New Approaches to
Pest Control and Eradication

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INTRODUCTION

Pesticides in general have been the target for considerable criticism, especially in recent months. Critics and outright opponents of pesticides stress their harmful effects on the wildlife of both forest and stream and allude to human health hazards in their application and as chemical residues in our food.

On the other side are proponents of pesticides, who point out their many benefits in enhancing the quality and quantity of our agricultural production and in protecting the health of the public, especially from insect-borne diseases. Insecticides have made it possible to combat malaria, a disease formerly responsible for half of the world's deaths. Results have been so successful that now one third of the world's population is entirely free of malaria and another third has eradication within its grasp. Insecticides have also scored great successes in controlling other devastating insect-borne diseases such as typhus and yellow fever. Without insecticides we would have insect-infested vegetables and fruits, and grain and other stored products. For most of these substandard commodities, we would necessarily have to pay high prices because of scarcity. We would have to put up with the depredations of grasshopper outbreaks, infestations of the Mediterranean fruit fly, the cattle grub, ticks, and lice—to name only a few. Opponents of insecticides often say in rebuttal that they would not try to eliminate their use entirely, but use only certain insecticides (especially botanicals) and these only in a limited way. Unfortunately, this would not begin to answer the many problems we have in the control of insects.

While there are some real problems to be solved which involve residues, insect resistance to insecticides is the really big problem. Curiously, the opponents of pesticides fail to measure it in its full dimensions. Resistance of insects to insecticides is a truly growing problem. As long ago as 1908 the repeated use of lime-sulfur sprays in orchards in Clarkson Valley, Washington, selected out a resistant strain of the San Jose scale, which spread and reached southern Illinois orchards in 1920. Then there followed three species of scale insects

in California which were very resistant to hydrogen cyanide. The codling moth, peach twig borer, and two species of cattle ticks developed resistance to arsenicals. Other species were selected out that resisted the killing action of tartar emetic, cryolite, selenium, and rotenone. But during the period 1908 to 1945 only 13 species of insects or ticks had developed recognizable resistance. Now the picture is different. The total number of resistant strains has risen to what A. W. A. Brown, a noted authority on resistance, calls the "appalling figure" of 137 species. This period commenced with development of resistance to DDT in the housefly and was quickly followed by resistance to BHC, chlordan, and dieldrin. Seventy-two species of insects of public health importance are involved, 58 showing resistance to dieldrin, 36 to DDT, and 9 to organophosphorus insecticides. Among agricultural insects, 65 species of plant-feeding arthropods have developed resistant strains, 19 to DDT, 16 to dieldrin, and 20 to organophosphorus insecticides.

This whole problem calls for the utmost resourcefulness and good research planning, if we are to keep ahead of it. There is no single answer to the resistance problem. We can meet some parts of this problem by shifting to other types of insecticides; this is being done where feasible. Where the shift is from a chlorinated hydrocarbon to an organophosphorus insecticide, some residue problems may incidentally be solved. In some sectors there is a shift over to carbamate-type insecticides. But this is not enough, nor does it, by any stretch of the imagination, answer the resistance problem. Cross resistance between insecticides is more the rule than the exception.

Resistance hits hard in agriculture. For example, the boll weevil, which showed a pronounced resistance to toxaphene and other chlorinated hydrocarbons in 1955, affects 80% of the total cotton acreage and more than 95% of all cotton producers in the United States. As resistance builds up it affects not only the grower, particularly of cotton, but the pesticide chemical industry. Costs of development of a pesticide range from $500,000 to perhaps something over $3,000,000. The chemical industries may very well question the development of a new chemical that may have a relatively short market life expectancy because of resistance. Here the opponents of pesticides can score with ample justification. What do the proponents have to say? First of all, substitute insecticides must be tested to the utmost; this has yielded some very definite successes. Then all other means must be explored. Basic research, however, is sorely needed. What causes resistance? We are learning something about it and there are

indications that we may learn how to combat it, but the answers will not come overnight. Progress here is very slow.

More serious consideration is being given to the eradication of an injurious species, where this is feasible, before the resistant strains are selected out and become dominant. This makes sense. It is generally not easy to eradicate with insecticides, but it has been done and can be done in certain instances. In the case of the Mediterranean fruit fly in Florida, an insecticide did the eradicating with the help of a specific insect attractant to tell us exactly where and when and about how much to spray to stamp out the infestation. Another insect eradication program was achieved without the use of an insecticide. I refer to the highly successful sterile-male eradication of the screwworm in Florida and the Southeast. Insects rendered sterile by gamma irradiation are systematically released to outnumber the fertile insects in the natural environment. Now a more extensive program in Texas and the Southwest is meeting with the success which was expected of it. Surely we must all stand in admiration at the boldness and imaginativeness of this scheme of insect eradication which reckons on turning the sex drive and enormous reproductive capacity of a destructive insect against itself. Here you will find no problems of killing wildlife or beneficial insects or of residues or of selecting out resistant strains! The females lay their eggs normally, but the eggs never hatch. Without killing a single insect the population of the pest is pushed down and down and down until finally it disappears. Is this not a beautiful method? Most surely it will get more attention in future years for other injurious insect species and pests that reproduce sexually.

Another approach (the newest of all) to pest control and eradication is with chemosterilants. A chemosterilant—a coined name given to a chemical causing sexual sterility in an insect—may perform a task that would not be feasible by the irradiation and release method. As an example, the boll weevil simply cannot take 5000 or so roentgens of gamma irradiation and come out feeling fit and eager to mate. A chemosterilant is not as rough on an insect and can perform its task simply and cheaply. Chemosterilants are strictly in the research stage; they certainly do function, and their potentialities are being tested experimentally with suitable baits. Results are promising, but we have much to learn about developing safe procedures for the ultimate use of chemosterilants. There are definitely no recommendations for general use.

