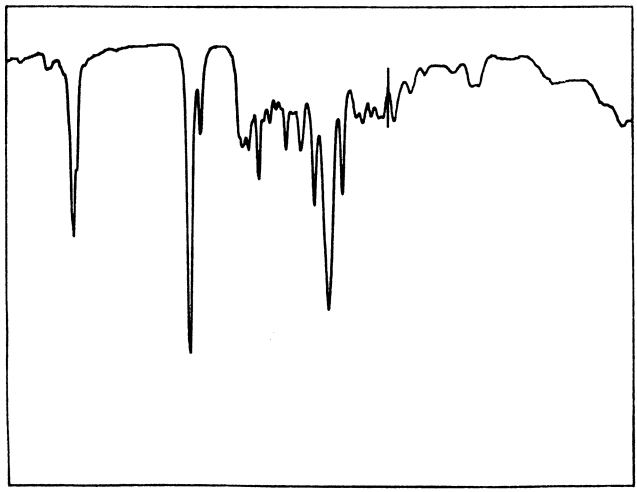

Figure 8. Infrared spectrum of a cinerin I type Fraction isolated from Celite-acetonitrile-hexane partition column and rechromatographed on a silicic acid–nitromethane-hexane (with 5% acetone) partition column. Gas chromatography indicates purity of 98%

A solvent system consisting of hexane–ethyl acetate–benzene (75–15–10) was found the most appropriate for resolving the components of pyrethrum. When a pyrethrum concentrate was spotted and developed, a series of separated components was observed. The observation was made possible by using a viewing cabinet fitted with long- and short-wavelength ultraviolet lamps. From seven to nine constituents were observed, depending on which lamp was used. All of the same components were not necessarily observed with each lamp. One of the most interesting observations was that of a brilliant red spot with an R_f of about 0.85, which was observable only under the long-wavelength lamp. The spot was bright and tight even at a

relatively high R_f value. The compound has not been identified. It is not one of the known pyrethrin or cinerin compounds, however.

The multiplicity of responses makes thin-layer chromatography not particularly suited for pyrethrum analysis, either qualitative or quantitative. It did confirm, however, that the crude oleoresin contains several pyrethroid compounds in substantial quantity, as previously shown by gas chromatography work.

Thin-layer chromatography was also used in an attempt further to purify fractions that had been separated by column or gas chromatography. Rather precise R_f values for several compounds were

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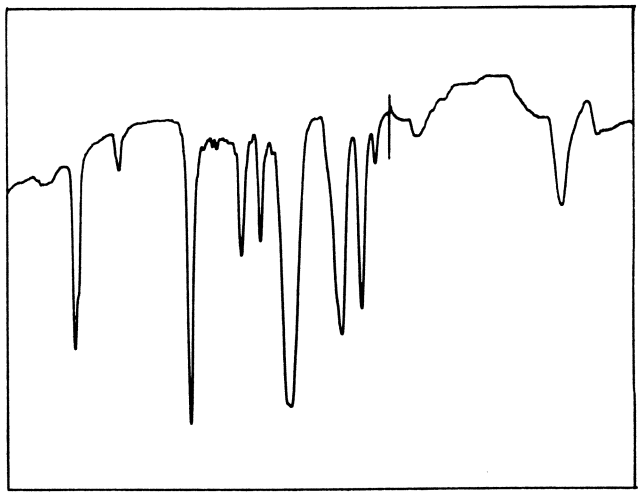


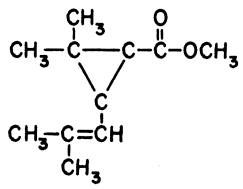
Figure 9. Infrared spectrum of pyrethrin I decomposition product isolated from thin-layer chromatographic plate

determined, but complications were injected into the total separation picture. Whenever, for example, pyrethrin I was spotted on a plate and the chromatogram developed, two spots were observed: the original material and a decomposition product of higher R_f (*see* Figure 9). The decomposition product rechromatographed without change, but the recovered pyrethrin I on rechromatography again showed further decomposition. It was determined that this degradation took place in air and was not caused by ultraviolet radiation.

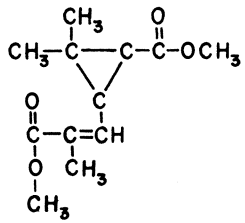
Quantitative Gas Chromatographic Method

One purpose of this work was to develop a gas chromatographic procedure based on separation and quantitation of each of the com-

ponents known to be present in the mixture. This rather ambitious goal has not yet been reached. As an outgrowth of this work, however, a quantitative analytical method has been developed, based on gas chromatography. The procedure starts with the AOAC (*8, 11*) hydrolysis to yield the chrysanthemic and pyrethric acids. The acids are recovered, esterified with diazomethane, and determined by gas chromatography. Since pyrethrin I and cinerin I give the same monocarboxylic acid, and pyrethrin II and cinerin II give the same dicarboxylic acid, quantitation is based on the same criteria as the AOAC method. It is now suspected that other components of the



METHYL CHRYSANTHEMATE



DIMETHYL PYRETHRATE

mixture contribute to the total monocarboxylic and dicarboxylic acids found.

Table II. Determination of Total Pyrethrin I and Cinerin I

Sample No.	*% PI by*		*% Recovery, GC/AOAC*
	AOAC	*Gas chromatog.*	
1	11.38	11.03	97.0
2	6.1	6.14	100.7
3	11.38	11.29	99.5
4	6.1	6.13	100.3
5	9.83	9.94	101.0
6	11.38	11.00	96.8
7	11.81	11.98	101.4
8	10.98	10.40	94.7
9	9.97	9.58	96.0
10	11.38	10.38	98.6
11	11.38	10.73	94.5
12	9.83	9.57	97.4
13	11.81	11.25	95.3

Table II presents some data using the gas chromatographic procedure and comparing the results to the standard AOAC procedure. The samples of pyrethrum concentrate and AOAC analyses were supplied by several of the principal pyrethrum producers from the United States and abroad.

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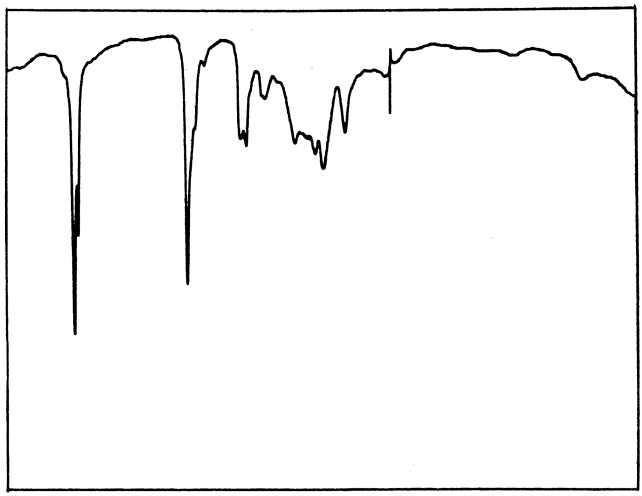

Figure 10. Infrared spectrum of long-chain ester recovered from gas chromatography after hydrolysis and methylation of pyrethrum concentrate

The columns labeled PI reflect the total of pyrethrin I and cinerin I just as in the AOAC procedure. The gas chromatographic results are in terms of the total amount of the mixture but were analyzed as the methyl ester of chrysanthemic acid. The present state of the determination of PII (pyrethrin II plus cinerin II) is not complete because of the erratic extractability of the dicarboxylic acids from the hydrolysis mixture. The gas chromatographic pattern is distinct and straightforward. As the extraction procedure for PII is improved, the gas chromatographic method will be more applicable. The present recovery of PII is in the range of 80 to 90%. The average of the values shown in Table II for PI is 98.0%.

It was recently found that the free monocarboxylic and dicarboxylic acids derived from pyrethrum hydrolysis will pass through the gas chromatograph and present suitable responses. When this is followed up, it is expected that the method will be simplified and provided better over-all recoveries.

Discussion

Figure 1 presents the gas chromatographic responses obtained from a pyrethrum concentrate. Peak I has been identified as cinerin I, peak II as a cinerin-type compound, peak III as pyrethrin I, peak

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IV as a cinerin-type compound, peak V as cinerin II, and peak VI as pyrethrin II. It is evident that peak IV also contains another component. The peaks beyond peak VI are known to be of the pyrethrin type as they respond to the color test. This gas chromatography pattern was obtained with a 6-foot ¼-inch column packed with 20% SE-30 on 40-60-mesh Chromosorb P. Later work provided good resolution of the peaks with a 2-foot ¼-inch column packed with 20% Dow-11 silicone oil on 45-60-mesh Chromosorb P.

Figures 2 through 9 are infrared spectra of fractions collected from partition columns, gas chromatography, thin-layer chromatography, or a combination of these separation techniques. Figure 10 is the infrared spectrum of a compound isolated by gas chromatography after hydrolysis of a pyrethrum concentrate. In this case the compound is a long-chain ester. All the infrared spectra were made with a Perkin-Elmer Model 221 instrument. The following operating parameters were used. A liquid demountable cell with a 0.01-mm path length was employed.

Resolution	927
Response	11.00
Gain	4.2
Speed	16
Suppression	10
Scale	1×
Prism	NaCl

The identity of certain of the compounds was confirmed by available infrared data (7, *12*).

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Crystalliferous Bacteria as Insect Toxicants

R. F. Anderson and M. H. Rogoff

Bioferm Division, International Minerals and Chemical Corp., Wasco, Calif.

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Bacillus thuringiensis *produces a variety of organic compounds which are toxic to the larvae of certain susceptible insect hosts. Among the toxic entities are proteinaceous crystals, probably three soluble toxins, and certain enzymes. The protein material is the major toxin active in killing lepidopterous larvae. The protein is formed by the cells apparently in close synchrony with sporulation, and its nature is a constant function of bacterial strain. The mode of action of the protein is under study. The sequence of events in the pathology observed is probably solubilization of the crystal (enzymatic or physical)⟶liberation of toxic unit⟶alteration of permeability of larval gut wall⟶change in hemolymph pH⟶invasion of hemolymph by spores or vegetative cells of the bacterium.*

Reports that crystalliferous bacteria of the genus *Bacillus*, and *Bacillus thuringiensis* in particular, are pathogenic for a variety of lepidopterous insect species can be found in the literature as early as 1915 (*4*). The interesting characteristic of these insect pathogens is that for certain of their insect hosts infection, in the true sense of the word, is not required for a kill. At the time of sporulation, the bacillus produces a protein, characteristic for its species, in such quantity that it crystallizes out within the bacterial cell. On ingestion by susceptible lepidopterous larvae, this protein crystal inclusion can kill the insects via a characteristic pathology. The usual course of infection—involving reproduction of bacterial cells within the host tissues, production of invasive factors (enzymes, etc.) by the bacilli, and toxin production by the bacteria within the host—need not be

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Advances in Chemistry; American Chemical Society: Washington, DC, 1966.

followed to produce a toxemia. Ingestion of the externally formed (from the host) toxin can be sufficient to cause death.

With certain Lepidoptera, the crystalline protein alone is not sufficiently toxic to cause a quick kill, and the normal course of tissue invasion by viable bacilli must take place to kill the host. Indeed, this is probably required for the bulk of susceptible Lepidoptera. However, the ability of crystal alone to produce kill (and similar singular action of another toxin produced by these bacilli on flies) places the compounds within the realm of true toxicants and mandates their consideration as chemical entities.

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Composition of a Bacillary Microbial Insecticide

Microbial insecticides are very complex materials in their final formulation, because they are produced by fermentation of a variety of natural products. For growth, the bacteria must be provided with a source of carbon, nitrogen, and mineral salts. Sufficient nutrient is provided to take the strain of choice through its life cycle to complete sporulation with concomitant parasporal body formation. Certain crystalliferous bacilli require sources of preformed vitamins and/or amino acids for growth. Media for growing these bacilli may vary from completely soluble, defined formulations, usable for bench scale work, to rich media containing insoluble constituents for production situations (*10*, *27*). Complex natural materials such as cottonseed, soybean, and fish meal are commonly used. In fact, one such commercial production method (*25*) is based on use of a semisolid medium, a bran, which becomes part of the final product.

The bacterial culture converts a portion of the supplied nutrient into vegetative cells, spores, crystalline protein toxin, soluble toxins, exoenzymes, and metabolic excretion products by the time of complete sporulation of the population. Although synchronous growth is not necessary, nearly simultaneous sporulation of the entire population is desired in order to obtain a uniform product. Depending on the manner of recovery of active material for the product, it will contain the insolubles including bacterial spores, crystals, cellular debris, and residual medium ingredients plus any soluble materials which may be carried with the fluid constituents. Diluents, vehicles, stickers, and chemical protectants, as the individual formulation procedure may dictate, are then added to the harvested fermentation products. The materials are used experimentally and commercially as dusts, wettable powders, and sprayable liquid formulations. Thus, a

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very complex material is finally presented to its prospective insect target. The product must also be attractive to its intended insect host, since it is not a contact poison and must be consumed by the larvae.

Toxic Materials Produced by Crystalliferous Bacilli

Materials produced by crystalliferous bacilli which elicit a toxic response in susceptible insects may be separated into two types. The first type, the true toxins, include the crystalline protein inclusion body [the parasporal body of Hannay (*14*)], a heat-stable, water-soluble exotoxin active against flies, a heat-stable, dialyzable water-soluble exotoxin, toxic to Lepidoptera on injection (*23*), and a heat-labile, water-soluble, filterable exotoxin, toxic toward larch sawfly larvae (Hymenoptera) which was reported by Smirnoff (*31*).

The second type of material includes spores, which may or may not produce disease symptoms but which can germinate in the insect gut and give rise to vegetative bacterial cells which in turn may produce, and exoenzymes such as phospholipases (lecithinases) or hyaluronidase. The phospholipases may produce direct toxic symptoms owing to their action on nervous or other phospholipid-containing tissue. Hyaluronidase breaks down hyaluronic acid and produces effects on animal tissue which are morphologically similar to the breakdown of insect gut wall in the presence of microbial insecticide preparations.

It is not implied that all of the above-mentioned entities are present simultaneously in a given microbial insecticide preparation. Certain of the toxins may or may not be produced, dependent on make-up and constitution of the media used for growth of the bacillus. Treatment of material in harvesting and formulation procedures may lead to loss of heat-labile materials, and separation of fermentation solids prior to formulation results in loss of soluble material present. The enzymes, for example, are products only of the vegetative cell and, because of their inherent instability under normal processing conditions, are probably never carried to product in sufficient quantity to have any bearing on insect activity. Finally, production of the toxin complement is a function of the particular strain of bacillus used, while the toxin spectrum produced is the result of its genetic capacity in both the quantitative and qualitative aspects. At this juncture, we may proceed to consider the toxic components separately, in some detail, within the limits of current knowledge.

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Crystalline Protein. MICROBIOLOGICAL ASPECTS. If the bacillus is cultivated in a medium supplying all necessary growth requirements with some single component adjusted to provide sufficient material for maximum growth but no more, the entire culture should sporulate on exhaustion of the limiting nutrient. On visual examination, simultaneous with the appearance of spores in the bacterial cells, the crystalline parasporal bodies appear. These apparently are formed in close proximity to nuclear elements within the cell, a conclusion based on electron-microscopic observations by Norris and Watson (*30*). When the sporangium undergoes autolysis, both mature spores and the crystals are released into the surrounding medium. The protein of the crystal does not have to be synthesized directly from the nutrients in the medium by the cell. It can be demonstrated experimentally that if a culture is harvested at a point just prior to sporulation, washed free of medium, and resuspended in distilled water, or water plus specific compounds required for sporulation, spores and crystals are then formed in the endotrophic system. The synchrony of spore and crystal formation can be upset by rigorous environmental conditions. For example, Smirnoff (*33*) has demonstrated inhibition of crystal formation with continuing spore formation in the presence of high concentrations of urea. He also demonstrated the reverse effect on prolonged culture at low temperature, obtaining crystal formation without concurrent spore formation (*32*). Neither of these experiments, however, precludes the possibility of definitive relationships between the sporulation process and the crystal-forming protein synthetic process. Monro (*28*) demonstrated that crystal protein antigens were absent from vegetative cells of a crystalliferous bacillus and, in fact, were absent until the time of optically visible crystal structures in sporulating cells. Repression of the crystal protein synthesizing system (enzyme?) until the occurrence of sporulation indicates some relationship between the two anabolic processes, at least on control mechanisms for the synthetic pathway. We would expect the crystal protein, as a characteristic cell constituent, to be under control of all internal regulatory mechanisms normally operative in a bacterial cell.

The crystal formed by any given strain of crystalliferous bacterium is a constant and a strain function. Constancy of insect susceptibility spectra for given bacillus strains tells us this, and differences between strains indicate uniqueness. Krywienczyk and Angus (*22*) demonstrated that dissolved endotoxins of strains of *B. thurin-*

giensis var. *thuringiensis*, *sotto*, and *entomocidus* had a common toxic component but that the gross compositions were serologically different. The situation is apparently not too different from that of other protein or polypeptide materials with physiological activity which are produced by bacilli (bacitracin). These are not single molecular entities but rather families of related compounds. Physiological activity may vary with particular molecular type, and this is also true of the crystal proteins. Superficially identical crystals from different strains may have widely different activities on a given host. Shape of a given strain's crystal is fairly constant, but size may vary, perhaps even as a function of medium composition. We may assume that availability of any individual amino acid might directly affect crystal synthesis. The weight of crystal protein produced by crystalliferous bacilli probably amounts to about one-third of the total dry cell weight. Thus, Fitz-James *et al.* (*12*) analyzing sporulated cultures of *Bacillus cereus* var. *alesti* found that 32% of the dry weight of the cell material could be accounted for as crystal protein.

There are indications that the crystal protein is subject to proteolytic enzymes when separated from the sporangium. The crystal protein has also been shown to be degradable by fairly nonspecific proteases such as pepsin and trypsin.

Physicochemical Aspects. Other than toxicity toward insects, the protein of the crystals has no unique characteristics not shared by other protein molecules. It contains between 17.5 (*15*) and 18% (*1*) nitrogen, present in 17 to 19 amino acids (Table I), none of which are unusual or present in any unusual concentration. The protein is sensitive to heat and when dissolved (0.01*N* hydroxide) absorbs characteristically at the 280-mμ maximum displayed by aromatic amino acids.