An approach that is receiving much more emphasis is the development and use of insect attractants. A number of these have been found

by the empirical screening and synthesis method. For example, the attractant used in the eradication program of the Mediterranean fruit fly in 1956 was found in this way. Thousands of traps were maintained at strategic locations in Florida for this purpose. When the medfly invader turned up again early in 1962, the infestation was quickly stamped out with a minimum use of insecticide at an estimated saving of $9,000,000 over the previous campaign, which did not have the benefit of the early warning system of attractants. Potent attractants have also been found for the melon fly and for the oriental fruit fly. Sex attractants are receiving much attention since the determination of the structure and synthesis of the gypsy moth sex attractant. A synthetic homolog of this attractant, called "gyplure," is available in sizable quantity and its possible use for control or eradication is under investigation. Many insects, especially among the Lepidoptera, have sex attractants that enable the male to find the female for mating and reproduction. A sex attractant has been found in the pink bollworm, southern armyworm, tobacco hornworm, and the European corn borer. When the pure compounds are isolated, the chemical structures determined, and synthesis accomplished, ways will be devised to use these powerful materials that nature provided for reproduction so as to turn this extraordinary force against the insect. This goal may be achieved by male annihilation of the harmful species or possibly by coupling a chemosterilant with a sex attractant.

The discovery of "antifeeding" compounds which prevent chewing insects from feeding on a crop may offer promise; it is too early to assess the significance of this development.

There are many other approaches of a long-term nature that have been explored (but too little) for a good many years. The development of insect-resistant crop varieties has sometimes been used when all other methods of control have failed. This is the case with the wheat stem sawfly. The development of sawfly-resistant wheat varieties permits the profitable growing of wheat on some 2,000,000 acres in Canada and on more than 600,000 acres in the North Central United States. Varieties of wheat bred for resistance to the Hessian fly are now grown on 4,500,000 acres in 26 states.

Breeding field crops for disease resistance has had similar successes. While the examples in which parasites and predators have been used to achieve pest control are numerous, it is evident that not enough support has been given here in the past and it is virtually certain that this will receive far more exploration in the future. Other biological

control methods, such as the use of specific pathogens for insects and other pests, are also being slowly developed today.

Conventional pesticides will undoubtedly be needed for many years to come, despite the fact that they bring problems in their wake. Resistance and residues are the main problems. Shifting to other types of insecticides will answer some of the problems in both these categories as well as some of the problems involving losses of wildlife. To maintain our progress we must gain more basic knowledge about mode of action of insecticides and how to combat resistance. We must also explore all other profitable approaches to pest control. Biological control methods in general cannot take the place of insecticides tomorrow, or next year, or the year after that—perhaps not for decades; but there will surely, even if slowly, be real gains in these other approaches in proportion to the work that is done in exploring them. There is a strong and healthy trend toward eradication of certain important pests rather than year-in and year-out control to try continually to keep them in check. We thus find ourselves in a situation where great progress has been made, where we are struggling to consolidate this progress and to clean up problems that have come with it. Most important of all, we are planning to move ahead faster through new types of research keyed to basic studies in biology and chemistry. If we were not on the move, our progress would surely be cancelled out by the dynamic forces of nature.

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Recent Progress in the Chemistry of Insect Sex Attractants

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Insect sex attractants are probably the most potent physiologically active compounds known today. Although only two have as yet been completely identified and synthesized, the chemistry of a number of others is now under investigation. Developments in these investigations during the past 5 years are reviewed. The use of insect sex attractants in insect survey and control is discussed, the gypsy moth sex attractant being used as an example.

It is a remarkable fact that a force of nature as potent as that which enables a male insect to track down the female of its species to a distance measurable in miles in some observations should have received so little scientific study. The fact remains that the study of insect sex attractants is quite recent.

The literature on the chemistry of insect sex attractants prior to and including 1957 has been adequately covered by Karlson and Butenandt (*28*), and this presentation is limited to work reported subsequently.

Sex attractants are known by several names. Kettlewell, in England, prefers the term "female assembling scents" (*29*), but Karlson and Butenandt, in Germany, prefer the term "sex pheromone," from the Greek "pherein" (to carry) and "horman" (to excite, to stimu-

late) (*28*). Whatever the term used, the potency of these materials is almost fantastic; infinitesimal amounts lure males from a distance, depending upon the order and species.

Gary (*14*) has reported experimental evidence demonstrating the existence of volatile chemicals functioning as mating attractants in the queen honey bee (*Apis mellifera* L.). Drones in flight are attracted to the flying queen by substances present on or in her mandibular glands. Fractionation of the gland lipides, obtained by extraction with ether, yielded "queen substance" (*trans*-9-oxodec-2-enoic acid), attractive to drones at 0.1 mg. per assay tube, and at least two other substances with some attractiveness. However, reconstitution of the lipide complex resulted in considerably more attractiveness than was shown by individual fractions. It was subsequently shown (*31*) that extirpation of the mandibular glands does not necessarily render a virgin queen incapable of mating, indicating that drones may utilize supplementary stimuli such as vision to locate queens in flight.

Attraction of worker bees to queens in the hive is also due to a mixture of volatile acids (containing "queen substance") extracted from queen mandibular glands; attractivity is lost by chromatography and can be recovered by remixing fractions (*33*).

In 1960, Coppel and his coworkers (*13*) reported on the sex attractant present in the female introduced pine sawfly [*Diprion similis* (Hartig)]. In field investigations virgin females were found capable of attracting exceptionally large numbers of males; one caged female attracted well over 11,000 males. An attempt to trap the attractant by passing air rapidly over virgin females and then through various solvents was unsuccessful, but the crude attractant was obtained by extracting crushed whole females with acetone or benzene and by rinsing, with ether, glassware that had contained the live or dead females.

Few sex attractants have been reported in insects of the order Hemiptera. Butenandt and Tam (*11*) isolated from certain abdominal glands of males of the tropical water bug [*Lethocerus indicus* (Lepeletier and Serville) (=*Belostoma indica*)] a substance which is believed to make the female more receptive to the male. The substance, possessing a cinnamonlike odor, was identified as the acetate of *trans*-2-hexen-1-ol. A homolog of this, the acetate of *trans*-2-octen-1-ol, was isolated from the bronze orange bug [*Rhoecocoris sulciventris* (Stal.)] in 1962 by Park and Sutherland (*34*), who suggest that this substance may be a sex attractant for this insect.

Virgin female sugar beet wireworms [*Limonius californicus* (Man-

nerheim)] attract numerous males upwind from distances up to 50 feet. Laboratory and field tests of ethyl alcohol extracts of the head, thorax, and abdomen showed that only the abdomen was attractive. Paper chromatography of the crude abdominal extract with 0.1*N* NH_4OH or EtOH-NH_3 (95:5) gave spots at R_f 0.9 and 0.85, respectively, that were attractive to males in laboratory tests. The attractant is highly specific and quite stable at room temperature (*30*).

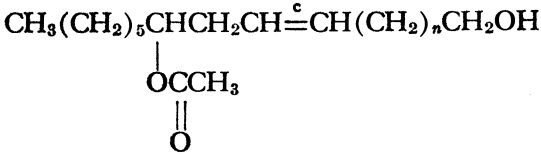
Wharton *et al.* (*42*) showed that filter paper over which virgin female American cockroaches [*Periplaneta americana* (L.)] had crawled was highly attractive to males, and these investigators have very recently reported that they obtained from such papers 28 μg. of an attractant which they were unable to identify. As the result of an independent investigation which was not sufficiently completed to be reported at the symposium held in September 1962 in Atlantic City, Jacobson, Beroza, and Yamamoto (*24*) isolated the sex attractant and identified it as 2,2-dimethyl-3-isopropylidenecyclopropyl propionate. The pure attractant, which elicits a response from males at levels below 10^{-14} μg., was obtained by condensing the air stream passed over the virgin females in metal containers and fractionating the condensate (*43*). It causes intense excitement, wing raising, and copulatory attempts in males.

Sex attractants among insects of the order Lepidoptera have received the most detailed chemical study to date. This work has centered mainly on the sex attractants of the silkworm moth [*Bombyx mori* (L.)] and the gypsy moth [*Porthetria* (=*Lymantria*) *dispar* (L.)]. In both species the attractant is formed in the lateral glands of the virgin female abdomen. The female is able to protrude and retract these glands, and in this manner she regulates the release of the attractant.

The pioneering detailed chemical studies on the gypsy moth sex attractant were conducted by Haller and Acree, and are adequately reviewed by Jacobson, Beroza, and Jones (*24*) and Holbrook, Beroza, and Burgess (*18*). Stefanovič (*39*, *40*), working with a hydrogenated benzene extract of the female abdominal tips, obtained a bright yellow oil that was highly attractive to the males.

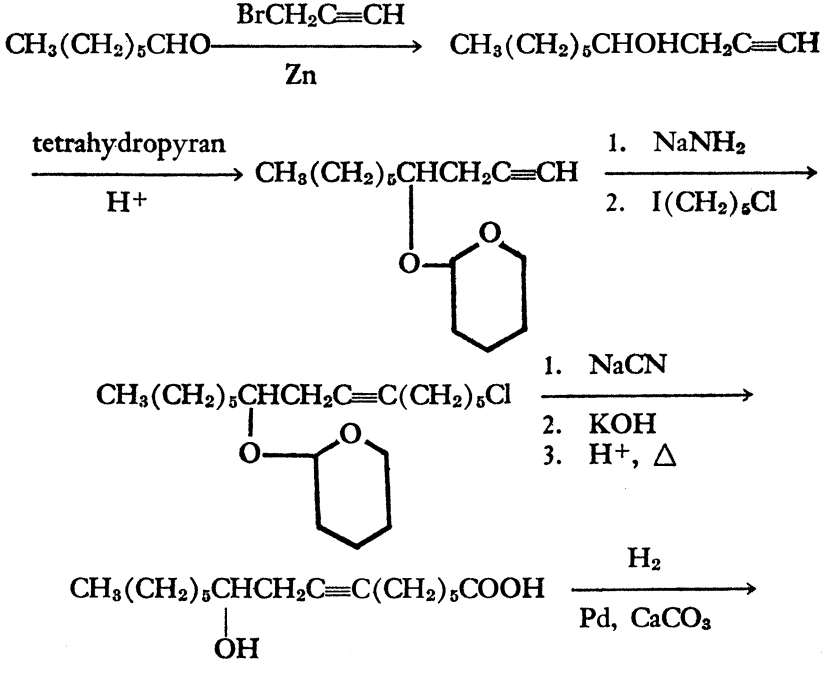
After 30 years of study, the gypsy moth sex attractant was isolated, characterized, and synthesized in 1960 (*25*). To isolate the attractant, it was necessary to clip the last two abdominal segments of many virgin female moths, separate the neutral fraction from a benzene extract of the abdomens, and either chromatograph by a tedious process on adsorbent columns, or what is more satisfactory, dissolve the

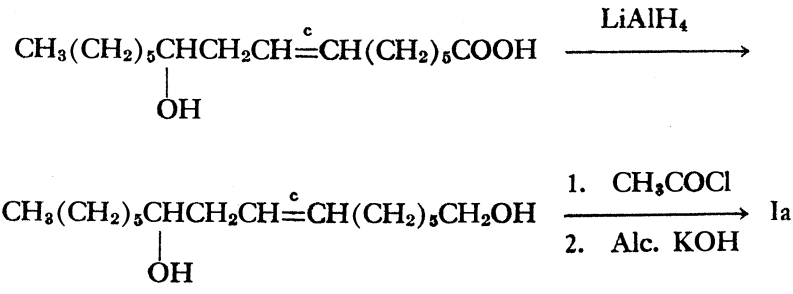
neutral fraction in acetone, precipitate out the inactive solids, and subject the attractive yellow oil to paper chromatography. Of the five spots obtained, only one was attractive to males, and this was separated into a highly attractive colorless liquid (the major attractant) and a solid of much lower activity. A total of 20 mg. of pure major attractant was isolated from 500,000 females; its structure has been identified by synthesis as *dextro*-10-acetoxy-*cis*-7-hexadecen-1-ol (Ia).



Ia. $n = 5$
Ib. $n = 7$ (gyplure)
Ic. $n = 9$

The *dl*-form of the attractant, identical in all respects save optical activity with the natural attractant, was synthesized in 0.2% over-all yield by the steps shown below (*24*, *25*).





Fractions were bioassayed in field traps (*18*) or in the laboratory (*4*). In the latter method, gypsy moth males imprisoned by their wings and exposed to the scent on a rod or filter paper exhibited curving of the abdomen and copulatory attempts.