Its solubility characteristics in aqueous systems are such that retention of toxicity to insects by dissolved crystal protein is always suspect, and loss of activity on dissolution owing to denaturation is often observed. The protein is soluble only in relatively strong aqueous alkali. Thus, it has been variously reported to be soluble in 0.01*N* to 0.05*N* sodium hydroxide (*1*) and alkali at pH 10.5 in the presence of thioglycollate (*35*); we have also observed its solubility in alkali at pH 9.5 in the presence of urea and potassium borohydride. One difference between the characteristic proteins produced by various strains of crystalliferous bacilli is observed in the degree of alka-

Table I. Amino Acid Composition of Solubilized Crystalline Toxins of Crystalliferous Bacillus Species

Amino Acid	*Bacillus thuringiensis*	
	var. sotto[a]	*var.* thuringiensis
	Amino Acid, Gm./100 Gm. Protein	
Arginine	9.6	0.7
Lysine	3.6	1.0
Cysteine (incl. cystine)	1.2	0.8
Histidine	2.7	0.9
Aspartic acid	9.6	18.4
Glutamic acid	11.8	17.6
Glycine	3.2	5.7
Serine	4.8	6.4
Alanine	2.8	5.7
Proline	7.5	5.6
Tyrosine	6.8	3.2
Threonine	4.5	8.8
Methionine	1.3	1.3 (1.4[b])
Phenylalanine	8.6	0.6
Valine	5.3	7.5
Leucine/isoleucine	11.2	8.6
Norleucine	–	–
Tryptophan	2.6	0.1

[a] After Angus.
[b] Including 0.08 gm./100 gm. methionine sulfoxide.

linity required for their dissolution. Solution of the protein at neutrality has not been found in any solvent or solvent system tested to date. Hannay and Fitz-James (*15*) reported a pH of 11.8 necessary for dissolution of *B. thuringiensis* var. *thuringiensis* crystals, Angus (*1*) reported pH 10.0 for var. *sotto*, and Fitz-James *et al.* (*12*) reported pH 11.0 to 12.2 for var. *alesti* crystals. Once in solution, the protein is reported to have an isoelectric point somewhere between pH 4.9 and 5.2 (*15*). Precipitation was accomplished by reduction of the pH to a value of 4.0 and was also observed on addition of trichloroacetic acid. We have found that dissolved toxin precipitates at pH 4.6. The dissolved protein is also precipitated by addition of 2% ammonium sulfate to an alkaline solution (*1*) and by dialysis of the alkaline solution. In all cases, the recovered precipitate is amorphous.

Homogeneity of the protein in the crystal is questionable at this juncture. Based on physicochemical and immunochemical studies, it seems probable that the crystal contains more than one component,

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albeit a single toxic entity may be responsible for the pathological changes it causes in insects. Dissolved protein was studied for electrophoretic characteristics by Angus (*1*). Angus observed the behavior of *B. thuringiensis* var. *sotto* protein in an electrophoretic field with a 0.1*N* tris buffer at pH 9.0. A single fraction was seen, accompanied by a slow-moving minor shoulder. In our laboratories, using partially solubilized material (pH 9.5) and a paperionophoresis system in 0.6% acetic acid, five differentiable ninhydrin-positive zones are observable. Immunochemical studies on highly purified material (*28*) indicated at least two, and perhaps more, distinct components in the crystals of *B. thuringiensis* var. *thuringiensis*. Similarly, Krywienczyk and Angus (*22*), studying crystal protein of *B. thuringiensis* var. *thuringiensis*, *sotto*, and *entomocidus*, report several components, one of them common to all three preparations. Their observation is plausible; in fact, it would be surprising were minor differences not observed among the crystals produced by distinctly different strains of bacilli.

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Entomological Aspects. The gross pathology associated with ingestion of crystal protein toxin by a susceptible host insect (larva) is characterized by a rapid paralysis of the midgut. Guts of some susceptible insects, such as the silkworm, Chinese oak silkworm, tobacco hornworm, and tomato hornworm, show the characteristic paralysis within 20 to 30 minutes after consumption of crystals of *B. thuringiensis*. Effects of the toxin may be seen even prior to gut paralysis. This is reflected in the initiation of an increasing alkalinity of the blood of silkworms some 10 minutes after crystal ingestion. This rise in pH continues until an increase in blood pH of 1.0 to 1.5 units is reached. This rapid pH change brings on total paralysis within 1 to 7 hours, which eventually results in death of the insect, usually within 24 hours.

Observation of change in blood pH following crystal ingestion raises the question of site of action of the toxin. A second question, whether or not the protein itself actually is a toxin, is raised in view of the observation by Angus (*3*) that the protein material, so potent on ingestion, was nontoxic when injected into the hemolymph of silkworm larvae. Several experimental findings considered together form a picture which is representative of the present viewpoint on the mode of crystal action. The extremely rapid initiation of change in blood pH correlates to histological evidence obtained from studies of several susceptible insects. It was found that on ingestion of toxin,

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cells of the midgut become free from one another and also from the basement membrane of the gut. In the silkworm, for example, relaxation of the midgut and disorganization of the midgut epithelium are apparent only 45 minutes after ingestion of the toxin (*16*). The permeability of the midgut is thus affected, resulting in leakage of the alkaline, highly buffered midgut contents into the hemocoel of the insect, causing the aforementioned pH change and complete paralysis. Heimpel suggests that the cell-cementing substances are the sites of toxin action. Their precise nature is unknown, as is the precise mode of action of the toxin.

The second question concerns the active form of the toxin. It had been proposed (*18*) that the crystal toxin had to be in solution for expression of the toxin action. Indeed, toxicity of the crystals correlates well with a high midgut pH in the susceptible hosts although there are notable exceptions. In general, Lepidoptera with a gut pH of 9.0 to 10.5 are highly susceptible, and solubility of crystal at these pH values in the presence of reducing substances is probable. Heimpel and Angus (*19*), however, noted the presence of enzymes in silkworm foregut which broke down dissolved crystal protein to amino acids. Based on this observation, they suggested that the crystal might be a protoxin with the actual toxic entity being a smaller, enzymatically formed subunit of the protein. Some light on both questions has been shed by a recent study by Angus (*2*). First, he demonstrated that clarified silkworm gut juice contained no toxic principle, such as is present in *Pieris brassicae*. He then exposed a relatively pure preparation (98%) of *B. thuringiensis* var. *sotto* crystals to the gut juice. The filtered reaction mixture was then fed or injected. Those larvae receiving solubilized crystals orally exhibited normal paralytic symptoms; those receiving 10 times the amount of toxin by injection into the hemocoel were not so affected. This indicates not only that incubation with gut juice yields a soluble toxin active by the normal oral route, but its inactivity on parenteral injection supports the proposed mode of action on midgut epithelium, since the general paralysis must then be caused by other than soluble toxin itself. The alkaline midgut content leakage may well be the answer. One final bit of evidence (*3*) is that spores from which crystal has been removed by alkali leaching do not produce any pathology in the insect when taken by the oral route. However, on injection into the hemocoel, septicemia is observed within 12 hours, thus indicating that without

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concomitant action of crystals, the spore or its derived vegetative cell cannot pass through the gut wall; it has retained its integrity.

Water-Soluble Exotoxins. In this area, we enter into the slightly muddy waters of the lower molecular weight compounds which elicit toxic responses in susceptible host insects. Without reference to molecular identity, it is possible to sift through the various reports on the effects of bacillus-produced soluble exotoxins and recognize three possible types of material which are produced under appropriate conditions by specific strains of bacilli:

Type 1. A heat-stable material, toxic on ingestion, which prevents completion of adult development in the pupal stage in certain susceptible Muscidae (*6*).

Type 2. A heat-stable material, toxic upon injection, which produces a mortality in various Lepidoptera (*23*).

Type 3. A heat-labile material, toxic on ingestion to several species of larch sawfly (*31*).

Of these, the first, often referred to as the "fly factor or fly toxin," has received the most attention and has, to date, been most clearly defined. In fact, some investigators (*9*) feel that the McConnell-Richards factor (Type 2) and the "fly factor" are identical. Part of the confusion stems from the early work in which certain insect species were tested for susceptibility by parenteral injection. The particular insects studied [wax moth, cockroach, corn borer, mosquito, flesh fly (*23*)] were not susceptible to the toxin via the oral route. Later studies by various investigators demonstrated susceptibility of housefly, silkworm, flour moth, etc., to the soluble toxin on ingestion (*6*, 7, *13*). Thus, the mode of introduction of toxin to host indicated a difference between toxins which may not actually exist. Again, there are differences between the opinions of various investigators as to precisely which strains of bacteria produce the soluble toxins. At present, it appears to be only strains of *B. thuringiensis* var. *thuringiensis* and possibly var. *galleriae*.

The toxin kills larvae and/or pupae of some Diptera and Lepidoptera and acts primarily by prevention of completion of pupation. It is produced in cultures prior to sporulation (*9*, *26*) and remains in the supernatant liquid of sporulated cultures. Its chemical structure is not known, but initial isolation and purification studies are under way (*9*). One cannot, however, leave a discussion of this toxin with a feeling of certainty. Burgerjon and deBarjac (7) and Krieg and Herfs (*20*) reported the above-mentioned effectiveness of the soluble

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toxin against Lepidoptera, yet Cantwell *et al.* (*9*) obtained no mortality on certain Lepidoptera fed up to 50 mg. of a semipurified toxin preparation. Whatever opinions are held in regard to the soluble toxins of *B. thuringiensis* should all be held to be strictly of the order of "working hypotheses."

The exotoxin reported by Smirnoff (*31*) is definitively different from other soluble toxins, as indicated by its reported heat lability. This soluble toxin was obtained from the supernatant of a sporulated *B. thuringiensis* culture. In testing, it was found to be very toxic by ingestion to 18 species of larch sawfly larvae. No further studies on this toxin have been reported at this time.

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Phospholipase (Lecithinase) Enzymes. Lecithinase enzymes produced by various crystalliferous and noncrystalliferous (*B. cereus*) spore-forming bacilli have been proposed as having some role in the causation of insect mortality by the bacteria. Indeed, a number of investigators have demonstrated death of test insects, such as wax moth (Lepidoptera) and larch sawfly (Hymenoptera), on injection or ingestion of lecithinase (*5, 17, 21, 34*). The lecithinases catalyze reactions in which lecithins are cleaved at various positions on the molecule, each particular enzyme (lecithinases A, C, and D and lysolecithinase) being specific for site of action. Since, histologically, damage to the midgut cells of infested larvae (larch sawfly) involves parts of the cells of which phospholipids are known constituents, lecithinase or other phospholipase activity might be involved in the observed pathology. Lecithinase-catalyzed reactions could conceivably lead to disruption of the gut wall by dissolution of phospholipid cementing material, thereby permitting invasion of the hemocoel by spore or vegetative forms of the pathogen. The end results would be death by a typical bacteremia.

The importance of the phospholipases in the pathology produced by the crystalliferous bacteria, particularly as regards their use as toxicants, must be relegated to a relatively minor position in view of several considerations. First, it has not been demonstrated that any relationship exists between phospholipase production in a given strain and its effectiveness as a toxicant for lepidopterous insects. Second, phospholipases must be produced by vegetative cells of the invading pathogen. In regard to most of the insect hosts against which *B. thuringiensis* activity is directed, the die is cast at the time of ingestion of crystal; vegetative cell factors are not required for kill–, i.e., crystal alone results in midgut epithelium disruption. Thus, any

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lecithinase effects would be only accessory and additive in small part to the massive damage caused by crystal. Finally, in most susceptible Lepidoptera, the midgut pH is higher than the pH values of 6.8 to 7.4 reported as optimum for the lecithinases (*5*). The larch sawfly, with its more neutral gut (pH 7.0 to 7.8), may well be a more receptive host to lecithinase action. In short, if any role is played by the phospholipases produced by *B. thuringiensis* in causation of the usual pathology observed, it must be relatively minor. Even in the case of the Lepidoptera in which spores are known to germinate in the midgut with active vegetative cell multiplication taking place, the gut contents do not leak into the blood (*16*), indicating no lecithinase activity of any consequence occurring. The various toxins are listed in Table II.

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Table II. Components of a Crystalliferous Bacillus Insecticide Which May Contribute to the Observed Pathology in Insects

Component	*Action or Pathological Symptom*
Crystal	Midgut paralysis in susceptible Lepidoptera
Spore	Septicemia following penetration to hemocoel
Fly toxin	Prevention of emergence of certain fly pupae
McConnell-Richards factor	Mortality in *Anagasta kuhniella*
Heat-labile soluble toxin	Mortality in larch sawfly larvae
Exoenzymes (phospholipases, hyaluronidase)	May be partially responsible for gut cell destruction
Vegetative bacterial cells	May produce toxins and enzymes above, plus other metabolic products on growth in host gut and hemocoel

Characteristics of a Crystalliferous Bacillus Intoxication

As in any biological system, there are no sharp breaks in the responses of various hosts to the action of crystalliferous bacilli. The entire range of reaction from exquisite sensitivity to complete resistance is encountered. The responses of Lepidoptera can be broadly broken down into two major groups, insects susceptible to crystal action alone and those giving little or no response to the crystal. The unifying characteristic of the response of those insects relegated to the first group is the onset of the gut paralysis previously described. General paralysis following gut paralysis then divides the crystal-suseptibles into two subgroups.

The general paralysis is symptomatic in a restricted group of insects whose larvae are characterized by a comparatively high gut pH. Following crystal ingestion, changes in the permeability of the gut wall can be observed (*11*); the high gut pH then falls because of leakage of gut contents into the hemocoel. The pH of the hemolymph concurrently rises as a result of the same phenomenon. The general paralysis takes place as the hemocoel pH rises; the same effect can be demonstrated by simple injection of buffer into the hemocoel, raising the pH to 8.0 (*29*). Thus, the general paralysis is only a secondary effect of toxin action, in reality an indicator of degree of gut will damage. Lepidoptera displaying the general paralysis symptom are called Type I insects by Heimpel (*16*, *19*), who classifies insects into four groups on the basis of response to the crystal toxin. Representatives of this extreme sensitivity group are the silkworm, the Chinese oak silkworm, and the tobacco and tomato hornworms.

The Type II insects, which represent the majority of the Lepidoptera according to Heimpel (*16*), encompass the group mentioned here as the remaining crystal susceptibles. In these larvae, no leakage of gut contents, no rise in pH of the hemolymph, and no general paralysis are observed. Following ingestion of the crystal, the onset of gut paralysis is observed in a few hours, feeding ceases, and a slow decrease in pH of the gut occurs. A similar decrease in pH is normally seen on starvation of healthy larvae. Here, apparently, death is the end result of starvation, toxin action being insufficient physiologically to cause the secondary symptoms observed in the Type I host, but it must be recognized that toxin action is the prime factor in the kill.

Norris (*29*) broadens this concept a little by considering that when the gut pH falls, it goes sufficiently low to allow initiation of germination of ingested spores and rapid multiplication of vegetative cells. He feels that growth of bacteria in the gut results in breakdown of the wall and bacterial penetration of the hemocoel. In the course of this type of infection, general septicemia develops and the insect, when it succumbs, is packed with spores and crystals. He does, however, raise the question as to just how much of a role crystals *vs.* toxins of the growing cell (lecithinases) play in attack on the gut wall. The proportional role of each may well prove to be a function of the particular host species.

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Table III. Summary of Heimpel and Angus' *(19)* Host Classification Based on Susceptibility to Crystalliferous Bacilli

Insect Type	*Primary Responsible Agent*	*Representative Hosts*	*Symptoms*
I	Crystal	Silkworm Chinese oak silkworm Tobacco hornworm Tomato hornworm	Rapid midgut paralysis with lowering of pH of midgut contents. Rapid midgut disorganization Leakage of midgut contents to hemocoel with rise in hemocoel pH General paralysis in 1 to 7 hours
II	Crystal	Bulk of susceptible Lepidoptera	Midgut paralysis Slow drop in midgut pH (starvation) No histopathological changes in midgut epithelium; general paralysis or rise in blood pH Germination of spores and multiplication of vegetative bacilli
III	Soluble toxins	*Anagasta kuhniella*	No gut or general paralysis Death in 2 to 4 days
IV		*Leptinotarsa decemlineata* *Euxoa segetum* *Lycophotia sancia*	Not susceptible

Heimpel and Angus' (*19*) third group of insects is most probably represented by only one lepidopterous host, *Anagasta kuhniella.* In this case, neither spores nor crystals alone killed all the insects tested (spores 4 to 8% mortality, crystals 12% mortality). However, ratio of original culture spore to crystal of 1 to 1 caused 80 to 90% mortality. This appeared to be a synergistic effect hitherto unreported. Heimpel now feels that this unique reaction may be due to the action of the aforementioned McConnell-Richards toxin complex which is reported as a soluble component found in the culture fluid. This material, if a true toxic entity dried with spores and crystals, might well be responsible for the *Anagasta* response. Other investigations on the effect (*8, 24*) tend to support Heimpel's hypothesis.

The fourth group of insects are the resistant or nonsusceptible Lepidoptera. This group would include such species as *Agrotis,*

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Peridroma, *Euxoa*, and *Mamestra*. This fourth classification is an addition to the scheme by the French workers in the field (7). The above-described classification is summarized in Table III.

Conclusions

It is somewhat difficult to summarize the heterogeneous information presented here. An attempt would be to state that we are dealing with a complex biological activity of some unique organic compounds, plus the activity of a living entity, the *B. thuringiensis* cell itself. The crystalline protein toxin is the prime factor in the effects, although its relative efficacy remains a function of the host insect. However, since all components of the system can play a role in the host's death, it is desirable that the *B. thuringiensis* preparations used as toxicants contain crystal, spore, and all soluble toxins, characterized or not.

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8

Some Antifungal Factors in Barley

ALBERT STOESSL

Research Institute, Research Branch, Canada Department of Agriculture, University Sub Post Office, London, Ontario, Canada

An antifungal factor known to be present in young barley plants has been isolated and fractionated into a number of chemically related bases. Of the two substances obtained in a state of high purity, one was shown to be p-*coumaroylagmatine, which exhibits only slight antifungal activity. The other, a very active substance, is a considerably more complex molecule. It appears to incorporate a substituted* p-*coumaric acid residue, while the basic functions are due to two agmatine residues. A glucose moiety is a further constituent which can be removed without affecting the antifungal activity of the compound.*

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Helminthosporium sativum has attracted considerable attention as a pathogen of a number of graminaceous and other plants (*1*). [This is the common name of the fungus. A referee has pointed that it is more correctly described as *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur, (Imperfect state *Bipolaris sorokiniana* (Sacc. in Sorokin) Shoemaker.)]

Several aspects of the relationship between the fungus and young barley as its host were studied in detail in these laboratories. They led to the discovery, isolation, and structure determination of a toxin, helminthosporal (*2*), which is released by the fungus to render the host susceptible (*5*) to invasion. A further discovery of interest was the fact that young shoots resisted infection until 5 to 6 days old, after which they became susceptible (*6*). The presence of an antifungal agent was probable and confirmed provisionally, by the observation that 5-day-old plants yielded aqueous extracts which exhibited fungistatic activity. In contrast, shoots old enough to be

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subject to infection yielded a water extract which was inactive itself but from which a fungistatic butanol extract could be prepared. With this apparent anomaly, the clues to further progress were provided. It was thus found that the protection against fungal invasion ceased with advancing age of the barley shoots not because of destruction of the antifungal factor, but because of its neutralization by a number of cations, among which Ca^{+2} and Mg^{+2} were the most effective (*6*). Further work on this interesting situation has been deferred until more is known about the chemistry of the antifungal factor. The present contribution describes some studies on its chemical structure which is, of course, also of intrinsic interest.

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After some misleading earlier results, it became evident that high antifungal activity is associated with a basic fraction which is obtained from 5- to-6-day-old barley shoots by extraction with hot water and a subsequent ion-exchange procedure. The complex mixture of inorganic and organic acetates thus prepared can be fractionated by countercurrent distribution in 1-butanol–water (*4*), or more efficiently in 1-butanol–water–acetic acid (4:5:1). This leads to several fractions which are marked by strongly basic character, positive Sakaguchi reactions, and characteristic ultraviolet absorption, with an intense maximum near 300 mμ. Several of the fractions are clearly mixtures of compounds very difficult to separate. This point is illustrated in Figure 1, which reproduces a chromatogram of the products. Fortunately, one of the most biologically active fractions occurred in greatly preponderating amount and was obtained in a chromatographically homogeneous state. This fraction is referred to below as compound M. A second substance of approximately equal activity can also be purified to chromatographic homogeneity and is very similar to M in all its properties except for R_f. Its yield is approximately one tenth that of M, and since it offers no obvious advantages of study, further work on it has been deferred. Lastly, attention may be drawn to the compound marked CA in Figure 1, which it became desirable to isolate and study while the work on M was proceeding.

Compound M is obtained from the countercurrent distribution as the sirupy acetate which gives 100% inhibition of germination of *Monilinia fructicola* at 6 p.p.m. It is homogeneous in a large number of chromatographic systems, but a word of caution is in order. When the countercurrent distribution is followed by ultraviolet spectroscopy tube for tube, the spectra of the few fractions consisting ap-

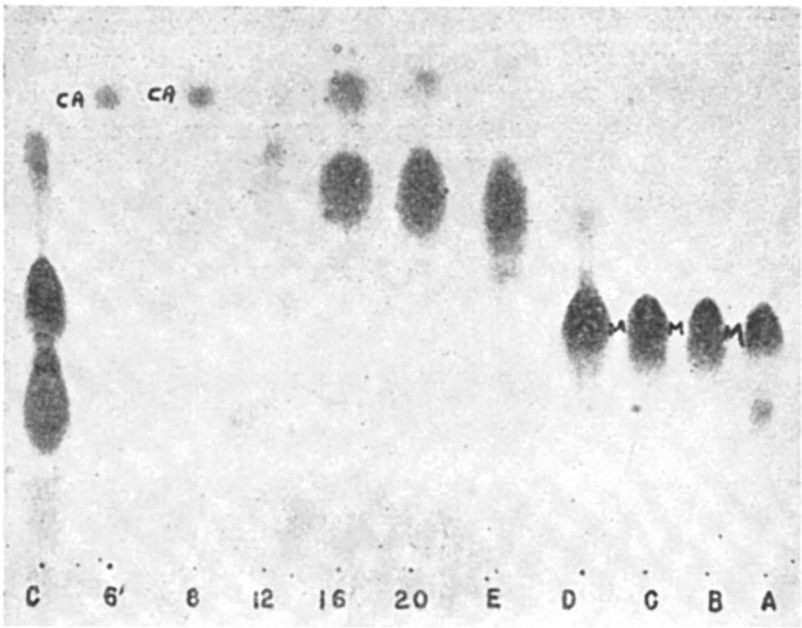

Figure 1. Chromatogram of fractions from countercurrent distribution of basic portion of barley extract

C. Crude basic product before distribution
6 to 20. ρ fractions collected 10 at a time; numbered in order of collection
E. r ***fractions 99 to 70***
D ***to*** **A. r** ***fractions 69 to 46***

Chromatography on thin layers of Avicel C (microcrystalline cellulose from American Viscose Division, F.M.C. Corp., Newark, Del.), in 1–butanol–water–acetic acid (4:5:1) with bromocresol green as indicator. Countercurrent distribution in same solvent by single withdrawal procedure with 299 transfers and 100 elements

parently only of M show slight but reproducible differences from one another. This may be caused, at least partly, by an isomerization of M, discussed below. However, the spectroscopic differences could indicate also that M is a mixture of two or more very closely related compounds. Obviously, it would be impractical to separate these on a preparative scale and it seemed advisable to proceed with the chemical investigation of M, cognizant of the special care that would be required in interpreting the experimental data.

Attempts to convert M into a crystalline salt were unsuccessful. However, an amorphous dipicrate could be purified by repeated precipitation and could also be converted into a clean though equally

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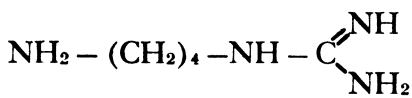
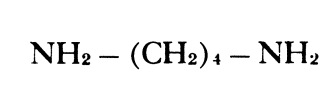
amorphous dihydrochloride. Both salts gave analyses which were in reasonably good agreement with a composition $C_{37}H_{58}N_8O_{11}$ for the free base. (M dipicrate: Found, C, 47.3; H, 5.2; N, 16.2; O, 31.6%. Calculated for $C_{49}H_{64}N_{14}O_{25}$, C, 47.4; H, 5.2; N, 15.7; O, 32.1%. λ_{max} 357 mμ, ϵ 31,300. Trimethylamine picrate had λ_{max} 358 mμ, ϵ 15,700. M dihydrochloride: Found, C, 51.4; H, 6.8; N, 12.5; O, 21.9; Cl, 8.1%. Calculated for $C_{37}H_{60}N_8O_{11}Cl_2$, C, 51.4; H, 7.0; N, 12.9; O, 20.4; Cl, 8.2%.)

Although the exact composition was and still is regarded as only tentative, all the subsequent evidence showed that the formula is at least approximately correct. In particular, ample proof will be found that the substance is a diacidic base.

The nature of the basic functions was defined by hydrolysis of M in 2*N* hydrochloric acid at 100°C. This furnished agmatine (I) which was characterized as its picrate and hydrochloride and also by degradation to putrescine (II).

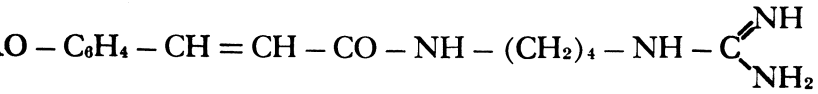


I



II

The positive Sakaguchi reaction of M indicated that the guanidino group of the agmatine moiety was free and this conclusion was confirmed and amplified by potentiometric titration of the metabolite. The curve obtained is shown in Figure 2, where it is compared with the corresponding curves due to agmatine and *p*-methoxycinnamoylagmatine (III).



III R = Me

VI R = H

Evidently, the titratable functions of M are two very strong bases, since the equivalent weight of M calculated from the curve was 391, corresponding to approximately half the molecular weight (791) calculated from the analyses discussed above. Apparently, one of the functions was due to the guanidino group of the isolated

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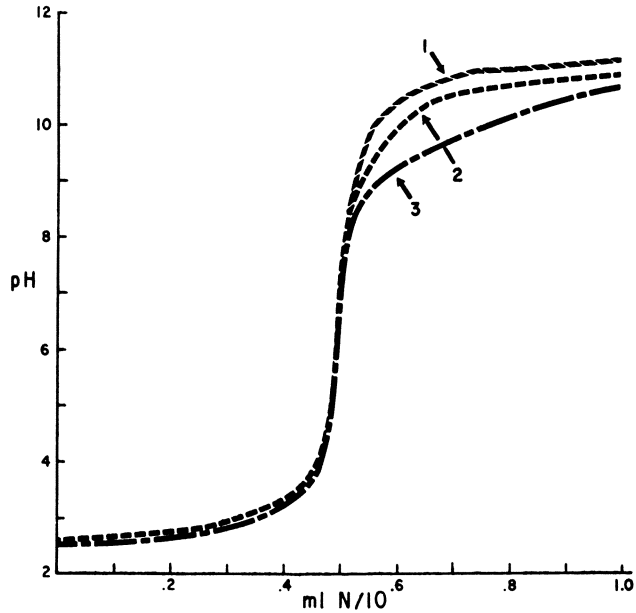

Figure 2. Titration of metabolite M and model compounds

1. 0.025 mmole metabolite
2. 0.05 mmole p-*methoxycinnamoylagmatine*
3. 0.025 mmole agmatine

Compounds converted into free base form by ion exchange and dissolved in standard hydrochloric acid. Titrant 0.1N sodium hydroxide

agmatine. The yield of this was about 0.9 mole under the hydrolytic conditions described. When M was hydrolyzed more energetically, however, the yield of agmatine was estimated to be 1.34 moles. Consequently, the second titratable function also was due to the guanidino group of a second agmatine residue. Finally, no other titratable function was discovered down to pH 2.5 and this indicates that the amino nitrogens of the agmatine moieties are neutral. The most probable explanation for this is that both are bonded in amide links. Inspection of Figure 2 shows that a basic amino nitrogen, as in agmatine itself, would have been revealed.

All the nitrogen atoms of M were thus accounted for, together with the provisional placement of two of the oxygen atoms. An acetylation experiment, under mild conditions, showed that the metabolite has five to six acetylable groups, at least four of which would be hydroxyl. This observation led to periodic acid oxidations which

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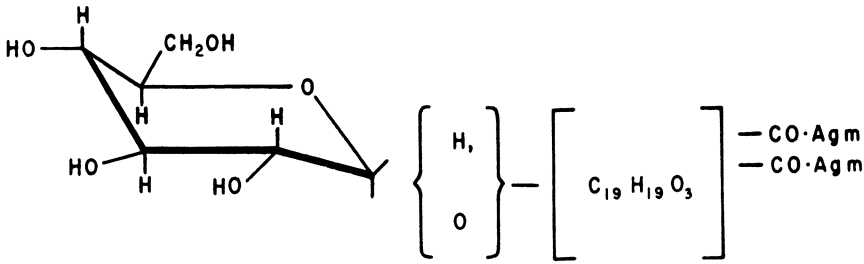
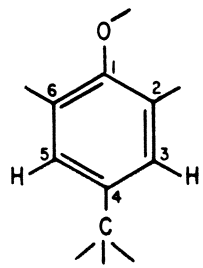
showed that 1 mole of metabolite consumes 2 moles of periodic acid and liberates 1 mole of formic acid. The site oxidized was defined when methanolysis of M with 2% dry hydrogen chloride in methanol gave α-methylglucoside in approximately 90% yield. Because of the stoichiometry of the periodic acid oxidation, the glucose residue must be attached to the metabolite in the pyranose form. We thus arrive at part structure IV for M.



IV

Alkali fusion of the metabolite furnished *p*-hydroxybenzoic acid in good yield as the only isolable product. Vigorous nitric acid oxidation of M gave a high yield of picric acid. Both degradation products must have arisen from the same site, which can be represented by part structure V. While positions 3 and 5 are probably unsubstituted, the vigorous nature of the degradations allows that those at 2 and 6 could bear carbon atoms.



V

The benzenoid residue thus established is necessarily part of the chromophoric system of the metabolite which could now be considered. It was soon evident that the spectrum of M exhibits the characteristic features of the *p*-coumaroyl chromophore but with

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some significant differences. The spectra of metabolite acetate and of *p*-methoxycinnamoyl agmatine acetate are shown in Figure 3.

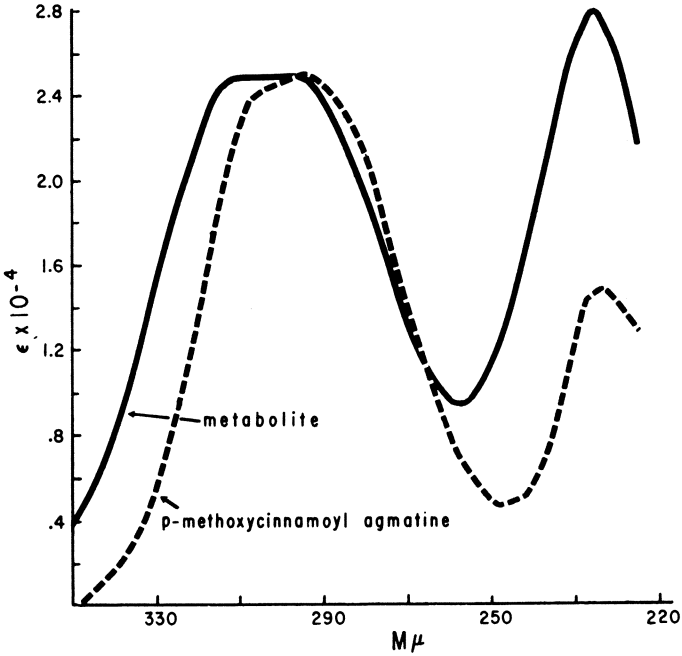

Figure 3. Ultraviolet spectra in water

p-Methoxycinnamoylagmatine hydrochloride, experimentally determined curve
Metabolite diacetate, curve replotted for comparison, to give ε = 24,500 at λ_{max} 298 (experimental value 20,500)

The greater intensity of the band of the metabolite at 220 mμ is probably due to the presence of a second, superimposed chromophore which could also account for the shift of the minimum. On the other hand, the band near 300 mμ has the expected intensity. Its broadness and displacement towards longer wavelength are probably due to the presence of a substituent on the double bond or benzenoid ring. That the assignment to a coumaroyl chromophore is essentially correct is evidenced by the fact that both M and the model compound underwent the same type of reaction on irradiation in the near-ultraviolet (Figure 4). The observed isosbestic points imply that the photoreaction is a simple one, such as A → B or A ⇄ B, and is obviously the well-known light-induced *trans-* to *cis*-isomerization (7) of cinnamic acid derivatives.

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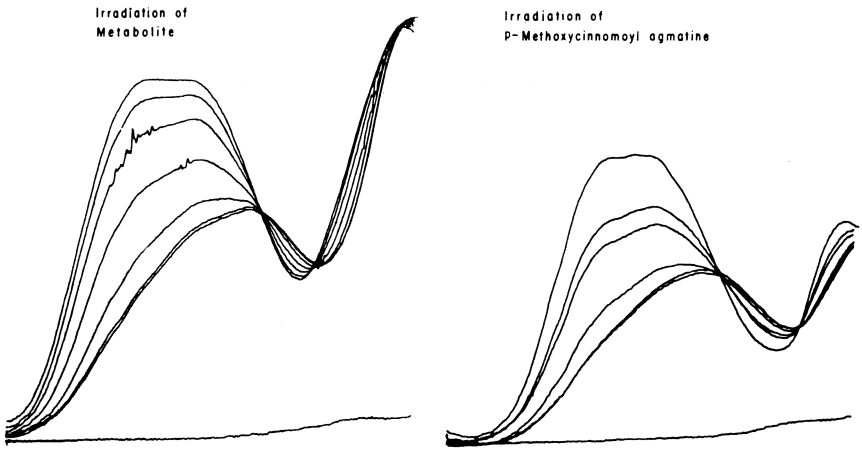

Figure 4. Photoisomerization of M and model compound Ultraviolet absorption curves obtained at intervals during irradiation of dilute aqueous solutions until constant (ca. 40 minutes) showing three (left) and two (right) isosbestic points

Attempts to isolate the postulated substituted *p*-coumaric acid by hydrolysis have been of no avail, probably partly because of the known proneness of coumaric acid to decarboxylation followed by polymerization (*3*). In addition, evidence is available that the coumaroyl residue is firmly attached to the as yet unexplored moiety of M.

Apart from methylglucoside, methanolysis of M furnished a compound, A, as the only other important fragment. A is also a sirup which cannot be crystallized. It is optically active and it has all the antifungal activity of M itself, giving 100% inhibition of germination of *Monilinia fructicola* spores at a concentration of 6 p.p.m. A is also a strong base, with two agmatine residues and the expected equivalent weight. Its ultraviolet spectrum is very similar to that of the metabolite and, in particular, the maximum at 300 mμ is insensitive to added base. The oxygen in the *p*-position of the coumaroyl moiety is therefore still bound to carbon. This coumaroyl ether link of A was cleaved when A was treated with dry hydrogen chloride in methanol under vigorous conditions. This gave 1 mole of agmatine and a compound B. B is still a guanidine and liberated agmatine in high yield on acid hydrolysis. The presence of two agmatine residues in M is therefore conclusively established. B absorbs intensely at 310 mμ in neutral or acid solution, but the maximum is shifted to 360 mμ on addition of alkali, indicating a free hydroxyl in the *p*-position of the coumaroyl residue. On the

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basis of these facts, if M were a derivative of an unsubstituted *p*-coumaric acid, B could only be *p*-coumaroylagmatine (VI). This, however, is definitely not the case, as evidenced by direct comparison with the latter compound. B undergoes a number of interesting reactions which are still under study.

The difficulties experienced with M and its derivatives prompted the isolation and purification of the barley metabolic product marked CA in Figure 1. This material was similar to M in giving a positive Sakaguchi reaction and in exhibiting a similar ultraviolet spectrum but with considerably increased intensity. This suggested that CA might be a smaller molecule which could be more readily studied and might furnish clues to the nature of its cometabolite, and this object was achieved by the preparation of a crystalline picrate. The work has been described in detail (*8*) and it will be sufficient here to state that CA was recognized to be probably *p*-coumaroylagmatine (VI). This was substantiated by the synthesis of the authentic compound from methyl *p*-coumarate and agmatine monohydrochloride, and also by methylation of both the metabolic and the synthetic products to the same methyl ether, independently prepared from *p*-methoxycinnamoyl chloride and agmatine sulfate.

p-Coumaroylagmatine exhibits only weak antifungal properties (24% inhibition at 56 p.p.m.). Its isolation, however, served a useful purpose in adding weight to the evidence which suggests that compound M is a derivative of a substituted *p*-coumaric acid.