The *dl*-form was successfully resolved by treating its acid succinate with L-brucine, separating the brucine salts by fractional crystallization from acetone, decomposing the salts, and saponifying the acid succinates with ethanolic alkali (*20*).

Characterization of the natural gypsy moth attractant resulted in the synthesis of a homolog, *dextro*-12-acetoxy-*cis*-9-octadecen-1-ol (Ib), which has been designated "gyplure" (*19*, *27*). This compound was prepared in high yield from ricinoleyl alcohol by acetylating both hydroxyl groups and then selectively saponifying the primary acetyl group with refluxing ethanolic potassium hydroxide. The secondary acetyl group is extremely resistant to saponification with ethanolic alkali, and it is necessary to use a diethylene glycol-potassium hydroxide mixture at 120° to break this linkage. This unusual stability appears to be due in some way to the position of the secondary acetyl group with respect to the double bond, since the same behavior was noted during the attempted saponification of the natural lure and of a higher homolog (*dextro*-14-acetoxy-*cis*-11-eicosen-1-ol) (Ic) (*27*).

The preparation and use of gyplure as a gypsy moth attractant have recently been patented (*22*, *23*).

Gyplure, highly attractive to male gypsy moths, is readily available commercially at low cost. If used for survey alone, one pound of this material at a cost of $10 is sufficient to bait 50,000 traps per year for 300 years. However, several commercial batches showed little or no attractiveness to males in field tests, whereas others were highly active. This lack of attractiveness has been traced to the content of inactive *trans*-gyplure in some batches, formed through the failure to control temperature and alkali concentrations in their preparation.

As shown in Table I, a concentration of 20% or more crude *trans*-gyplure in formulations of *cis*-gyplure is sufficient to cause inactivation; the mechanism of this inactivation is not known. The trans- form was prepared by elaidinization of the cis isomer with nitrous acid. The propyl and butyl analogs of *cis*-gyplure were completely devoid of activity in field tests. These results show that a cis double bond and an acetoxy group are necessary for activity (27).

Table 1. Number of Male Gypsy Moths Caught by Cis-Gyplure Containing Various Amounts of Trans-Gyplure

Sample No.	(25 μg./*trap*) % *trans*	*Moths Caught* (2 *Hours*)
639	0	19
640	1	15
641	3	14
642	5	22
643	10	13
644	15	25
645	20	4
646	30	0
647	40	0
648	50	0
649	100	2

The comparative attractancy of gyplure and its homologs and analogs to male gypsy moths is shown in Table II. The natural attractant is active at 10^{-12} μg. in the laboratory and 10^{-7} μg. in the field; *cis*-gyplure is active at 10^{-12} μg. in the laboratory and 10^{-5} μg. in the field. Conversion of the gyplure double bond to the trans form causes a tremendous drop in activity (27).

Table II. Comparative Attractancy of Gyplure and Its Homologs to Male Gypsy Moths

Compound	*Attractancy, μg.*	
	Laboratory	*Field*
d-Ia (natural)	10^{-12}	10^{-7}
dl-Ia (synthetic)	10^{-12}	10^{-6}
l-Ia (synthetic)	10^{-12}	10^{-6}
d-Ia (synthetic)	10^{-12}	10^{-7}
d-Ib (*cis*-gyplure)	10^{-12}	10^{-5}
d-Ib (*trans*-gyplure)	10^{4}	2.5×10^{5}
d-Ic	10^{-2}	10

Several other 16-carbon compounds attract male gypsy moths, but their activity does not approach that of gyplure (*21*). Examples are 1,2-epoxyhexadecane and its hydrolysis product, 1,2-hexadecanediol.

The gypsy moth is an excellent example of an insect whose sex attractant may be used for survey and possibly for control. For many years, the U. S. Department of Agriculture utilized a benzene extract of virgin-female abdominal tips in metal field traps to locate infested areas and to determine the size of the infestation by the numbers of males caught in these traps (*18*). With the ready availability of gyplure to replace the natural extract, the metal traps have now been completely supplanted by a disposable, economical cup-type paper trap (*12*). The gyplure is impregnated on small dental roll wicks at 25 μg. per trap, and an adhesive substance (Tanglefoot) is used to line the inside walls of the cup; baited traps are hung on the limbs of trees and attracted males enter through an opening in each end of the cup. To the present time, gyplure has been used in about 150,000 of these traps and found completely effective. Since the male gypsy moth is eliminated when it is lured to an adhesive-coated surface, it was proposed that a combination of gyplure and Tanglefoot coated on Celotex boards be tried for control. In limited field trials, the mixture containing 5% gyplure by weight was an effective luring device that resulted in large moth catches. In the future, consideration will be given to limited and large-scale field tests aimed at annihilating male moth populations through the use of gyplure with and without toxicants; thus, it remains only for the most efficient formulation to be determined.

The chemistry of the sex attractant of the female silkworm moth reported prior to 1958 has been reviewed by Karlson and Butenandt (*28*). In 1959, the pure attractant, designated "bombykol," was obtained as its 4′-nitroazobenzenecarboxylic acid ester and identified as 10,12-hexadecadien-1-ol (II) (*5*, 7). The extract prepared from 500,000 virgin-female abdominal tips with ethanol-ethyl ether (3:1) was saponified and the active neutral fraction was freed of sterols and esterified with succinic anhydride; saponification of the succinates, treatment with 4′-nitroazobenzenecarboxylic acid chloride, and chromatography gave 12 mg. of the attractant derivative, from which II was regenerated by saponification (*16*).

$$CH_3(CH_2)_2CH{=}CHCH{=}CH(CH_2)_8CH_2OH$$

II

Although the configuration of the conjugation in bombykol was at first thought to be cis,trans (7), subsequent synthesis of the four

possible geometrical isomers of structure II showed bombykol to possess the *trans*-10,*cis*-12 form (*6*, *10*, *17*, *41*).