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9

Virus Replication Inhibitors and Stimulators from Plant Sources

P. C. CHEO[1] and R. C. LINDNER

Tree Fruit Research Center, Washington State University. Wenatchee, Wash.

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Extracts from 152 plant species, representing 46 different families, were screened for effects on tobacco mosaic virus (TMV) replication in cucumber cotyledons. Twenty species have shown enough activity to warrant further study. Several members of the Caprifoliaceae *family increased virus replication. An extract of* Lonicera involucrata *enlarged the virus lesions in local lesion hosts and produced a thirtyfold increase in virus titer, but had no effect on virus replication in systemic hosts. The active material appears to affect the virus defense mechanism of local lesion hosts. An extract of common geranium is an active virus inhibitor. It inactivates TMV and TMV-RNA (ribonucleic acid) in vitro by forming noninfectious complexes. In vivo, it also inhibited starch lesion formation in cucumber cotyledons incited by TMV infection.*

The abundance of organic materials that occur in the plant kingdom offer an opportunity for searching for specific compounds interfering with virus replication in plants. It is hoped that such compounds, if discovered, could be used efficiently for virus control and also serve as tools for our understanding of the complex virus-host system.

In our screening program for virus inhibitors and stimulators we used cucumber as an assay host, in which starch lesions are formed in proportion to the concentration of virus in the inoculum.

[1] Present address: Department of Arboreta and Botanic Gardens, Arcadia, Calif.

In Natural Pest Control Agents; Crosby, D.;
Advances in Chemistry; American Chemical Society: Washington, DC, 1966.

Cucumber cotyledons were inoculated with purified tobacco mosaic virus (TMV) 20 to 24 hours before vacuum infiltration with different concentrations of crude water extracts of plant leaves (*4*). After 7 days, inoculated leaves were harvested and stored 24 hours in the dark in a moist chamber to remove excess starch. Starch lesions were counted after clearing with alcohol and staining with an iodine-potassium iodide-lactic acid mixture. The inhibitory effects of various extracts were demonstrated by comparing lesion counts of treated cotyledons to counts on control cotyledons.

To date, 152 species of flowering plants belonging to 46 different families, as well as a few species of mushrooms, mosses, ferns, and lichens, have been screened. Extracts from 20 seed plants have shown enough inhibitory activity to warrant further study. Leaf extracts of members of three families, *Liliaceae*, *Pinaceae*, and *Labiateae*, show a high incidence of antiviral agents. Several members of the *Caprifoliaceae*, on the other hand, contain virus-stimulating agents.

A twinberry (*Lonicera involucrata*) extract, as well as snowberry (*Symphoricarpas albus*) and several other species of common honeysuckle (*Lonicera spp.*), markedly increased the TMV lesion

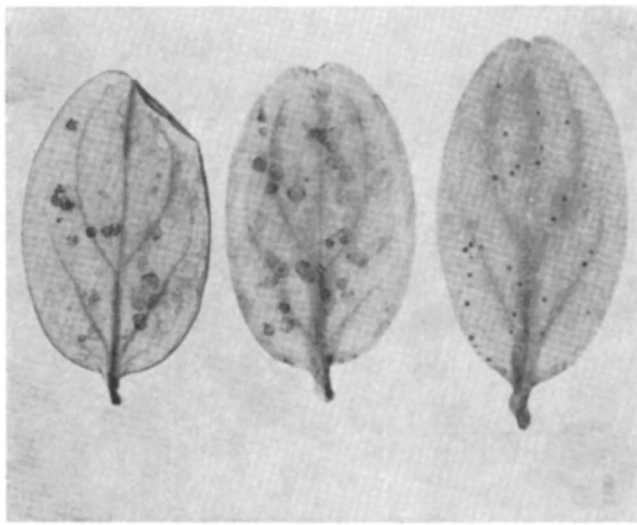

Figure 1. Effect of twinberry leaf extract infiltrated 24 hours after virus inoculation on TMV starch lesion development on cucumber cotyledons

Right. Control
Middle and left. Treated with twinberry extract

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size in cucumber (Figure 1) and other local lesion hosts such as pepper (*Capsicum frutescens*) and *Nicotiana glutinosa*. The effect was obtained by infiltrating cucumber cotyledons 20 to 50 hours after virus inoculation. The ordinary dotlike starch lesions became expanded and sometimes formed two to three layers of concentric ringlike lesions with a thirtyfold increase in virus titer.

Experimental

The active ingredient is soluble in water, ethanol, methanol, acetone, dioxane, and pyridine and can be extracted from an aqueous solution with *N*-butyl acetate, chloroform, and acidified ether (H_2SO_4). The alcohol extract of the twinberry leaves was evaporated to near dryness over a hot plate. The residue was taken up in distilled water, and the water-insoluble materials were removed by low speed centrifugation (10 min. at 3000 r.p.m.). The aqueous solution was treated with 40% lead acetate until no further precipitate was formed and then with 20% dibasic sodium phosphate to precipitate excess lead in the solution. Five per cent by weight of activated charcoal (Nuchar) was added and mixed to adsorb the active component, and the adsorbed charcoal was then shaken in a flask with acidified ether (3 drops of concentrated H_2SO_4 per 50 ml. of ether for about 2 days). After evaporating the ether, it was redissolved in water and passed through a column of diethylaminoethylcellulose (DEAE) previously equilibrated with 0.01*M* phosphate buffer at pH 7.5. The column was 1 cm. in diameter and 30 cm. in length. Fractions of 5 ml. each were collected. The active component from tubes 6 to 9 were pooled and then lyophilized. The dry powder was again extracted with ether, and after evaporation of the ether a colorless final product was obtained.

Results

This active substance gives a maximum absorption at 233 mμ. On paper chromatograms, it gave negative reactions with ninhydrin, $FeCl_3$, Sudan IV, silver nitrate, and aniline sugar reagent. It passes through a cellophane membrane.

The treated cucumber cotyledons showing enlarged ringlike lesions were macerated in the presence of neutral phosphate, and the crude juice was inoculated into healthy cucumber cotyledons. Only dotlike small lesions were produced from such infections, indicating

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that the ringlike large lesions were not a mutated virus lesion resulting from the treatment of twinberry extracts. Systemic hosts of TMV, *Physalis floridana*, and spinach (*Spinacia oleracea*) were also treated by infiltrating twinberry extracts 24 hours after virus inoculation. After 1-week and 2-week intervals following virus inoculation, leaf samples from treated and untreated plants were collected, homogenized, and heated (25 minutes at 60°C.), followed by two cycles of differential centrifugation. The total virus content per fresh weight of leaf samples was then determined based on the readings at 260 mμ in a spectrophotometer (Beckman Model DU). These results indicated no significant increase in total virus content of virus-infected tissue subsequently treated with twinberry extract over water-treated controls.

Consequently, the stimulating effect of twinberry extract on TMV is shown only in local lesion hosts, which exhibit a defense mechanism in limiting virus invasion. Holmes (*3*) has suggested that local lesion formation is the result of host hypersensitiveness to virus infection. The reaction of the first invaded cells is so severe that they die before the virus has time to move from them. Farkas *et al.* (*2*) have further suggested that the activation of phenol-oxidizing enzymes resulting in the formation of toxic quinones is responsible for the formation of necrotic lesions. These papers (*2, 3*) presented the evidence of metabolic activities following virus infection and subsequent effect on host tissue; nevertheless the mechanism of preventing further virus spread in local lesion hosts is not satisfactorily explained. In cucumber, TMV infection is normally limited to an area of about 200 cells around the infection site (*5*). There is no further enlargement of the virus lesion. TMV is a very stable virus, and the virus particles are highly active in these lesion areas. It would be reasonable to assume that some chemical defense mechanism in the surrounding healthy tissue rather than the necrosis of the virus-infected tissue prevented the spread of virus. This was suggested by Loebenstein in 1963 (*6*). Our study on the effect of aqueous geranium (*Pelargonium hortorum*) extract (*1*) further supports such an explanation.

Aqueous geranium extract inhibited TMV starch lesion formation in cucumber cotyledons. Starch lesions were completely inhibited by vacuum infiltrating effective dosages at any time between 1 and 33 hours after virus inoculation. Between 33 and 72 hours, inhibition decreased progressively. The active ingredient in the geranium extract was identified by means of ultraviolet absorption spectrum and

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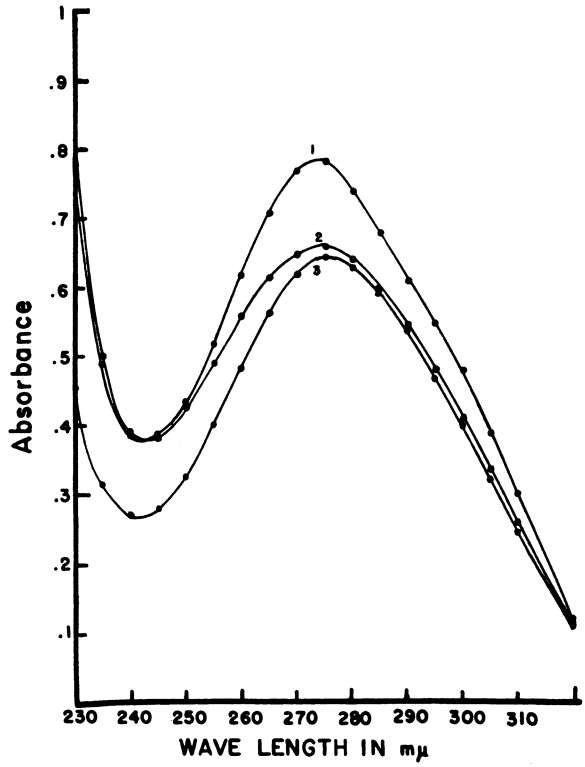

Figure 2. Ultraviolet absorption spectra
1. *Geranium leaf extract, 1 to 1500 dilution*
2. *Active TMV starch lesion-inhibiting component isolated from geranium leaves on paper chromatograms*
3. *Commercial tannic acid, 15 μg. per ml.*

paper chromatography as a tannin related to tannic acid (Figure 2). Geranium leaves contain relatively large amounts of tannin. Assays based on a standard ultraviolet absorption curve of commercial tannic acid indicated an average of 2% tannin in fresh geranium leaves. Infiltration of 0.06% commercial tannic acid completely inhibited development of starch lesion, and one half of the lesions were inhibited at 0.024%.

Lesion formation represents a host response to virus infection. Factors preventing the formation of starch lesions incited by the virus do not necessarily mean that virus replication is inhibited at the same time. Therefore, the total virus titer in cotyledon samples treated with different concentrations of tannic acid was determined (Figure

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3). The two highest concentrations of tannic acid (0.051 and 0.034%) resulted in a linear increase of virus titer up to 21 days after inoculation, even though the reduction of starch lesion formation was 91 and 64%, respectively. Thus, the virus must have replicated beyond the limitation of starch lesions. Further experiments indicated that a systemic spread of the virus into the primary leaves in cucumber plants could be obtained by daily brushing the noninoculated primary leaves (only the cotyledons were inoculated) with tannic acid following a vacuum infiltration of whole plants with 0.051% tannic acid 24 hours after virus inoculation. Primary leaves were shielded by tinfoil during the inoculation of the cotyledons to prevent accidental infection. Aerosol O.T. (0.1%) was incorporated in tannic

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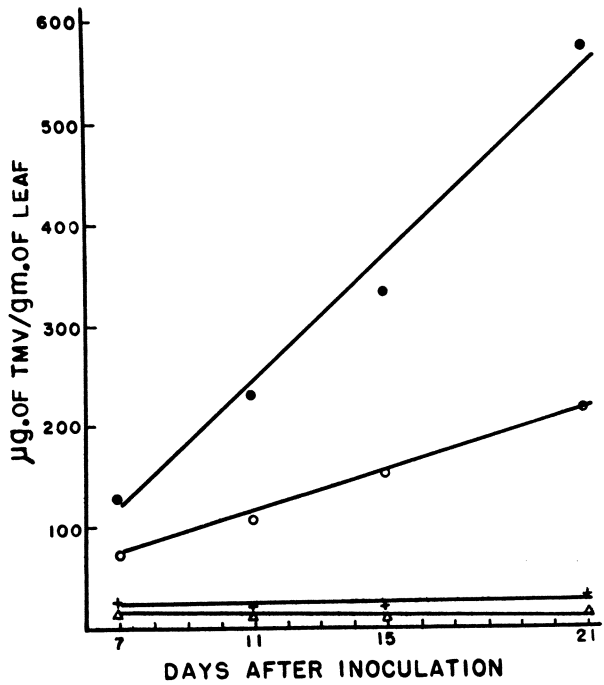

Figure 3. Virus titers of infected cucumber cotyledons infiltrated with different concentrations of tannic acid at various times after inoculation

● ***0.051% tannic acid***
O ***0.034% tannic acid***
\+ ***0.017% tannic acid***
△ ***Distilled water***

acid solutions to ease the surface spread for the brushing. Both tannic acid and aerosol O.T. inactivate TMV in vitro, so there is no chance of infecting primary leaves during brushing.

However, in a systemic host of TMV (*P. floridana*) the infiltration of tannic acid (0.034%) 24 hours after virus inoculation reduced virus titer about 75% during the first week after infection. After two weeks there was no significant difference in total virus content between tannic acid-treated and water-treated samples. Thus tannic acid does interfere with virus synthesis at an early stage in a temporary way.

Therefore, in the cucumber-TMV system, tannic acid treatment had no effect on the establishment of infection; it merely suppressed the expression of starch lesions and at the same time interfered with the defense mechanism of the host, permitting systemic spread of the virus.

Tannic acid is a strong inhibitor of virus particles in vitro. It inactivated both TMV and TMV-RNA by forming noninfectious complexes (1). TMV-RNA was much more sensitive to inactivation than was whole TMV. It would thus appear that tannic acid could possibly inactivate TMV by reacting with either the protein coat or the RNA core.

In both cases, a seeming virus stimulator (twinberry extract) and a virus inhibitor (tannic acid) operated in a more or less similar way in the cucumber-TMV system. They both affect the host defense mechanism against virus infection. The active component in twinberry extract exhibits a mild and temporary interference, thus permitting virus to make further rounds of gain (ringlike patterns) while tannic acid produces a strong and permanent interference.

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10

Chemistry and Physiology of Abscisin II, an Abscission-Accelerating Hormone

F. T. Addicott, Kazuhiko Ohkuma,[1] O. E. Smith[2] and W. E. Thiessen

University of California, Davis, Calif.

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Abscisin II is a plant hormone which accelerates (in interaction with other factors) the abscission of young fruit of cotton. It can accelerate leaf senescence and abscission, inhibit flowering, and induce dormancy. It has no activity as an auxin or a gibberellin but counteracts the action of these hormones. Abscisin II was isolated from the acid fraction of an acetone extract by chromatographic procedures guided by an abscission bioassay. Its structure was determined from elemental analysis, mass spectrum, and infrared, ultraviolet, and nuclear magnetic resonance spectra. Comparisons of these with relevant spectra of isophorone and sorbic acid derivatives confirmed that abscisin II is 3-methyl-5-(1-hydroxy-4-oxo-2, 6, 6-trimethyl-2-cyclohexen-1-yl)-cis, trans-*2, 4-pentadienoic acid. This carbon skeleton is shown to be unique among the known sesquiterpenes.*

Abscisin II is an abscission-accelerating hormone present in young cotton fruit. It is now the best known example of the naturally occurring abscission-accelerating substances discovered by Osborne in 1955 (*19*) and shown by subsequent investigations to be widespread among the higher plants (*1*). They are found in senescent or aborting organs; there is little evidence of their presence in vigorous, healthy organs. Apparently, these endogenous substances function in the control of abscission, interacting with other hormonal and

[1] Present address, Institute of Physical and Chemical Research, Tokyo Japan.
[2] ARS, U.S. Dept. of Agriculture, based at Davis.

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physiological factors. The agricultural importance of the regulation of abscission—of being able either to accelerate or retard the shedding of leaves, flowers, or fruits—has given impetus to research on the chemistry and physiology of the abscisins. Further, the investigations reviewed below indicate that abscisin II exerts a very wide range of physiological effects, retarding growth and accelerating aging and senescence.

Physiology

The investigation of abscisin II had its inception with the discovery that diffusates from young fruit of cotton (*Gossypium hirsutum* L.) contained a substance which inhibited the growth response of *Avena* coleoptiles to indoleacetic acid (IAA) (*3*). The hormonal nature of the substance was suggested by the observation that a peak of growth-inhibiting activity in the diffusates occurred during the period of young fruit abscission. Subsequent experiments with purified ether extracts of young fruit showed that the extracts contained a substance which accelerated the abscission of young fruit, of defruited pedicels, and of debladed petioles of cotton. Recent experiments with crystalline abscisin II showed that it was responsible (largely, if not entirely) for the growth inhibition and abscission acceleration induced by the diffusates and extracts (*1*). Work now in progress indicates that the yield of abscisin II is maximal during the period of young fruit abscission (*27*). Collectively, the evidence indicates that abscisin II has the physiological characteristics of a typical plant hormone: its production in the young fruit is correlated with the period of young fruit abscission; it moves some distance to a site of action (the abscission zone at the base of the fruit pedicel); it influences a particular physiological process (abscission); and it is active in very low amounts (0.01 to 10 μg. per abscission zone).

Although our supply of crystalline material has been very small, a portion of it was committed to determining the spectrum of biological activities of abscisin II. Much of this work was conducted in our laboratory, but certain experiments were conducted by L. G. Paleg, L. T. Evans, M. J. Chrispeels, and J. E. Varner, whose experience with specialized techniques led to valuable results. These investigations provided important information on the physiological role of abscisins in plants and indicate possible agricultural applications as well. The first experiments showed that abscisin II was active in accelerating petiole abscission of beans, *Citrus*, and *Coleus*, as well

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as cotton (*26*). These observations have a number of implications, among them that the abscission process is controlled by essentially the same mechanism in the different species, and that the naturally occurring abscission-accelerating substances in the different species may be chemically similar to abscisin II. In the detached radish leaf test, abscisin II accelerated senescence; its effect was opposite to that of the kinins, which retard senescence. Alone, abscisin II was inactive as an auxin (in the growth of *Avena* coleoptiles) and as a gibberellin [in the growth of dwarf maize or dwarf peas (*26*) or in the barley endosperm assay (*20*)]. However, when applied simultaneously with representatives of these classes of hormones, abscisin II counteracted their stimulatory action (*1*). For example, it counteracted IAA (an auxin) in the growth of *Avena* coleoptiles (*1*), and Paleg (*20*) found that it counteracted the gibberellic acid (GA_3)-induced hydrolysis of starch in barley endosperm. Further, Chrispeels and Varner (*4*) found that abscisin II inhibited the GA_3-induced synthesis of α-amylase in barley aleurone layers. In *Lolium*, where flowering can be induced by exposure of one leaf to one long day, Evans (*10*) found that abscisin II inhibited this flowering response. In none of the experiments did abscisin II show any sign of toxicity to the plant tissues.

Related investigations by Van Steveninck have disclosed a substance in lupin fruit which is physiologically similar to abscisin II, in that it accelerates abscission and counteracts auxin-induced growth (*29*). The chemical information on this substance obtained by Rothwell and Wain (*24*) is in every respect consistent with the structure of abscisin II. Another related series of investigations has involved a substance, in the leaves and buds of *Acer* and *Betula*, tentatively named "dormin" by Eagles and Wareing (*8*). It was obtained in greatest amounts from plants under short photoperiods (autumnal conditions); when applied to growing seedlings the purified extracts arrested growth and induced dormancy (*8, 9, 30*). The extracts also counteracted auxin-induced growth, and counteracted gibberellin-induced growth and amylase synthesis (*9, 28*). Recently Cornforth *et al.* (*6*) determined that dormin is chemically identical with abscisin II.

Isolation

The extraction and purification, leading to the crystallization of abscisin II, were guided by an abscission bioassay (*1*). Seedlings of

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cotton (*Gossypium hirsutum* L., cultivar Acala 4-42) were grown under fluorescent lamps until 14 days old. The cotyledonary nodes were excised and trimmed so that the petiole and stem stumps were 3 mm. long and the total axial length (stem plus hypocotyl) was 15 mm. These explants were placed upright in stainless steel holders in Petri dishes containing a 5-mm. layer of 1.5% agar. Fractions to be tested were applied to the cut surface of the petiole stumps in 5-μl. droplets of 1% agar. Explants were kept in the dark at 30°C. and tested for abscission at least once daily by application of a force of approximately 5 grams to the petiole stumps. The method permitted the detection of several degrees of abscission acceleration (as well as abscission retardation).

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For the extraction approximately 86,500 cotton fruits, 4 to 7 days old, weighing 225 kg., were collected at the U. S. Cotton Field Station, Shafter, Calif. The fruits were quick-frozen on solid carbon dioxide, stored at −5°C., and later lyophilized to a moisture content of approximately 10%. They were extracted with 80% acetone overnight at approximately 22°C. The extract was concentrated, adjusted to pH 2.0 with dilute hydrochloric acid, and then extracted with ethyl acetate. The ethyl acetate fraction was then extracted with 2% sodium bicarbonate. This in turn was acidified to pH 2.0 and extracted with ethyl acetate. After the removal of ethyl acetate the acid fraction weighed 147 grams. This fraction was further purified by adsorption chromatography on a carbon-Celite column. The column was eluted with increasing concentrations of acetone in water. The abscission-accelerating activity was found in the 50 and 60% acetone eluates, from which 4.15 grams of oily material was obtained. This material was applied to a silicic acid–Celite column, which was eluted with increasing concentrations of ethyl acetate in chloroform. Peaks of abscission-accelerating activity were found in the 10 to 30% and in the 50 to 60% ethyl acetate eluates. The activity of the latter eluates was only moderate and they were set aside. (Later investigation showed that their activity was due largely to gibberellins.) Only the highly active fraction in the 10 to 30% ethyl acetate eluates was purified further. These combined eluates were evaporated to dryness and the residue was taken up with chloroform, from which 238 mg. of oily material was obtained. This material was applied to Whatman 3-mm. chromatographic paper and the chromatogram developed with isopropyl alcohol–ammonia–water (10:1:1, vol./vol.). The methanol eluates from R_f 0.4 to 0.8 were active and had a combined weight of 66 mg.

This material was applied to a silicic acid–Celite column. The 10% ethyl acetate (in choloroform) eluate from this column yielded

23 mg. of semisolid oily material. After two recrystallizations from chloroform–petroleum ether, 9 mg. of a crystalline substance were obtained. The substance behaved homogeneously in thin-layer and paper chromatography with nine solvent systems; it was very active in the acceleration of abscission and was named abscisin II (*18*).

A second isolation using slightly modified procedures yielded 7 mg. of crystals. Thus, a total of only 16 mg. of abscisin II was available for our initial chemical and physiological investigations.

Chemical Structure

The small amount of available crystalline abscisin II limited this investigation to the measurement and interpretation of elemental analysis, mass spectrum, and infrared, ultraviolet, and nuclear magnetic resonance (NMR) spectra (*17*).

The combustion analysis and mass spectrum indicated the molecular formula $C_{15}H_{20}O_4$ for abscisin II. The infrared spectrum revealed functional groups containing all four of the oxygen atoms: an alcoholic hydroxyl group, a conjugated carboxyl group, and a conjugated keto group. This high degree of conjugation was reflected in the ultraviolet spectrum, which could be interpreted as the superposition of the absorption curve of an $\alpha, \beta, \gamma, \delta$-unsaturated carboxylic acid upon that of an α, β-unsaturated ketone.

The 100-mc. NMR spectrum of abscisin II is displayed in Figure

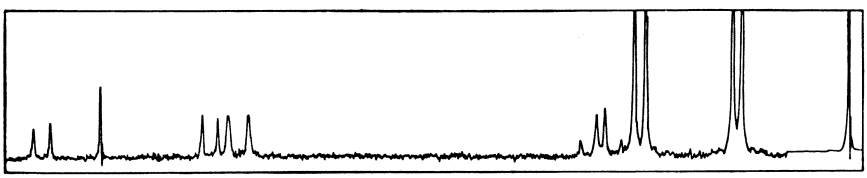

Figure 1. Nuclear magnetic resonance spectrum of abscisin II in $CDCl_3$

1. Proceeding downfield from the reference signal of tetramethylsilane, it contains two singlets corresponding to two methyl groups at saturated carbon, two slightly broadened singlets corresponding to vinylic methyl groups, and a nonequivalence quartet whose area and field position suggest a methylene group adjacent to a carbonyl group. In the vinyl proton region there are two slightly broadened singlets of unit area and two doublets, each of unit area, separated by the signal of the $CHCl_3$ impurity in the $CDCl_3$ solvent. The two doublets have a spin-spin coupling constant of 16 cycles per second. The signal

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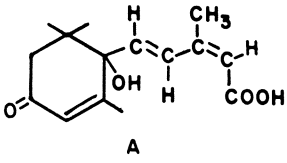
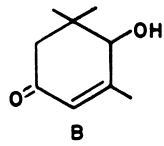
corresponding to the hydroxyl and carboxyl protons occurs at very low field and is not seen in this spectrum.

These data are consistent in every respect with structure A. Moreover, comparison of these spectral characteristics with those of



A



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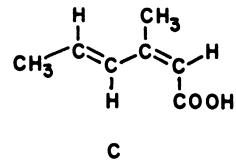

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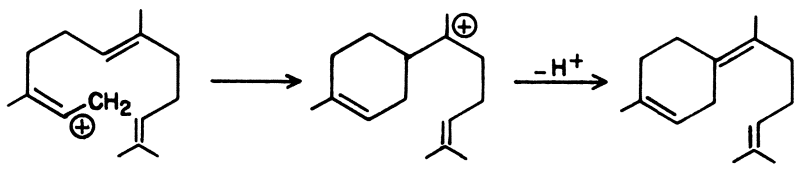

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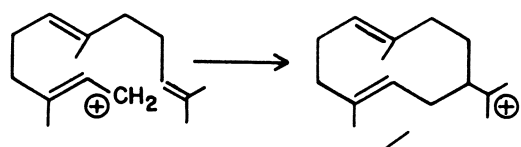

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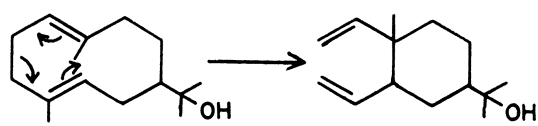

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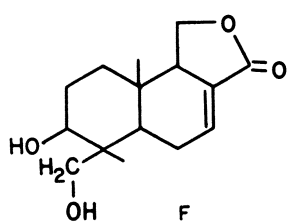

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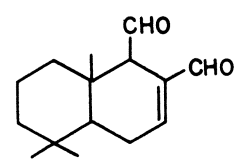

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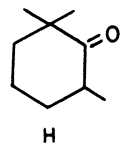

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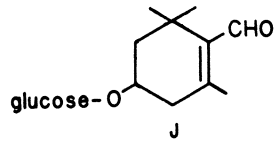

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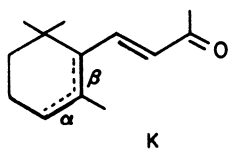

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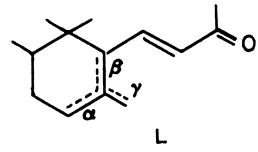

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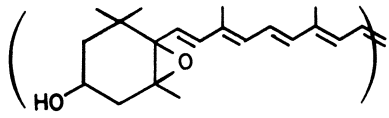

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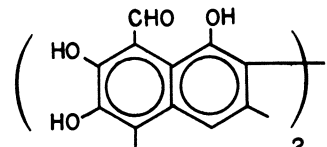

N

each "half" of the molecule—i.e., hydroxyisophorone (B) and *cis*, *trans*-β-methylsorbic acid (C), showed such similarities as to leave little doubt of the correctness of the assignment of structure A to abscisin II (*17*). Cornforth *et al.* (*5*) have recently confirmed this structure by total synthesis.

As far as we are aware, abscisin II is the only known sesquiterpene possessing the carbon skeleton of structure A. All the other monocyclic sesquiterpenes are thought to arise by way of ion D (the example given is bisabolene) or E (the example given is elemol) (*21*).

The only sesquiterpenes which appear to arise by cyclization initiated by external electrophilic attack (as is common in the di- and triterpenes) are iresin (F) by attack of OH^+ and polygodial (G) by attack of H^+ (*2*, *7*, *22*, *23*).

Abscisin II may be viewed as a monocyclic analog of these two anomalous sesquiterpenes. Alternatively, it may be one of a larger group of miscellaneous compounds which do not necessarily possess exactly 10 or 15 carbon atoms but may be looked upon formally (and perhaps actually) as degradation products of the carotenoids. This view is favored by the fact that the carotenoids are the only class of plant products in which cyclization of the type found in abscisin II is very common. Other examples of these compounds in-

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clude: 1, 1, 3-trimethylcyclohexan-2-one (H) from *Cistus* spp. (*11*); picrocrocin (J) from *Crocus sativus* (*12, 13*);α- and β-ionones (K) from *Boronia megastigma* (*16*) and Costus oil (*15*); and, if an extra methyl group be allowed, α-, β-, and γ-irones (L), the odor constituents of *Iris* spp. (*25*).

The functional groups of abscisin II could easily arise from a precursor like violaxanthin (M), a carotenoid of widespread occurrence (*see 11*). Whatever the genesis of abscisin II, it is clear that it must be different from that of the bis-sesquiterpenoid, gossypol (N), which also occurs in the cotton plant and has received considerable attention.

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11

Biologically Active Agents from *Helminthosporium sativum*

E. Y. Spencer and R. A. Ludwig[1]
Research Institute, Canada Agriculture, London, Canada
P. de Mayo, R. W. White[2], and R. E. Williams
Department of Chemistry, University of Western Ontario, London, Canada

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The fungus Helminthosporium sativum *associated with seedling blight and root rot of cereals was shown to produce a toxin among several metabolites. Isolation of the pure crystalline toxin from a synthetic medium analyzed for* $C_{15}H_{22}O_2$ *and gave a bis-oxime. Ultimate degradation and conversions indicated it to be a sesquiterpinoid dialdehyde, 1,7-dimethyl-4-isopropylbicyclo [3.2.1] oct-6-ene-6,8-dicarboxaldehyde (helminthosporal). The monoaldehyde-monoalcohol (helminthosporal) has also been isolated in small quantities, as well as possible precursors of both. Biosynthesis studies with labeled mevalonic acid suggested a possible mechanism via a farnesyl cyclization.*

The importance of toxins in disease development has been demonstrated with increasing frequency (*10*). The fungus *Helminthosporium sativum,* associated with such diseases of cereals as seedling blight, head blight, leaf spot, and foot and root rot, was shown to produce toxin which was essential in disease development of barley seedlings (*11*). Later a number of nutrient media were examined for toxin production by a number of strains of the fungus. Three methods of assay were initially developed: extent of growth

[1] Present address, Plant Research Institute, Canada Agriculture, Ottawa, Canada.
[2] Present address, *Chemical Abstracts*, Columbus, Ohio.

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inhibition of 10-day-old barley plants, inhibition of barley seed germination after overnight soaking in assay solutions, and inhibition of spore germination using *Monilinia fructicola.* Since the results of all three methods correlated well and the last was the least time-consuming, it was normally employed.

The fungus was found to utilize both organic and inorganic nitrogen (*9*), but the rate of growth was much more rapid with the former. Maximum toxin production coincided with an inhibition of fungal growth and diminished on exhaustion of the sugar. It appears that toxins are produced until they limit the growth of the fungus and then are gradually destroyed.

The sensitivity of barley to the toxin increases as the stage of germination advances. This may be partially explained by the presence of antifungal agents in the young plant whose activity is reduced with age (*8, 12*). Further progress on the chemistry of this material is reported by Stoessl (*14*).

The toxin is less effective on oats and wheat and does not have the specificity reported for the toxin from *Helminthosporium victoriae* for oats. A metabolite isolated from another strain of *H. sativum* in Japan by Tamura *et al.* (*15*) has a marked elongation effect on the shoots of rice seedlings. The structure of this metabolite is closely related to that described here.

Optimum toxin production was found in a stirred, aerated culture medium consisting of potato infusion and sucrose after 3 to 5 days' growth. The toxin was adsorbed on charcoal from the culture filtrate and eluted with chloroform. The red-brown residue remaining after evaporation showed little or no absorption in the carbonyl region of the infrared and only weak absorption in the ultraviolet. However, on mild treatment with acid, base, or heat two carbonyl peaks appeared at 1715 and 1685 $cm.^{-1}$ in the infrared and at 266 $m\mu$ in the ultraviolet (*3*).

The crystalline material was shown to be modified sesquiterpinoid (*5*) containing two aldehyde functions, one of which is α,β-unsaturated (Ia). It analyzed for $C_{15}H_{22}O_2$ and formed the dioxime. It readily took up oxygen on standing and was converted to the diol on treatment with lithium aluminum hydride. The bisdinitrobenzoate of this diol with osmium tetroxide yielded the tetraol, bisdinitrobenzoate, which was not readily acetylated.

The dialdehye, helminthosporal, was converted to the monoacid (Ib) with silver oxide. Infrared and nuclear magnetic resonance

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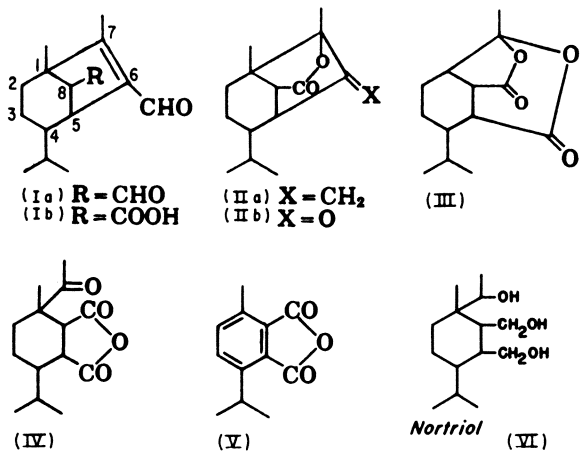
spectra indicated that the aldehyde conjugated with the double bond was unaffected. Reduction with sodium borohydride yielded an unexpected lactone (IIa) (*4*). Its formation evidently involved an allylic rearrangement with the production of an exocyclic methylene group, while ozonolysis produced a γ, δ-dilactone (III). Reduction with lithium aluminum hydride resulted in a diol, different from that obtained directly from helminthosporal. The nor-γ, δ-lactone (III) was also produced by ozonolysis of the γ-lactone with the simultaneous yield of formaldehyde, while treatment with osmium tetroxide and periodate produced the cyclopentanone (IIb). The



nortriol (VI) was produced with lithium aluminum hydride while alkaline hydrolysis followed by acetic anhydride yielded the ketoanhydride (IV). Selenium dehydrogenation resulted in a compound identified as 3-isopropyl-6-methyl phthalic anhydride (V).

From the above data and nuclear magnetic resonance and infrared spectra the structure of helminthosporal was postulated as 4-isopropyl-1,7-dimethylbicyclo [3.2.1] oct-6-ene-6,8-dicarboxaldehyde (Ia). The bridged system in helminthosporal defines the stereochemistry in all points except the isopropyl group with respect to the carbon bridge and the configuration of the saturated aldehyde (VII). The points of fusion of the five-membered ring to the six must be axial. Formation of γ-lactone indicates that the saturated aldehyde must be equatorial. Examination of NMR spectra supports the equatorial configuration of these two groups. Synthesis by Corey and Nozoe (*1*) has confirmed the absolute stereochemistry.

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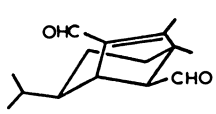
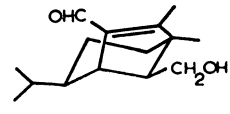


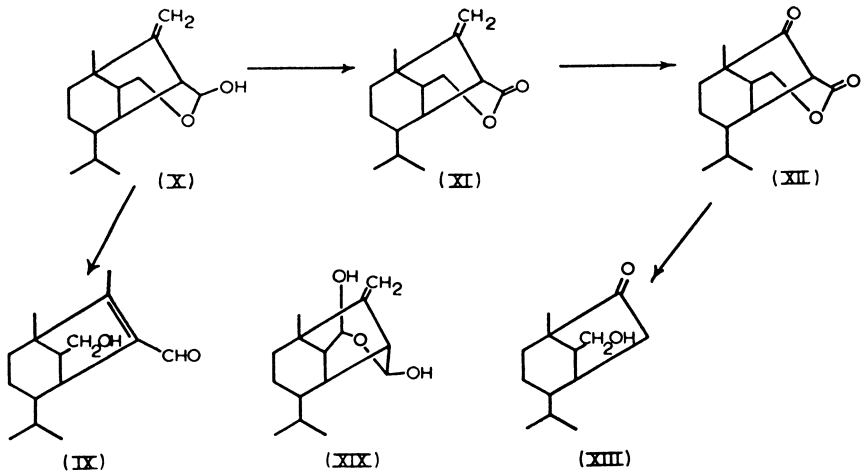
(VII)



(VIII)

During the course of this work Tamura *et al.* (*7, 14, 15*) isolated a metabolite from another strain of *Helminthosporium sativum.* By comparison with the bis-3,5-dinitrobenzoate of the diol from helminthosporal they identified it as the unsaturated aldehyde–saturated alcohol, helminthosporol (VIII, IX).