The four geometrical isomers showed the following attractiveness to male silkworm moths (μg./ml.) in laboratory tests (*9*): *cis*-10,*cis*-12, 1; *cis*-10,*trans*-12, 10^{-3}; *trans*-10,*cis*-12 (bombykol), 10^{-12}; *trans*-10,*trans*-12, 10. The laboratory bioassay was based on the behavior of the male moth when confronted with the attractant. A whirring vibration of the wings and typical circling dance (called "schwirrtanz") are elicited by an active compound (*16*).

The synthesis of straight-chain, doubly conjugated primary alcohols with 14 to 18 carbon atoms and their use as insect sex attractants have been patented in Germany (*8*, *15*).

Although Anders and Bayer (*2*, *3*), as a result of an independent investigation of the gas chromatography of the female silkworm extract, reported the presence of three materials attractive to males, only one of these was highly active. Schneider and Hecker (*37*) had previously shown that several unsaturated alcohols were attractive to males in varying degree, but the most potent of these was only $1/10^6$ times as strong as the natural attractant.

Ouye and Butt (*32*) have recently shown that a stable sex attractant for males can be extracted from copulating pairs of pink bollworm moths [*Pectinophora gossypiella* (Saunders)], with ether or methylene chloride, and Allen and coworkers (*1*) have reported the extraction of a potent sex attractant from the abdomens of female tobacco hornworm moths [*Protoparce sexta* (Johannson)] with these same solvents. The chemical nature of these attractants as well as that of the southern armyworm moth [*Prodenia eridania* (Cramer)] is at present under investigation in U. S. Department of Agriculture laboratories.

The sex attractants of *Bombyx* and *Porthetria* (and probably those of most Lepidoptera as well as of insects of other orders) are received by olfactory receptors of the male antennae (*38*). Removal of one male antenna does not prevent detection of the sex scent, but complete removal of both antennae results in loss of such reception. Schneider and coworkers (*35*, *36*) have taken advantage of this antennal reception in developing an electrophysiological method for the bioassay of insect sex attractants which is approximately 1000 times more sensitive than any known behavioral method. They found that stimulation of the sensory cells in the male antenna by exposure to the female sex scent set up a local electrical potential (receptor potential) whose amplitude is dependent upon the intensity of the stimulant. The

nerve impulses thus released are amplified and recorded on an oscilloscope to give typical "electroantennograms" (EAG's). The greatest advantage of the electrophysiological method of bioassay lies in its extreme sensitivity.

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2

Synthetic Chemicals as Insect Attractants

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An approach to the control of insects that is gaining acceptance is based on the use of synthetic chemicals as insect attractants. Because many insects depend for their survival on odors which may lead them to food, water, the opposite sex, or oviposition sites, they can frequently be attracted by means of a chemical to a trap for detection purposes, or to a toxicant that destroys the insect. This paper deals with some of the known insect attractants, describes how they are found, discusses uses and kinds of attractants, and shows how attractants can increase the efficiency of insect-control operations.

Insects have been evolving for some 300,000,000 years. They have managed to persist in hostile surroundings because they have developed extraordinary adaptations or abilities, one of which is a highly specialized sense of smell. Some insects have the ability to follow an odor trail successfully to a source of food, to host plants and animals, to the opposite sex, or to the right place to lay eggs. We are attempting to utilize this highly efficient apparatus to combat injurious insects on a large scale, an approach that has already proved fruitful (*10*, *20*, *83*, *88*, *89*, *94*).

This paper reviews briefly some of the basic information on chemical insect attractants, and includes the more recent developments since the appearance of a comprehensive review by Green, Beroza,

and Hall of advances in this field up to 1956 (*53*). Other previous reviews, compilations, and articles dealing with insect attraction were published by Beroza (*17, 18*), Dethier (*38*), Hall, Green, and Beroza (*55*), Guillaume (*54*), Hecker (*59, 60*), Hocking (*63*), Hodgson (*64*), Meyer (*78*), and Schneider (*85*).

To our knowledge there are no powerful general insect attractants. The potent chemical attractants, which may be effective at distances of ½ mile or more, are usually highly specific; they attract only one or a few closely related species, and then only the males. The high specificity of these lures has been responsible for their major use—i.e., for the detection and estimation of insect populations. In traps the attractants assure early detection of an infestation before it can enlarge or spread. Several of the insect lures discovered by members of our division are now used at points of entry into our country to guard against the accidental introduction of certain agricultural pests (*100*). The Mediterranean fruit fly [*Ceratitis capitata* (Wied.)] or medfly is one of these (7). It was eradicated from Florida in 1957 (*94*), but in the summer of 1962 it was found there again. With the help of lures this infestation was eradicated in a few months.

For any eradication program a detection device is essential. Traps baited with attractants are one of the cheapest and most efficient devices that may be employed for this purpose. With this tool available entomologists are no longer scoffing at the prospects of eradicating a given species from a limited area.

Traps require good lures (*31*), but finding a good lure is expensive and often difficult. Nevertheless the search is worthwhile, because with a potent lure at hand and the exercise of reasonable care, the introduction of a new destructive species is not to be feared greatly. Actually, the tempo of international travel, air travel in particular, has been stepped up to such proportions that accidental introductions are inevitable (*101, 102*). The attractant or lure can help by delineating an infestation, making control and eradication measures very efficient, helping locate those last few hard-to-find insects, and showing when insecticide applications may be terminated in a given area. Money is saved because insecticides are not wasted; residue problems are held to a minimum.

Attractants may be combined with an insecticide for the direct control of insects (*32, 88, 89, 93*). Here we may have a means of eliminating a harmful species without affecting other insects or wildlife. Use of this combination makes it unnecessary to obtain complete

coverage, since the insect comes to the lure. In this application, narrow specificity and long-range action are not important, and the inexpensive food-based lures, which are usually easily found, work well. A protein hydrolyzate-insecticide combination was used successfully against the medfly (*83*, *94*). The potent lures may be used for detection, the weak lures for control. The weak lures can therefore supplement the more costly specific ones.

The use of chemical antifertility agents or chemosterilants with attractants is being explored. Insects made infertile by this combination would be more damaging to their species than if they were killed outright (*71*, *72*), since they would seek out and mate with other members of their species and thereby prevent them from propagating—setting up, in effect, an entomological chain reaction.

The use of attractants has been proposed for the timing of insecticide applications to give maximum effects (*82*). We may see more work along this line in the future.