Since the unpurified toxins show no aldehyde absorption, it was of interest to identify the precursors of helminthosporol and helminthosporal. Thin-layer chromatography of fresh chloroform extracts of the toxin that had been concentrated below 40° indicated several spots (*6*). One was identified as prehelminthosporol, a hemiacetal (X), from NMR spectra and chemical reactions. Refluxing with triethylamine yielded helminthosporol, identical with Tamura's prod-



uct. Oxidation with chromic oxide gave a δ-lactone (XI), which on ozonolysis produced a ketolactone (XII). Alkaline hydrolysis opened the lactone, resulting in decarboxylation to give the ketol (XIII).

Subsequent examination of a large spot yielded on careful fractionation a complex with a molecular weight approximately equivalent to double that of either toxin. The NMR spectrum was roughly compatible with that of the addition of the hemiacetal of helminthosporol to what would be expected for the hemiacetal of helmintho-

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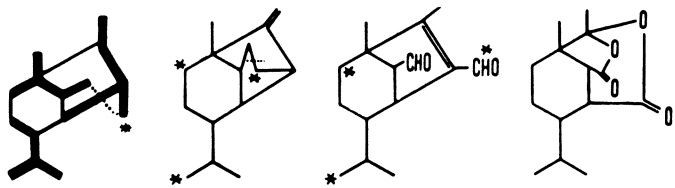
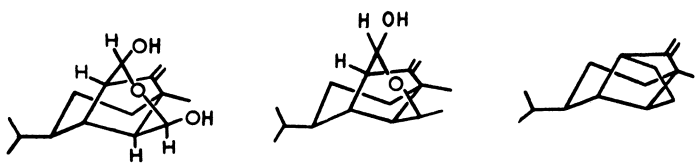
sporal. Passage through alumina yielded helminthosporal, prehelminthosporol, and traces of helminthosporol.

In view of the easy disruption of the nonaldehyde form of the toxin it seems probable that a loose complex in some form of acetal exists between the two hemiacetals (XIV, XV). When this is disrupted under mild conditions prehelminthosporol is formed and remains until converted under more vigorous conditions to helminthosporol, while prehelminthosporal (XIV, XIX) is too unstable and is converted to helminthosporal.



(XVI) (XVII) (XVIII) (III)

The carbon skeleton of helminthosporal can be accounted for from three isoprene units (XVI), but they cannot be formed by cyclization of a farnesol precursor, as the three units are not joined head to tail. There is the possibility that the aldehyde carbons were joined in the early stages of biosynthesis to form helminthosporane (XVII, XX) as an intermediate, followed by later oxidation to yield the dialdehydes. As a test of this hypothesis the actively growing fungus was fed mevalonic acid labeled with carbon-14 in the 2-position (mevalonic acid being a precursor of isoprene units) (*2*). Thus, three units of radioactivity should be incorporated, one being the



(XIV) (XV) (XX)

aldehyde conjugated with the double bond (XVIII). Following isolation and conversion to the more stable acid-aldehyde, it was recrystallized to constant radioactivity. Reduction with sodium borohydride to the γ-lactone followed by ozonolysis gave the dilactone with the loss of one aldehyde group (III). Recrystallization to constant melting point yielded material with 62% of the original

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radioactivity, agreeing well with the proposed loss of one of the three radioactive carbons.

In searching for earlier precursors of helminthosporal the culture medium was examined daily up to 5 days. The composition showed little change after the appearance of activity, although the total amount increased. The only unusual product was anthraquinone found in the first 2 days' growth.

Further work on the spectrum of toxicity of these toxins, as well as their mode of action in disease development, is under way.

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12

Regulation of Plant Growth by Constituents from Higher Plants

D. E. Moreland, G. H. Egley, A. D. Worsham, and T. J. Monaco

Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, and N. C. State University, Raleigh, N. C.

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Organic substances are released to the external environment by plants either directly as root secretions and foliar leachates or indirectly from the microbial decomposition of plant residues. Inhibition of plant growth by some of the released compounds, or their degradation products, has been established experimentally. Consequently, when present in the rhizosphere they have been implicated with "soil-sickness" and "replant" problems frequently encountered where specific crops are grown continuously and may have an ecological function of controlling plant succession and the phytosociological order of plant communities. Some of the released chemicals are postulated to function endogenously as inhibitors and regulators of seed germination, dormancy, root initiation, and other processes involved in plant growth and development.

The possibility that plants might secrete substances which would affect the growth of other plants was apparently recognized first by DeCandolle in 1832 (*33*). His postulate was developed from field observations, and he considered that such substances were important factors in controlling the distribution of plants. In subsequent years, the contribution of naturally occurring inhibitors in the regulation of plant competition was discounted, and emphasis was placed on the availability of inorganic nutrients. Schreiner and Reed (*129*)

In Natural Pest Control Agents; Crosby, D.;
Advances in Chemistry; American Chemical Society: Washington, DC, 1966.

re-examined the role of natural products as growth inhibitors in their studies on problems associated with unproductive soils. For the first time materials of plant origin were isolated, purified, and shown to possess growth-inhibitory properties. Many facets of the role which natural plant growth inhibitors play in controlling plant growth remain to be elucidated. Relatively few growth-regulatory chemicals have been identified to date, although there are numerous references in the literature to fractions characterized by various degrees of purity which have been shown to possess growth-inhibitory action. The physiological mechanisms through which control over growth is expressed have been considered for only a few of these chemicals.

The early literature on naturally occurring plant growth inhibitors and the influence one plant might exert on another by chemical means (allelopathy) is considered comprehensively in various reviews (*12*, *15*, *36*, *37*, *48*, *49*, *61*, *67*, *94*, *121*, *162*). Reviews of studies concerned with the influence of plant exudates on root-infecting fungi (*130*) and the effects of phytotoxins which arise as decomposition products (*113*) have appeared recently. Because of the excellent coverage of the topics by others, no attempt is made here to review the early literature exhaustively. Instead, consideration is restricted essentially to specific compounds and to some of the more recent literature.

The isolation and structural characterization of plant constituents are often difficult and time-consuming. Consequently, at the present time, only a small percentage of the total number of natural plant products has been identified. Early in the development of natural product chemistry it was apparent to many botanists and chemists that plants might be characterized and classified on the basis of their chemical constituents. The distribution of chemical constituents among plant families is presented by Swain (*139*) and Hegnauer (*66*).

Considering the multitude of diverse chemical structures known to be distributed throughout the plant kingdom, one could anticipate finding chemicals capable of adversely affecting plant growth and development. However, of the several hundreds of naturally occurring compounds which have been identified, only a limited percentage has actually been examined for antagonistic effects on growth and development of a very small number of plant species. There is no way of estimating what a systematic examination for interactions would uncover.

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Origin and Production of Phytotoxic Materials

Plants contribute to the soil, either while living or after death, the large diverse group of chemical constituents involved in their metabolism. Growth-inhibitory substances have been detected in all plant organs—i.e., leaves, coleoptiles, stems, shoots, tubers, buds, roots, stamens, pollen, fruits, and seeds (*67*). Compounds released to the soil must be sufficiently stable to be absorbed by the plant and translocated to the site at which inhibition is effected. These materials are also subject to decomposition and alteration through the action of biotic and abiotic agencies whereby many additional simple and complex chemicals could be produced. At any one time the soil environment could contain a variety of chemical constituents derived from plants. Conceivably, many of these could affect plant growth and development either by the direct action of a toxic material or indirectly by upsetting a delicate balance in a biochemical control system. Only fragmentary information is available as to the numbers of inhibitory materials formed, their chemical identity, whether they are formed in sufficient quantities to affect plant growth, whether they are sufficiently stable to affect plant growth, and the biochemical mechanisms through which effects are produced.

Intact root systems have been shown to exude a wide spectrum of chemicals: amino acids, simple sugars, glycosides, organic acids, vitamins, enzymes, alkaloids, nucleotides, phenolics, phloem-mobile herbicides, and inorganic ions. Evidence for the liberation of scopoletin from roots of oat seedlings under carefully controlled conditions has been given by Martin and Rademacher (*97*). The excretory process may be a normal physiological function of intact roots and the liberation of the various constituents may be the result of physical damage or the normal sloughing off and autolysis of root fragments such as cortical and epidermal cells, root caps, and root hairs associated with root extension. Injury may be an important contributory factor that enhances exudation. It can arise from mechanical abrasion; physiological disorders; insect, fungal, and microbial invasions; and toxins derived from the decomposition of plant residues and microflora.

The magnitude and composition of root exudates are a reflection of the physiological condition of the plant imposed by factors related to light (intensity, duration, and quality), temperature, soil pH, anaerobiosis, soil moisture, soil type, and nutritional status. The in-

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fluence of various genetic, environmental, and nutritional factors on the production of secondary plant products has been presented in some detail in reviews by Schroth and Hildebrand (*130*) and Flück (*39*). Fowden (*41*) specifically discussed variation in the nonprotein amino acid composition of plants as affected by the above factors.

Many materials have been reported to be leached from intact leaves of growing plants by the action of rain, mist, and dew; mineral nutrients as well as considerable quantities of organic metabolites are lost. Morgan and Tuckey (*103*) conducted one of the most qualitative studies of this type. Foliar leachates from seven plant species were fractionated on exchange resins and analyzed chromatographically. The following constituents were identified in the various fractions: 21 amino acids and amides, 14 organic acids including components of the Kreb's cycle, four free sugars (raffinose, sucrose, glucose, and fructose), polysaccharides, and pectinaceous materials.

Significance of Naturally Occurring Inhibitors

The liberation of organic substances, either directly from living plants or indirectly as decomposition products, has considerable economic and academic interest. A number of cases has been reported of influences exerted by one higher plant upon another which cannot be attributed to competition for mineral nutrients, space, water, and light. The term "allelopathy" has been used to describe effects for which products of plant metabolism might be held responsible. Although many observations have been made and hypotheses proposed on the nature of allelopathy, comparatively few well-founded experimental results are available. This subject has received more attention to date from workers in European countries than in the United States. The presence of substances which are toxic, stimulatory, or essential to other plants and to parasitic, saprophytic, or symbiotic soil microorganisms should be considered in the selection of agronomic practices such as intercropping and crop rotation.

In areas where particular crops are grown continuously, decreases in production with time have been noted. The condition is usually species specific, and the disorders which result are frequently referred to as "soil-sickness" or "replant" problems. Fruit trees are especially sensitive and the problem has been encountered with apples, peaches, grapes, cherries, plums, and citrus. In most situations, phytotoxicity has been related to the formation of toxic materials as a consequence of the microbial decomposition of plant remains.

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From an academic standpoint, liberated organic materials with inhibitory properties could have an ecological function of controlling plant dominance and succession. The sequence of events that occurs in plant successions when native vegetation is disturbed, or when cultivated fields are abandoned, has been actively studied by plant ecologists. Weeds comprise an early stage in plant successions which terminate with the climax vegetation characteristic of the area. The role that chemicals might play in controlling natural succession and the phytosociological order of plant communities has received limited attention. However, the contribution may be more important than is generally recognized (*61*). The fundamentals of plant ecology emphasize that plant successions occur in the direction of the climax vegetation rather than towards the growth of more productive economic crop plants. From an agricultural standpoint, it is essential that the plant succession trend be directed away from the climax vegetation. Various cultural, mechanical, biological, and chemical methods of weed control have been used since man first attempted to raise crops. In recent years, herbicides have demonstrated an outstanding potential for the selective control of weeds in crop production. Hopefully, a comprehension of naturally occurring inhibitors will provide leads for the development of new herbicides that may be more efficient and selective than their predecessors.

In addition to inhibitory chemicals which enter the plant from the external environment, many endogenous inhibitors appear to function as regulators of seed germination and plant growth and development. The interrelationships between endogenous inhibitors and growth promoters such as the auxins, gibberellins, and kinins remain to be elucidated.

Various assay methods have been used to detect the presence of inhibitory substances. These include some of the classical tests used by investigators of growth-promoting substances—i.e., the various *Avena* coleoptile assays which utilize intact, decapitated, or isolated cylinders; and the split pea stem test. Effects on seed germination and seedling shoot or root growth and development have also been measured in addition to other visible expressions of inhibition. Details of many of these tests have been compiled by Mitchell *et al.* (*99*). Tests have been carried out in Petri dishes, with various solution culture techniques, and by sand and soil culture. Effects so measured may or may not be similar to those obtained under field situations—i.e., the establishment of inhibition under controlled conditions pro-

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vides no definitive information on potential effects that might be expressed under more complex conditions.

A rigorous approach to studies of naturally produced toxic materials would involve isolation of the compounds in pure form and demonstration that the substances isolated were actually responsible for the observed inhibition. Synthesis and confirmation of activity of the synthetic *vs.* the natural material may also be required. These criteria have been met in relatively few cases. Unfortunately, studies with naturally occurring inhibitors have been made primarily by plant physiologists without sufficient assistance from chemists.

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Chemical Characteristics of Naturally Occurring Growth Regulators

For the purposes of this review, naturally occurring regulators such as indoles, gibberellins, and kinins are excluded. Attention is restricted to substances produced by one higher plant, which affect the growth and development of another higher plant of the same or different species. Products that are suspected of originating from the decomposition of higher plant residues are, however, considered.

Volatile Inhibitors. Of the volatile components that influence plant growth and development, ethylene has received the most attention. Literature concerned with the variety of effects produced by ethylene, factors which influence its production, and the mechanisms through which responses are expressed has been reviewed by Evenari (*37*). Other gaseous excretions with inhibitory effects considered by Evenari include hydrogen cyanide, ammonia, essential oils, and mustard oils (probably allyl isothiocyanate and β-phenethyl isothiocyanate).

Hydrogen cyanide, a strong inhibitor of germination and growth (*36*), is liberated enzymatically from cyanogenic glycosides present in seed and other tissue of various plant species. Amygdalin (a cyanogenic β-glycoside) is considered to be indirectly responsible for the peach "replant" problem because of its presence in the root wood and bark. Microbial degradation of amygdalin would release hydrogen cyanide in the soil (*111*). The hydrogen cyanide could escape as a gas or be adsorbed to soil colloids and, hence, might never accumulate to inhibitory levels in the soil. Jones *et al.* (77), however, demonstrated that extracts from buds, twig bark, and leaves of peach trees caused inhibition, on a straight growth bioassay with peas,

which was comparable to that produced by a sodium cyanide solution containing an equivalent amount of cyanide. In a subsequent study, Jones (*75*) examined the seasonal variation of cyanide in peach leaves and inferred that the level of cyanide might control dormancy in peaches.

Ammonia also strongly inhibits germination and growth, and is released enzymatically from nitrogenous substances contained in the pericarp of various dry fruits, and especially in sugar beet seed balls (*36*). Rehm (*123*) observed that free ammonia was formed from nitrate by reduction during the germination of beet seeds. The reduction of nitrate was attributed to the action of bacteria which developed in large numbers on germinating seeds. The toxic effects on the germinating seeds were observed in Petri dish germination tests, but not in the soil. Grodzinskiĭ *et al.* (*59*) studied the effects of volatile components released during the decomposition of plant residues on seed germination and photosynthesis. Some of the inhibition observed was attributed to ammonia.

Essential oils are known to have detrimental effects on plants. The inhibitory components have not been identified, but both aldehydic (benzol-, citrol-, cinnamal-aldehyde) and phenolic (thymol, carvacol, apiol, safrol) constituents are suspected. Muller *et al.* (*104*) demonstrated that volatile toxic materials localized in the leaves of *Salvia leucophylla*, *Salvia apiana*, and *Arthemisia californica* inhibited the root growth of cucumber and oat seedlings. They speculated that in the field, toxic substances from the leaves of these plants might be deposited in dew droplets on adjacent annual plants. In a subsequent paper, Muller and Muller (*105*) reported that the leaves of *S. leucophylla* contained several volatile terpenes, and growth inhibition was attributed to camphor and cineole.

A gaseous substance released from *Artemisia absinthium* L. (wormwood) was implicated in the stunting of *Foeniculum vulgare* (fennel) plants (*10*). The gas was condensed in a dry ice trap and was reported to be related to absinthin. Absinthin was considered to be secreted as a glucoside by the glandular leaf hairs and might be leached from the leaves and deposited on the soil by rainfall. Toxicity to several species of plants has been reported (*12*, *15*). Absinthin, according to Novotny *et al.* (*109*), is a dimeric guaianolide which contains two lactone rings and two hydroxyl groups and possesses the empirical formula of $C_{30}H_{40}O_6$.

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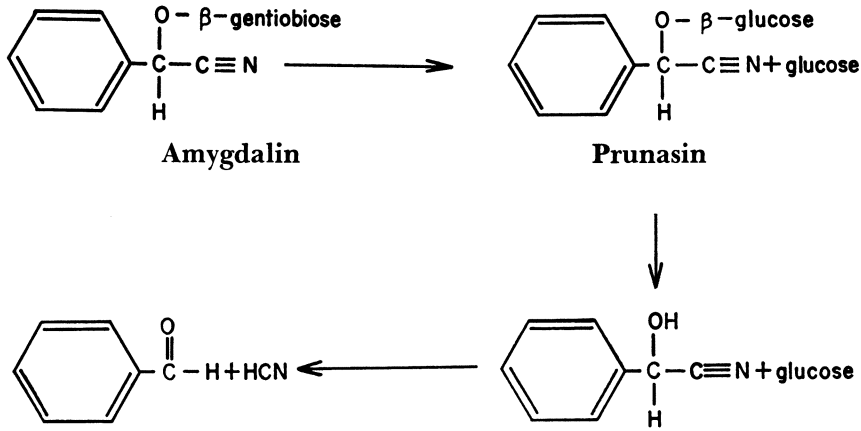
Nonvolatile Inhibitors. GLYCOSIDES A number of toxic constituents are known to be released by the enzymatic degradation of various glycosides. Some of the volatile components have been mentioned previously—i.e., isothiocyanates from mustard oil glycosides and hydrogen cyanide from cyanogenic glycosides.

Seeds and leaves of almond, cherry, peach, apple, and plum contain amygdalin, a cyanogenic glycoside, which upon hydrolysis yields hydrogen cyanide, benzaldehyde, and glucose. Jones and Enzie (*76*) have found both prunasin and mandelonitrile in dormant peach flower buds. Consequently, the degradation of amygdalin may occur in the following sequence:

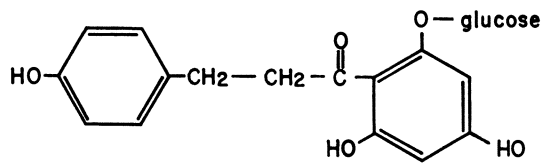


Amygdalin **Prunasin**

Benzaldehyde **Mandelonitrile**

Jones and Enzie (*76*) reported that mandelonitrile at $10^{-3}M$ completely inhibited the growth of pea sections but did not establish whether the inhibition was produced by mandelonitrile *per se* or by its breakdown products, hydrogen cyanide and/or benzaldehyde.

Phlorizin, the glucoside of the dihydrochalcone phloretin, is a constituent of the tissues of the apple tree. Its distribution and bio-



Phlorizin

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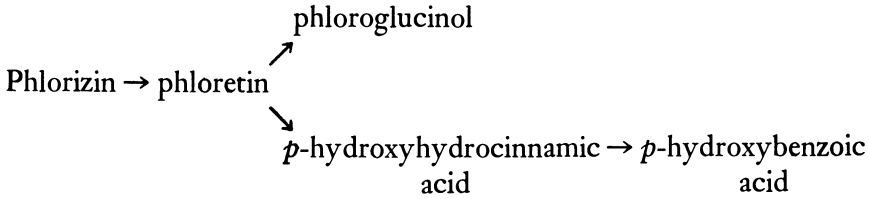
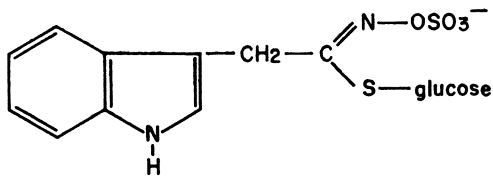
synthesis have been studied by Hutchinson *et al.* (*74*). Thimann and Marrè (*142*) cited references which reported inhibitory effects imposed by phlorizin on growth of wheat seedlings, growth of tomato roots, and respiration of *Elodea*. The apple replant problem may be related to the breakdown of phlorizin by soil microorganisms (*13*) and the following degradation pathway was postulated:



Börner (*13*) conducted solution culture experiments with apple seedlings and studied the inhibitory effects of each of the components. The following order of decreasing inhibitory effectiveness was reported: phlorizin > phloretin > *p*-hydroxybenzoic acid > *p*-hydroxyhydrocinnamic acid > phloroglucinol. With the exception of phloroglucinol, all compounds retarded seedling growth at concentrations of $10^{-4}M$. Hancock *et al.* (*64*) also observed that phlorizin was more effective as an inhibitor of coleoptile elongation than any of its breakdown products.

Glucobrassicin is a mustard oil glucoside found in various *Brassica* spp. (*50*). 3-Indoleacetonitrile (IAN) is released from glucobrassicin by the action of the enzyme myrosinase at pH 4.0. How-



Glucobrassicin

ever, at pH 7.0 cleavage by myrosinase produces 3-indolemethyl isothiocyanate, which is unstable and decomposes to form 3-hydroxymethylindole. The latter can dimerize to 3,3′—diindolemethane or can condense with ascorbic acid to produce ascorbigen (*50*). Kutacek (*82*) found that glucobrassicin at 10^{-3} *M* and IAN at 10^{-4} *M* inhibited the growth of both wheat and clover, but only IAN inhibited growth of Savoy cabbage. He suggested that internal

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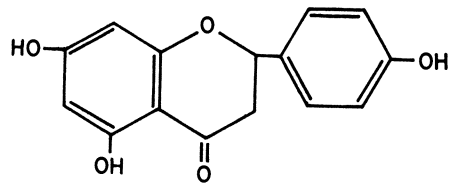
factors present in Savoy cabbage tissue favored the formation of growth-inactive materials when glucobrassicin was degraded.

Naringenin (5, 7, 4′-trihydroxyflavanone), isolated in pure form from dormant peach flower buds, strongly inhibited the growth of *Avena* coleoptiles at 4.6×10^{-4} *M* (*71*). Naringenin is the aglycone of the glycoside naringin.



Naringenin

Saponins are glycosides, with the aglycone being either a triterpenoid alcohol or a spiroketal steroid. Steroidal saponins are most common in the *Liliaceae*, *Amaryllidaceae*, and *Dioscoreaceae* (*125*). Nord and Van Atta (*108*) isolated a triterpenoid saponin from the seed coat of *Atriplex canescens* (fourwing saltbush). They reported that the germination of fourwing saltbush and *Purshia tridentata* (antelope bitterbush) seed was significantly depressed with 1 and 5% solutions of the saponin and considered that saponins were among the factors responsible for seed dormancy.

PHENOLIC ACIDS. Growth and germination inhibitors have been extracted from the straw of barley, rye, and wheat by many investigators [reviewed by Evenari (*37*) and Börner (*12*)]. Toxic constituents isolated include *p*-hydroxycinnamic acid, *p*-hydroxybenzoic acid, ferulic acid (4-hydroxy-3-methoxycinnamic acid), *p*-coumaric acid (4-hydroxycinnamic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), caffeic acid (3,4-dihydroxycinnamic acid), protocatechuic acid (3, 4-dihydroxybenzoic acid), and salicylic acid (*o*-hydroxybenzoic acid). The same phenolic acids and their derivatives have been isolated and identified as the growth inhibitors present in various dry fruits (*80, 151*) and in seed balls of the sugar beet (*157*). Phenolic growth inhibitors have also been extracted from willow, cabbage, and wheat tissue (*147*); tomato roots (*136*); and silver maple buds (*83*).

Yields of flax were reduced when a small percentage of *Camelina alyssum* plants was present. Grümmer and Beyer (*62*) reported that at least four, and possibly six, substances of phenolic configuration

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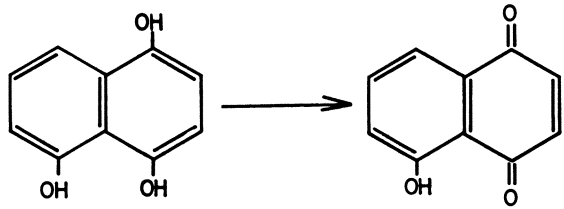
were washed out of *Camelina* leaves by rain in concentrations sufficient to inhibit the growth of flax. *p*-Hydroxybenzoic acid and vanillic acid were identified positively, and ferulic acid also appeared to be present. Grümmer (*61*) subsequently estimated that the phenolic derivatives comprised less than 10% of the total toxic complex. The most important toxin was not identified, but the data suggested that it could be an alkyl derivative of isothiourea and that more than a single such derivative might be present.

The toxic influence exerted by *Juglans nigra* (black walnut) on other plants has been attributed to 5-hydroxy-1,4-naphthoquinone (juglone) (*11*, *31*). Hydrojuglone is present in the root bark, leaves, and fruit husks. This compound is not considered toxic, but it is oxidized to the toxic juglone upon exposure to air (*57*). Rainfall



Hydrojuglone **Juglone**

can wash the toxicant from the leaves onto the soil. Davis (*31*) synthesized juglone and indicated that its toxicity was similar to that expressed by the natural product. Bode (*11*) also reported antagonism between the action of juglone and indoleacetic acid.

Amino Acids. Early observations on the liberation of amino acids by plant roots were reviewed by Loehwing (*94*), Rademacher (*121*), and Börner (*12*). Free amino acids have been isolated from soil fractions (*119*), and the excretion of a variety of ninhydrin-positive compounds by plant roots has been demonstrated under controlled conditions by Katznelson *et al.* (*78*), Rovira (*127*), and Pearson and Parkinson (*115*).

Various investigators have emphasized the significance of antagonism and balance in nutrient media with respect to amino acids. Some of this literature has been reviewed by Waris (*155*). Imbalance is manifested by morphological deviations and is considered to be the result of alterations imposed on pathways involved in the synthesis of nucleic acids and proteins. Two physiological disorders of plants manifested by growth reduction and morphological abnormalities

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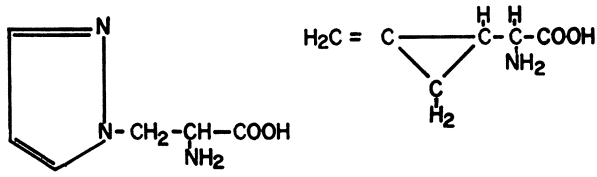
have been simulated by the addition of amino acids to the root zone. Steinberg (*134, 135*) produced "frenching" symptoms in tobacco with alloisoleucine and isoleucine which were similar to those produced by a toxin released in the soil by *Bacillus cereus*. Woltz and Jackson (*161*) were able to induce symptoms characteristic of the yellow strapleaf disease by applying DL-alloisoleucine, D- and L-isoleucine, and L-leucine to the soil surface around chrysanthemum plants. Woltz (*160*) subsequently reported that four of 20 protein amino acids (L-isoleucine, L-leucine, L-methionine, and L-valine) applied to the root zones of chrysanthemum plants produced significant growth-modifying effects. Several structural analogs of protein amino acids were also found to alter leaf morphology and the effects could be alleviated by application of the normal metabolic form of the acid.

Gressel and Holm (*56*) attributed delayed germination of tomato seed and inhibited elongation of the seedling to unidentified ninhydrin-positive compounds present in the seed and foliage of *Abutilon theophrasti*.

Studies on the intermediary nitrogen metabolism of plants resulted in the identification of more than 60 amino acids that were not considered to be normal components of protein (*41, 42*). These nonprotein amino acids have a restricted distribution and are considered to exist in the free state. Their function in plant metabolism and growth is for the most part unknown. They may be implicated directly in the biosynthesis or biodegradation of some of the protein amino acids, they may represent storage or transport forms of nitrogen, or they may function as regulators in the synthesis and degradation of protein amino acids. Structures for four of these nonprotein amino acids are given in Figure 1.



β-Pyrazol-1-ylalanine **α-(Methylenecyclopropyl)-glycine**

Figure 1. Structures of some nonprotein amino acids isolated from plants

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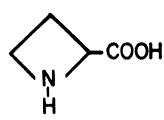
Azetidine-2-carboxylic acid, the lower homolog of proline, has been isolated from *Convallaria majalis* (lily of the valley) (*40, 44*), *Polygonatum officinalis* (Solomon's seal) (*153*), and *Polygonatum multiflorum* (*45*). Fowden and Steward (*47*) surveyed plants from 56 genera for nitrogenous compounds and found azetidine-2-carboxylic acid to be restricted to members of the *Liliaceae*. In some species it was identified in leaf, stem, and root but was more commonly found in the seed. In *Polygonatum*, azetidine-2-carboxylic acid accounted for 75% or more of the total nonprotein nitrogen in the rhizome (*45*). There was no evidence that it occurred as a constituent of protein.

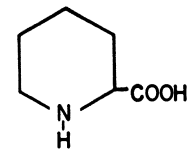
Pipecolic acid has been isolated and identified by various researchers from several legumes, hops, mushrooms, potato tuber, green pepper, tulip, celery, asparagus, Rhodesian teak, barley, and coconut milk. Grobbelaar *et al.* (*58*) isolated large quantities of free (-)pipecolic acid from *Phaseolus vulgaris*.

β-Pyrazol-1-ylalanine, an isomer of histidine, was isolated from *Citrullus vulgaris* (watermelon) seed and its structure was confirmed by comparison with synthetic material (*107*). It was the major free amino acid in the dormant dry seed but was present in only trace amounts in vegetative tissue. While present in seed extracts of other members of the *Cucurbitaceae*, it has not been identified as occurring in members of other plant families.

L-α-(Methylenecyclopropyl)glycine was characterized as a component of the seed of *Litchi chinensis* (*55*). It is spatially intermediate between leucine and isoleucine and is the lower homolog of hypoglycin A [β-(methylenecyclopropyl)alanine]. Both cyclopropyl derivatives have been found only in fruits of members of the family *Sapindaceae*.



Azetidine-2-carboxylic acid



Pipecolic acid

Fowden and Richmond (*46*) found that azetidine-2-carboxylic acid was growth-inhibitory to *Escherichia coli*, but no inhibition was observed when both DL-proline and the homolog were present in the

culture. They estimated that azetidine-2-carboxylic acid replaced nearly 50% of the proline residues in protein synthesized in the absence of proline.

Fowden (*43*) and Fowden and Richmond (*46*) also found that azetidine-2-carboxylic acid inhibited the growth of *Phaseolus aureus* (mung bean) seedlings. The amino acid was dissolved in the water imbibed by dry seeds during the first day of germination. A concentration of 2 mg. per gram dry weight of seed was lethal. In high sublethal concentrations the analog was estimated to have replaced nearly all of the proline residues in the newly synthesized protein molecules of the radicles. The inhibitory effect was partially reversed when proline was supplied to the seeds simultaneously with the homolog. The authors suggested that incorporation of azetidine-2-carboxylic acid would produce a polypeptide with an altered tertiary structure. The altered stereostructure of the homolog-containing protein molecules could be expected to express impaired biological activity.

Fowden (*43*) also found that α-(methylenecyclopropyl)glycine strongly inhibited the radicle growth of mung bean seeds. This analog has spatial characteristics between those of leucine and isoleucine. However, leucine was more effective than isoleucine in partially reversing the inhibition caused by α-(methylenecyclopropyl)glycine. No evidence was obtained for incorporation of the analog into newly synthesized radicle protein, but an effect on some stage of protein biosynthesis was suggested. Pipecolic acid and β-pyrazol-1-ylalanine were much less inhibitory to mung bean radicle growth than azetidine-2-carboxylic acid and α-(methylenecyclopropyl)glycine (*43*).

Some members of the *Liliaceae* accumulate free azetidine-2-carboxylic acid in a much higher concentration than that found to be lethal to mung bean seedlings, but it is not incorporated into their proteins. Fowden (*43*) postulated that these plants either had a proline-incorporating system which was more specific than that found in other species, or some subcellular mechanism operated to prevent the homolog from reaching the sites involved in protein synthesis. Data which supported the first suggestion were subsequently obtained (*116*).

In the soil, an imbalance of amino acids, the presence of D-amino acids derived from microbial metabolism, or analogs and homologs of constituent protein amino acids could produce a deviation in the

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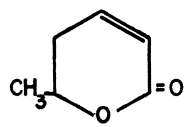
protein metabolism of plants which would be reflected in altered growth patterns. There is sufficient experimental evidence to suggest that this occurs under certain conditions.

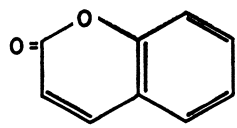
LACTONES. Physiologically active lactones such as parasorbic acid, coumarin, scopoletin, and protoanemonin occur in many plant families (Figure 2). The lactones may perform a regulatory function in the plant, and have been shown to inhibit germination and to repress root growth [reviewed in detail by Hemberg (*67*), Evenari (*36, 37*), and Börner (*12*)].

Parasorbic acid (Figure 2) was isolated from fruits of *Sorbus aucuparia*. Germination of mustard seed (*Sinapis alba*) was affected adversely by parasorbic acid at 3.5×10^{-3} *M* and growth of excised tomato roots was inhibited at approximately 8.5×10^{-4} *M* (*25*). The acid also antagonized indoleacetic acid (IAA) in the *Avena* assay. Cornman (*29, 30*) reported that parasorbic acid slowed down mitosis. Metaphase stages were observed to accumulate, but abnormalities were not detected.

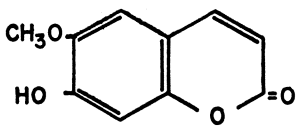
Coumarin, the lactone of *o*-hydroxycinnamic acid, and some of its derivatives have been isolated from many plant species (*51*). Thimann and Bonner (*141*) attributed the growth-inhibiting effects of coumarin to its action on enzyme sulfhydryl groups. Inhibitory effects of coumarin on *Avena* coleoptiles and pea stem sections could be overcome by 2,3-dimercaptopropanol (BAL). Coumarin has also been reported to disrupt mitosis (*29, 30*).



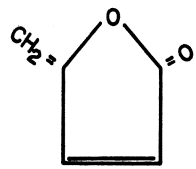
Parasorbic acid



Coumarin



Scopoletin



Protoanemonin

Figure 2. Structural formulas of some lactones isolated from plants which have a growth-inhibiting action

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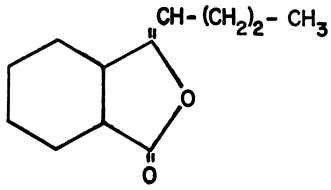
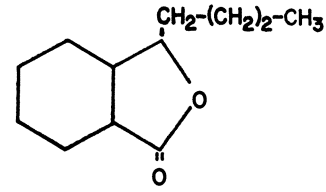
The action of coumarin and many of its naturally occurring analogs such as umbelliferone, aesculetin, daphnetin, scopoletin, aesculin, and limettin on root growth has been compared by several investigators (*7, 51, 118*). In all cases coumarin was the most inhibitory.

Protoanemonin, which has been isolated from *Anemone pulsatilla* and *Ranunculus* spp., was reported to inhibit root growth by slowing down metabolism and blocking mitosis (*35*). Erickson and Rosen (*35*) observed cytological effects in corn root tips at concentrations of 10^{-3} *M* and lower. Cells undergoing division appeared to accumulate in the interphase or prophase stages. Metaphase, anaphase, and telophase stages were not observed. Cytoplasmic and vacuolar structures were disturbed and the presence of mitochondria could not be demonstrated in treated tissue. Thimann and Bonner (*141*) reported that protoanemonin was 10 to 30 times more inhibitory than coumarin in coleoptile and split pea stem tests, and that BAL prevented the inhibitory action.

Germination inhibitors present in many seeds of the family *Umbelliferae* have been identified as phthalides (*106*). Two of the most active compounds isolated were 3-*n*-butylhexahydrophthalide and 3-*n*-butylidenehexahydrophthalide (*102*).