Attractants have also been used to advance research on insect ecology and behavior by helping to estimate and sample populations. Data on flight habits and longevity have been obtained with radiolabeled or marked insects (*65*, *91*, *95*), the released insects being subject to recall with lures. Attractive chemicals can be used in the search for better insect repellents (*15*, *16*).

Attractants have been classified into three categories:

1. Sex lures
2. Food lures
3. Oviposition lures

Sex Lures

The type of lure is inferred or deduced from insect behavior and the assignment is frequently uncertain. If exposure to a chemical causes a male insect to assume a mating posture, the chemical is probably a sex attractant, even if it is a synthetic and unrelated to any natural lure. For example, in 1932 Lehman found that caproic acid induced sexual behavior in Pacific Coast wireworms (*Limonius canus* LeConte) (*75*).

Some entomologists believe that methyleugenol, the attractant for the oriental fruit fly (*Dacus dorsalis* Hendel), is a sex attractant because the chemical attracts only the male and its action is so powerful (*89*). However, it appears to be a food lure, because the flies avidly devour the chemical (*89*).

Food Lures

The use of food-based or fermenting lures has a definite place in control operations. Disadvantages of these materials for detection include lack of specificity (traps fill with many kinds of insects), attraction over only a short distance (*90*), rapid deterioration (especially of fermenting lures), and frequently inconsistent performance. Still, such lures are useful for detection when no other effective lure is available.

A suggestion for future research that might be given serious consideration is the development of a synthetic food lure—one that might be combined with an inexpensive carrier and toxicant and be used in place of fermenting or cereal-based attractants. Such a lure might not have the fault of variable and short-time attraction that is characteristic of most of the food-based lures. Chemicals that prevent deterioration of food-based lures would also be useful.

Oviposition Lures

In regard to oviposition lures, gravid females have been induced to lay their eggs on, or in the vicinity of, certain chemicals (*34*, *35*, *36*). Materials that release ammonia are known to encourage oviposition in houseflies (*Musca domestica* L.) (*80*, *81*, *103*). The apple maggot [*Rhagoletis pomonella* (Walsh)] is attracted to decomposing proteins, such as egg albumin, the individuals attracted in one reported instance being 83% females (*37*). A species of *Sarcophaga* oviposits on skatole (*51*). Petroleum oils have attracted female biting midges (*8*). Ammonium carbonate, indole, and skatole have been used as oviposition lures in a study of the green bottle fly [*Phaenicia sericata* (Meigen)] (*61*, *62*).

Volatility of Attractant

Volatility of the attractant molecule is an important consideration. The effectiveness of some lures over great distances implies an appreciable degree of volatility, a property that is, in part, tied to the size of the molecule. At first we believed that a compound with 11 or 12 carbon atoms was about the optimum size and this idea seemed to work with some of the fruit fly lures. A compound of this size is volatile enough to be detected but not so volatile that it would last only a short time. The finding of gyplure, the synthetic sex attractant of the gypsy moth [*Porthetria dispar* (L.)], which has 20 carbon atoms

and is extremely effective (*30, 68*), showed that generalizations on the molecular size or volatility of attractants may not be readily made.

For compounds that are too volatile, use of a wick and a reservoir (*74*) or partial covering of a wick with metal foil will extend effectiveness (*87*).

Compounds like sucrose are not attractants (*39*) because they have practically no volatility. Should a fly find sucrose, it will stop and feed, but it will not be attracted because of sugar odor. Such materials are known as "arrestants" (*40, 41*).

A mixture of geraniol and eugenol is more attractive to the Japanese beetle (*Popillia japonica* Newman) than either chemical alone (*42, 73*). This might be called odor synergism. Very little work has been done along this line.

In searching for attractants we must recognize that some species may have a very poor sense of smell or none at all. The possibility of finding good attractants for these insects would, of course, be remote.

Isolation Approach

A key question is: How do we find lures? There are two main routes. In one of these—the isolation approach—an attempt is made to isolate a natural substance known to be an attractant, determine its chemical structure, and synthesize the active ingredient. This approach is exemplified by the work relating to sex attractants of the gypsy moth (*1-4, 25, 30, 56, 68, 70*) and the silkworm moth [*Bombyx mori* (L.)] (*27, 28, 29, 57, 58*). However, the attractant may be a host plant (*43, 97, 98, 99*), or animal (*22, 26*), or even a substance like petroleum oil (*8, 86*).

The task of isolating and identifying minute amounts of an active ingredient in a complex natural mixture is usually formidable. If we consider the natural sex lures, only two have been identified thus far and these only after 20 years of effort. However, new techniques promise to facilitate future exploits of this kind. With the many new forms of chromatography—adsorptive, partition, paper, gas, and thin-layer—infrared and ultraviolet spectroscopy, x-ray diffraction, mass spectrometry, and now nuclear magnetic resonance spectrometry, a wealth of information may be collected on minute amounts of material, and the material is frequently completely recoverable.

Even if we identify a lure, we are not always able to synthesize it; but if the synthesis defies us, we can still make simpler analogs and test them. On the basis of these results, the chemist may be able

to decide which grouping in the molecule imparts the attractive quality. This knowledge may help him prepare a lure more potent than the one found in nature.

Research on lures found in nature is important because it provides the attractive structures around which we can synthesize. We can have high hopes in the area of natural products, particularly since we are learning how to handle minute amounts of compound. When we unlock some of the secrets of the insect, we may find the means by which these pests may be destroyed.

Volume Screening

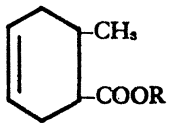
The second approach to finding insect attractants is by volume screening, or the synthetic approach. A large number of compounds are screened to find a lead, even a weak attractant; related compounds are then obtained or synthesized in an attempt to get a better lure. This procedure is the same as that used to find new insecticides, herbicides, pharmaceuticals, and a host of other physiologically active agents. To date our entomologists have run about 20,000 lure tests against about 15 insect species. (The results of these tests are being compiled for publication.) They have turned up potent and useful attractants for a number of economically important insect pests.

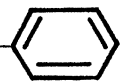
Chemicals reported to be lures are tried first; but shelf chemicals, intermediates or commercial chemicals, compounds from any source are all tried. The greater the volume, the greater the variety of chemicals subjected to test, the better are the chances of finding a good lure.

An illustration of this approach is the study conducted on the Mediterranean fruit fly (medfly) (*50*). Table I presents the olfactometer ratings of some of the synthetics that were tested on this insect. It is apparent that a small difference in R in these methylcyclohexenecarboxylic acid esters may make a big difference in attraction. The ethyl ester was the first attractive structure turned up among these compounds. In olfactometer tests, the first screen, it rated best; but it was not the best in field trials. A comparison of field and olfactometer ratings (Table II) shows that the *sec*-butyl ester, known as siglure (*sec*-butyl-6-methyl-3-cyclohexene-1-carboxylate), is the best compound in the field (*49*). It is evident that agreement between olfactometer and field results is not good. However, the olfactometer serves an important purpose, in that it permits the rapid screening of many compounds. By eliminating unattractive materials, only the attractive ones need be subjected to field tests, which are very time-consuming.

The final evaluation must be made in the field, preferably under actual conditions of use.

Table I. Olfactometer Ratings[a] of Siglure Analogs



R	*Olfactometer Rating*
—CH_3	48
—CH_2CH_3	122
—$CH_2CH_2CH_3$	96
—$CH(CH_3)_2$	100
—$CH_2CH_2CH_2CH_3$	71
—$CH_2CH(CH_3)_2$	99
—$CH(CH_3)CH_2CH_3$	87
—$CH(C_2H_5)CH_2CH_3$	83
—$CH_2CH{=}CH_2$	107
—$CH_2C{\equiv}CH$	83
—CH_2CH_2Cl	86
—$CH_2CH_2OCH_3$	0
—CH_2— 	0

[a] Isopropyl ester arbitrarily rated 100.

The first commercial lots of siglure proved to be less attractive than our laboratory batches. An investigation of this discrepancy disclosed that siglure can exist in a cis and trans form as shown in Figure 1 (*52*) and that *trans*-siglure is much more attractive than the cis isomer (*92*). Figure 1 is an oversimplification. A more accurate representation of the cyclohexene ring is shown in Figure 2. Not the upper boat form but the two interconvertible half-chair forms below represent the stable configuration (*14*). A means of making the all-trans product was worked out and an infrared method of analysis that would ensure procurement of the proper isomer was devised (*52*).

An investigation was also undertaken to determine why siglure epimerizes, our main purpose being to avoid formation of the less

Table II. Field and Olfactometer Ratings[a] of Siglure Analogs

Ester	*Field*	*Olfact.*
sec-Butyl (siglure)	279	87
1-Ethylpropyl	231	83
Isopropyl	100	100
Butyl	98	71
Propyl	58	96
Allyl	53	107
Isobutyl	48	99
Cyclopentyl	47	79
2-Propynyl	40	83
Ethyl	38	122
2-Chloroethyl	29	86

[a] Isopropyl ester arbitrarily rated 100.

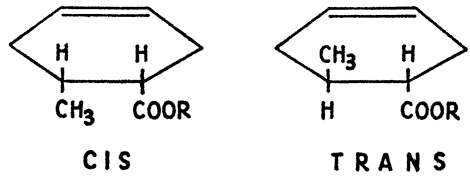


Figure 1. Siglure
R = sec-butyl

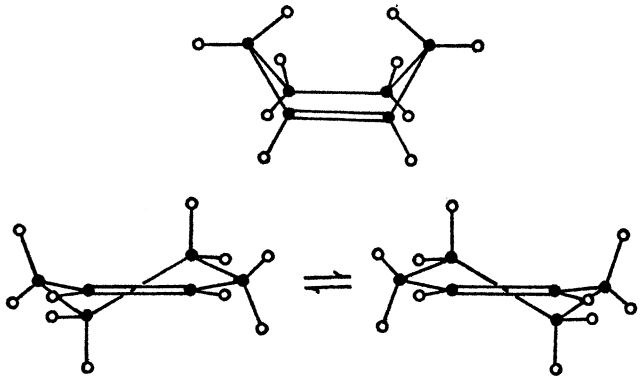


Figure 2. Cyclohexene structures

attractive isomer. In the course of this effort a means of making the hydrogen chloride adduct of siglure was discovered (*21*). There had been indications from earlier work that the hydrogen chloride adduct might be a good lure, but attempts to prepare the compound by conventional means had failed. When the compound proved to be much more attractive than siglure, 45 other derivatives were synthesized to find the most attractive analog.

The two best of the HCl-adduct esters are medlure and trimedlure, the *sec-* and *tert*-butyl esters of 4(or 5)-chloro-6-methylcyclohexanecarboxylic acid. Trimedlure (Figure 3) is the best attractant

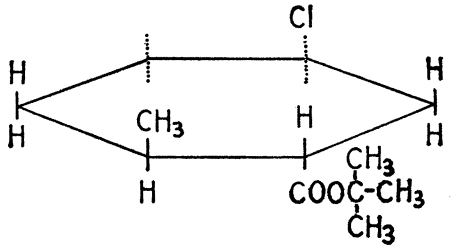


Figure 3. Trimedlure

known for the medfly. Now being commercially produced, it is the lure that helped eradicate the latest medfly infestation from Florida.

Trimedlure may exist in eight different isomeric forms, depending on which of the four dotted lines of Figure 3 the chlorine atom resides upon, and whether the methyl and ester groups are cis or trans. Our preparations of trimedlure are not pure compounds but mixtures of isomers. Trimedlure has been separated into two solid isomers and a liquid fraction. Tests at our Hawaii laboratory indicated that one of the solids and the liquid portion of trimedlure are attractive, but the other solid is not attractive (*91*). Apparently, stereoisomerism can play an important role in insect attraction.

Many of the compounds synthesized for medfly tests did not make the grade. A few general types of these structures are shown in Figure 4. Another group of compounds tested (dioxanes and dioxolanes) is shown in Figure 5. Practically every structure of this group that was not too complex was synthesized. Had not medlure and trimedlure been discovered, one of the dioxanes, better than siglure in field tests, might have supplanted siglure.