3-*n*-Butylidene-hexahydrophthalide



3-*n*-Butyl-hexahydrophthalide

Bennett and Bonner (*6*) surveyed 11 desert plants for the presence of compounds inhibitory to the growth of tomato plants. Three crystalline toxic furocoumarins were isolated from *Thamnosma montana*. Two were identified tentatively as byakangelicin [$C_{16}H_{15}O_6$-(OCH_3)] and isopimpinellin [$C_{11}H_4O_3(OCH_3)_2$]. The third and most active compound [$C_{16}H_{15}O_5(OCH_3)$] was lethal at 12 to 15 p.p.m. to tomato seedlings growing in solution culture, and was considered to be an isobergaptene derivative. The authors credited D. Crosby for the structural determinations.

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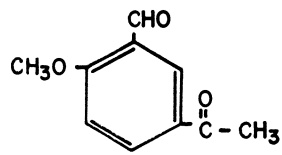
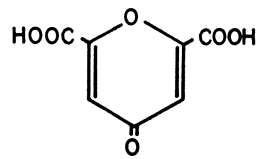
MISCELLANEOUS IDENTIFIED INHIBITORS. 3-Acetyl-6-methoxybenzaldehyde is present in the leaves of the desert shrub *Encelia farinosa*. It is apparently leached from the leaves and washed into the soil by rain. Concentrations of approximately 0.5 mg. per gram of dried leaf material have been measured. In sand culture studies, growth of tomato seedlings was inhibited by 50 p.p.m. while 115 p.p.m. reduced growth by 50% (*53*). A concentration of 250 p.p.m. killed the test plants within one day. The structure was confirmed by synthesis, and the synthetic material was shown to be as active as the natural product (*54*). Derivatives were also prepared in which a cyano, nitro, or amino group was substituted for the aldehyde moiety. The amino derivative was reported to be the most highly toxic.



3-Acetyl-6-methoxybenzaldehyde

Chelidonic acid has been found to exist in the free state in many plants (*122*). A concentration as high as 10^{-2} *M*, calculated on a fresh-weight basis, has been measured. Leopold *et al.* (*89*) found that chelidonic acid functioned as a growth inhibitor in the pea straight-growth test. Some inhibition was obtained at 10^{-5} *M* and 50% inhibition was measured at 10^{-4} *M*. Growth was promoted in the presence of IAA. This action is similar to that noted for coumarin and other unsaturated lactones. As with lactones, the inhibitory effect of chelidonic acid could be relieved by pretreatment of the tissue with BAL.

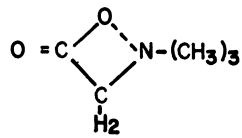


Chelidonic acid

trans-Cinnamic acid has been shown to be excreted by the roots of *Parthenium argentatum* (guayule) (*14, 16*). Growth reduction

of guayule plants was significant in solution culture experiments with concentrations of 1 p.p.m., and at 30 p.p.m. growth was reduced by 50%. The acid was reported to be rapidly degraded in nonautoclaved soils.

Betaine, a quaternary ammonium compound, has been isolated



Betaine

from mature sugar beet leaves and was reported to be present at concentrations of 9.6 mg. per gram fresh weight (*157*). Betaine at 150 p.p.m. decreased growth of sugar beet leaf discs 94% and that of dwarf French bean discs 81% relative to the water controls.

β-INHIBITOR COMPLEX. Paper chromatograms of extracts of various plant tissues have shown the widespread occurrence of acidic components which expressed inhibitory properties in the various *Avena* tests. Bennet-Clark and Kefford (*5*) first applied the name "inhibitor-β" to materials which migrated ahead of IAA in a 2-propanol-ammonia solvent system. Subsequent investigations have shown that many different chemicals are described by this inadequate definition. This mixture of inhibitory chemicals is now frequently referred to as the "β-inhibitor complex." Compounds associated with the β-inhibitor complex include coumarin, *o*- and *p*-coumaric acid, ferulic acid, salicylic acid, *m*-hydroxybenzoic acid, and cinnamic acid from fruit juices (*149*); cinnamic acid and salicylic acid from rice straw (*79*); uncharacterized phenols from tomato roots (*136*); scopoletin, azelaic acid, and an unsaturated polyhydroxy fatty acid from potato (*72*); possibly a β-hydroxy acid from *Acer pseudoplatanus* (sycamore maple) leaves (*124*); an undefined phenolic component from *Acer saccharinum* (silver maple) buds (*83*); and mandelonitrile (*76*) and naringenin from *Prunus persica* (peach) buds (*71*). β-Inhibitors have also been detected in the xylem sap of *Tecomaria capensis*, *Malus sylvestris*, and *Salix fragilis* (*32*), and have been extracted from the seed of *Striga lutea* (*158*).

Unfortunately, investigators have classified a component as a β-inhibitor if it was acidic and migrated at an appropriate R_f in the 2-propanol-ammonia solvent system. Robinson and Wareing (*124*)

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found phenolic compounds associated with the inhibitor complex which moved out of the inhibitor zone when it was rechromatographed with a second solvent system. Libbert (*93*) pointed out the need for additional studies on identification of inhibitors previously associated with the complex.

The components of the β-inhibitor complex, especially the phenolic constituents, have been implicated with the in vivo regulation of plant growth and plant dormancy (*68*, *154*). A positive correlation between a decrease in endogenous β-inhibitors and the breaking of dormancy has been reported by several investigators (*57*, *68*, *69*, *70*, *117*, *150*, *154*). Wareing *et al.* (*154*) were able to arrest the development of actively growing *Betula pubescens* seedlings with an inhibitor obtained from dormant tissues of the same species. They also extracted an inhibitor from sycamore maple leaves grown under short-day conditions and found that it completely inhibited growth of *Avena* coleoptile sections at a concentration of 25 p.p.m. [Subsequent to completion of the survey of literature, this inhibitor was reported by Cornforth *et al.* (*27*) to be identical to abscisin II (*1*) and its structure has been verified synthetically by Cornforth *et al.* (*28*)].

Unidentified Inhibitors. Many reports have been published relative to inhibitory responses which were obtained with extracts of plant tissue or from products associated with decomposition processes. For the most part, the inhibitory responses have been noted, but the inhibitors have not been identified chemically. Garb (*48*) tabulated approximately 25 references in which inhibitors were reported but were not characterized. These will not be relisted here. Uncharacterized inhibitors have also been reportd by Le Tourneau *et al.* (*91*), Patrick and Koch (*112*), Lapusan (*85*), Guenzi and McCalla (*63*), Lawrence and Kilcher (*86*), Grodzinskiĭ *et al.* (*60*), Brown (*24*), Patrick *et al.* (*114*), and Hoveland (*73*).

Conflicting observations have been reported relative to the existence of growth inhibitors in *Agropyron repens* (quackgrass) plants. Le Tourneau and Heggeness (*92*) and Kommedahl *et al.* (*81*) detected growth inhibitors in aqueous extracts prepared from the foliage and rhizomes, and Swietochowski and Gonetowa (*140*) reported that quackgrass plants excreted compounds into the soil which adversely affected the germination of rye and wheat seeds. However, Welbank (*156*) indicated that there was little direct evidence for the secretion of toxins by quackgrass and suggested that inhibitors

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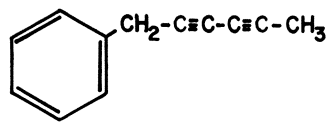
might arise from the decay of plant material in the soil. More recently Ohman and Kommedahl (*110*) also found no evidence for the secretion of phytotoxic substances from living quackgrass roots or rhizomes. They concluded that chlorosis and stunting of alfalfa and oats grown in soils which contained quackgrass residues might be caused by a temporary deficiency of available nitrogen—i.e., the decomposition of plant residues by microorganisms of the soil would temporarily complex available nitrogen. However, they were able to obtain evidence for the production of phytotoxic materials when aqueous extracts of quackgrass leaves and rhizomes were subjected to high temperatures during or subsequent to extraction. Extremely phytotoxic materials were extracted from soils that contained decomposing plant residues, especially when decomposition occurred under anaerobic conditions. Grümmer (*61*), in addition to finding that phytotoxic phenolic compounds were released from a variety of decomposing plant residues, identified agropyrene as the major constituent of the essential oils present in quackgrass. It had an antibiotic type of action against fungi and bacteria, but its effect on growth of higher plants was not established.



Agropyrene

Inhibition of tomato and barley plants growing in soils infested with *Centaurea repens* (knapweed) was reported by Fletcher and Renney (*38*). A toxic component was isolated in highest concentration from the foliage of knapweed. The inhibitor was considered to be an indole alkaloid or auxin precursor because of its ultraviolet absorption spectrum and the positive reactions obtained with Salkowski and Ehrlich reagents. The presence of the inhibitor was considered to explain partially the rapid establishment of *Centaura* spp. in almost pure stands.

Leaves of *Eucalyptus rostrata* were shown to contain substances that inhibited growth and germination (*90*). Four active fractions were obtained from the crude leaf extract. Inhibitory constituents were shown to contain a carbonyl group and a carbon-carbon double

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bond. Mesityl oxide, $(CH_3)_2C=CH-C(=O)-CH_3$, was found to possess properties similar to the natural leaf substances. The inhibitors present in the leaves did not appear to accumulate in the soil beneath the trees in inhibitory concentrations; hence, they may have no ecological significance.

Yokum *et al.* (*167*) have studied the properties of a germination and growth inhibitor produced by *Setaria glauca* (yellow foxtail). The inhibitor was heat-stable, dialyzable, neutral, and nonnitrogenous. The authors suggested that it was a carbohydrate.

Rothwell and Wain (*126*) have isolated in crystalline form a growth inhibitor from *Lupinus luteus* (yellow lupine) pods which they have partially characterized. Analytical data suggested that the inhibitor possessed the characteristics of an unsaturated hydroxyketo acid. Inhibition in the wheat coleoptile cylinder test was obtained with concentrations of 0.25 to 1.0 p.p.m.

Ailanthus altissima (tree of heaven) grows in pure stands, hence, secretion of a toxic principle is implied. Mergen (*98*) prepared aqueous extracts of the foliage and found growth inhibition produced in 45 of 46 species of test plants.

Miyamoto *et al.* (*101*) obtained four fractions from the seed coats of wheat which inhibited development of the wheat embryo. A component from one of the fractions responsible for 20% of the total inhibitor activity was crystallized but not identified.

Miyamoto (*100*) extracted and obtained "crude" crystals of a germination inhibitor from the bark of *Syringa vulgaris* (lilac).

Specific inhibitors have also been extracted, and studied, from *Cyperus esculentus* (*146*); *Artemisia vulgaris* (*87*); *Madia glomerata* (*26*); roots of *Chrysanthemum morifolium* (*4*); *Zinnia oligantha* (*145*); and *Vitis vinifera* (*133*).

UNIDENTIFIED STIMULATORS. Growth-inhibitory materials in, or secreted by, plants have for the most part been emphasized in this paper. However, root secretions of many plants also contain constituents required for the germination of seeds of angiospermous root parasites (*Striga*, *Orobanche*, *Alectra*, and *Sopubia* genera). This phenomenon has been reviewed by Brown (*17*). *Striga asiatica* was identified for the first time in the western hemisphere in 1956 in two

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southeastern states of the United States. The status of the problem and advances in witchweed control were reported by Shaw *et al.* (*132*). Williams (*159*) summarized efforts to isolate and identify the germination stimulants from *Striga* and *Orobanche* spp. While considerable progress has been made in purfication and characterization of the stimulants, chemical identities have not been established (*18, 21, 22, 23, 84, 95, 128, 138, 148*).

Results obtained by Worsham *et al.* (*165*) on the characterization of the *Striga asiatica* stimulant were in general agreement with those of previous investigators. Studies with concentrated but nonhomogeneous preparations suggested that the stimulant was neutral, had a low molecular weight (approximately 100 to 110), and showed bluish-white fluorescence under ultraviolet light. Results obtained with characterization reagents suggested that the stimulant possessed one or more hydroxyl or potential hydroxyl groups and an ester (lactone) bond. The *Striga* and *Orobanche* stimulants appear to be similar in many respects (*21, 23*). *Striga* germination stimulants from *Zea mays*, *Gossypium hirsutum*, and *Coleus* spp. have similar characteristics (*34*).

The germination stimulants are active at extremely low concentrations: 0.02 p.p.m. (*19*) and 0.001 p.p.m. (*165*). Sunderland (*137*) reported that the *Orobanche minor* stimulant from linseed promoted germination at concentrations of 10^{-8} or 10^{-9} *M*.

Thiourea and allylthiourea (*20*), D-xylulose (*22*), sodium diethyldithiocarbamate, L-methionine, and *n*-propyl-di-*n*-propylthiolcarbamate (*163*), 12 6-(substituted) purines (*166*), and two coumarin derivatives (*164*) have been found to promote *Striga* seed germination. None of these materials, however, appear to be constituents of the natural stimulant preparations.

Mechanism of Action of Naturally Occurring Inhibitors

A comprehension of the mechanism through which growth is controlled by naturally occurring inhibitors would hopefully assist in developing herbicides and growth regulators. Unfortunately, only limited information is available on specific interferences imposed at the molecular level by the natural inhibitors. High concentrations of an inhibitor can be present in an organism without apparently producing effects detrimental to that organism. Colchicine obtained from *Colchicum autumnale* and podophyllin from *Podophyllum*

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peltatum can produce polyploidy by interfering with the spindle fiber mechanism during cell division. However, there is no evidence that such an effect is imposed on the plant in which the materials are produced. Fowden (*43*) has reported that the nonprotein amino acid azetidine-2-carboxylic acid (the lower homolog of proline) was produced in considerable quantity by certain liliaceous plants. No evidence of its incorporation into protein in these plants was obtained. However, the homolog was found to be highly toxic to mung bean seedlings because it was incorporated into newly formed protein in place of proline.

Obviously, growth will be controlled only if an inhibitory molecule reaches the site at which its action is expressed. Not all of a chemical applied to the plant or soil, however, can be expected to participate in a growth-controlling reaction. Chemicals applied to the soil may undergo photochemical or chemical decomposition, may be lost through volatilization, may be adsorbed on soil constituents, or may be structurally altered by microorganisms. The action of microorganisms may activate an inactive material or inactivate an active one. Foliar applied material must be retained by the leaf, penetrate the cuticle, and be absorbed by the cells of the leaf. Morphological and physiological factors together with the inhibitor's chemical and physical properties will determine whether or not it is absorbed and translocated. Physical processes may inactivate the herbicide by causing adsorption to inactive sites and biochemical reactions may detoxify the chemical.

Relatively few sites of growth-modifying or growth-inhibitory action have been identified at the cellular and molecular level. Consequently, the exact action of, and relationships between, auxins, gibberellins, kinins, and growth inhibitors remain to be elucidated.

Naturally occurring inhibitors are considered to regulate correlative inhibition, dormancy, root initiation, and other processes involved in plant growth and development (*93*). Some of these inhibitors, especially phenolic components of the β-inhibitor complex, may control growth by affecting the level of IAA in the tissue (*65*, *144*, *169*). Genetic and environmental factors may indirectly regulate the level of endogenous inhibitors which in turn control the concentration of IAA in the cell via the IAA oxidase system. Some of the effects of nematodes (*152*) and plant pathogens (*131*) may also be imposed on the host plant through interference with IAA metabolism. The inhibitors may act directly by forming complexes with IAA or

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indirectly by affecting IAA synthesis or degradation. The formation of IAA complexes or conjugates with phenols (*88*), aspartic acid (*3*), and glucose (*168*) has been reported.

Various phenolic acids have been reported to inhibit IAA oxidase (*9*, *52*, *120*), and other phenols may act as cofactors of IAA oxidase (*144*). In general, the cofactors of IAA oxidase are monophenols, whereas the inhibitors of the system are polyphenols, with *o*-dihydroxyphenols being the most inhibitory (*52*, *65*). Unsaturated lactones have also been reported to inhibit IAA oxidase (*2*, *52*).

Inhibitory materials can be expected to modify and control plant growth by inhibiting essential physiological processes or by preventing the biosynthesis of metabolites essential to plant growth. The growth-inhibiting effects of coumarin, protoanemonin, and chelidonic acid have been attributed to their action on enzymic sulfhydryl groups because pretreatment of the tissue with BAL overcame the inhibition. Phenolic materials are known to uncouple oxidative phosphorylation and, conceivably, some of the phenols and other constituents of the β-inhibitor complex identified as toxic materials could function in this capacity. Marinos and Hemberg (*96*) found that the β-inhibitor complex extracted from potato peel stimulated oxygen uptake and decreased uptake of inorganic phosphate. Cinnamic acid had a similar effect.

In addition to effects on biochemical reactions, the inhibitors may influence the permeability of the various cellular membranes and through physical and chemical effects may alter the structure of other subcellular structures such as proteins, nucleic acid, and spindle fibers. Unfortunately, few definite examples can be listed. The action of colchicine and podophyllin in interfering with cell division is well known. The effect of various lactones (coumarin, parasorbic acid, and protoanemonin) on mitotic activity was discussed above. Disturbances to cytoplasmic and vacuolar structure, and the morphology of mitochondria imposed by protoanemonin, were also mentioned. Interference with protein configuration and loss of biological activity was attributed to incorporation of azetidine-2-carboxylic acid into mung bean protein in place of proline.

Information concerning the metabolic function of unusual naturally occurring plant constituents might be used to develop new growth regulators. An example of a development that arose in this way began with the observation by Tolbert (*143*) that as much as 30% of the total soluble phosphorus in plant roots, and sometimes in the leaves,

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was present as phosphorylcholine, $(CH_3)_3\,N^+\text{-}CH_2\text{-}CH_2\text{-}O\text{-}PO_3H^-$. In studies designed to learn about the role of this chemical, chlorine was substituted for the hydroxyl group of choline, a substitution that would prevent esterification of the molecule by phosphate. This analog, (2-chloroethyl)trimethylammonium chloride, was given the trivial name of chlorocholine chloride (CCC). Some 100 derivatives have been prepared and tested by Tolbert and his associates. Seed germination is inhibited by CCC derivatives. Foliar applications produce plants with short internodes, thick stems, and dark green leaves. In general, the growth responses produced by CCC derivatives appear to be the opposite from those induced by the gibberellins.

Inhibitors must possess chemical and physical properties that will ensure absorption by root tips or penetration by foliar surfaces, and translocation to the active site. Once there they must assume the precise spatial configuration required to complement the molecular architecture of the active center if they are to block the key reaction. A comprehension of comparative biochemistry and information on how plants differ in the architecture of the reactive sites should assist in developing truly selective herbicides.

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