Although methyleugenol, the potent oriental fruit fly attractant, was discovered in 1912 by Howlett (*66, 67*), it was not put to effective use until about 1950 (*88, 89*). This lure and a few closely related

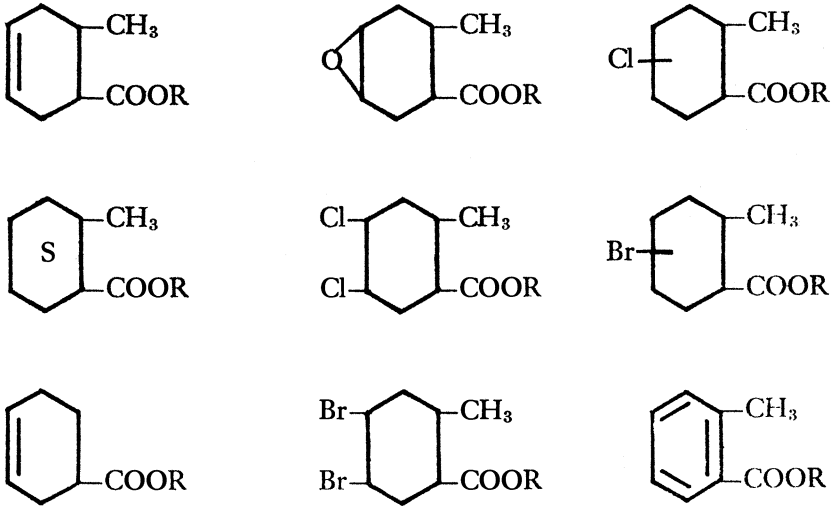


Figure 4. Structural types evaluated as medfly lures

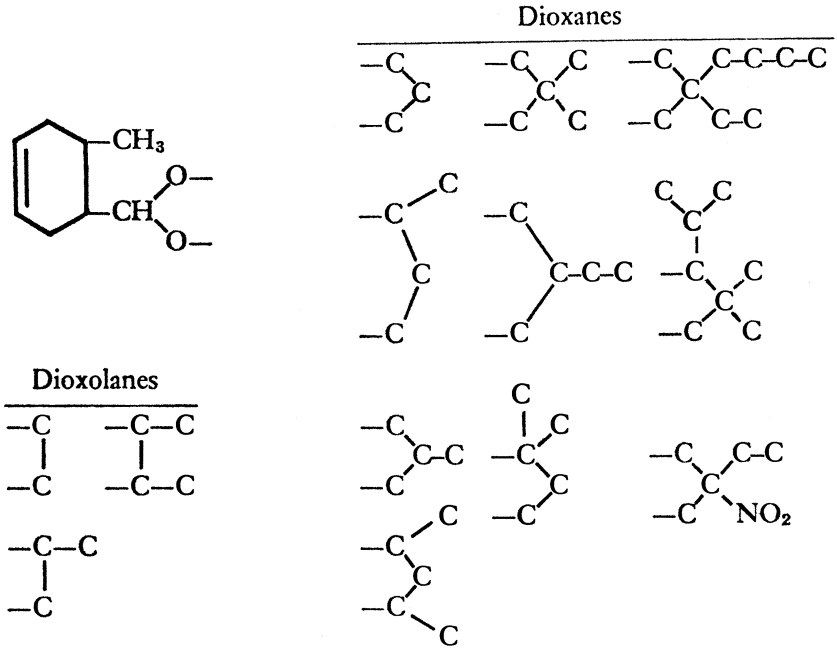


Figure 5. Dioxanes and dioxolanes tested as medfly lures.
Hydrogen atoms omitted for brevity

compounds that are much less active are shown in Figure 6. Our attempts to find an attractant better than methyleugenol for the oriental fruit fly have been unavailing.

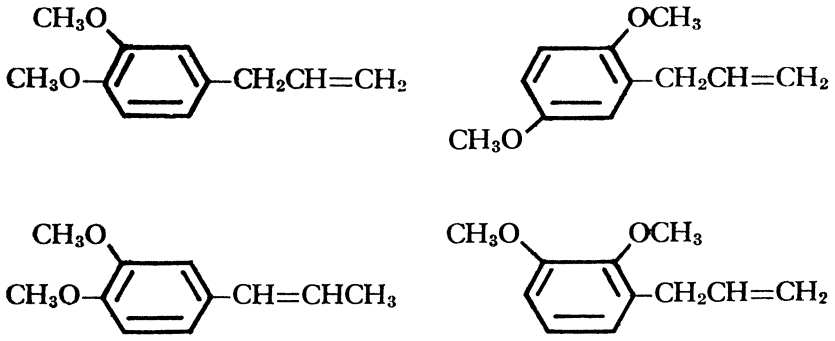


Figure 6. Methyleugenol (upper left) and analogs

Prior to the elucidation of the structure of the gypsy moth natural attractant, we tested about 2000 synthetics against this insect. Both the isolation and synthesis approaches were being explored. Several attractive compounds were turned up (*69*). Not so surprisingly, the attractive structures were later found to be similar to the natural attractant, in that they were 16-carbon-atom straight-chain compounds with a primary alcohol group, or at least a potential primary alcohol (epoxy) group.

A large number of compounds were tested as attractants of the melon fly (*Dacus cucurbitae* Coquillett). The first good attractants found for this species were benzylacetone and anisylacetone. Related compounds that were less effective are shown in Figure 7. The best melon fly attractant found thus far is the *p*-acetoxy derivative of benzylacetone (*9*, *19*), now known as cue-lure (shown in Table IV). Some idea of its activity compared with anisylacetone and other closely related compounds may be obtained from Table III. Cue-lure has a real advantage over anisylacetone, in that it attracts melon flies as soon as they emerge from pupation. Anisylacetone becomes attractive about 10 days after the fly's emergence. Cue-lure attracts *D. tryoni* (Froggatt), the Queensland fruit fly, in Australia (*104*); it also attracts *D. ochrosiae* Mallock (*91*). Cue-lure attracts several *Dacus* spp., but it does not attract *D. oleae* (Gmelin), the olive fruit fly. This serious pest is not at present found in the United States. A good lure is needed to make sure it does not enter and establish itself in this country.

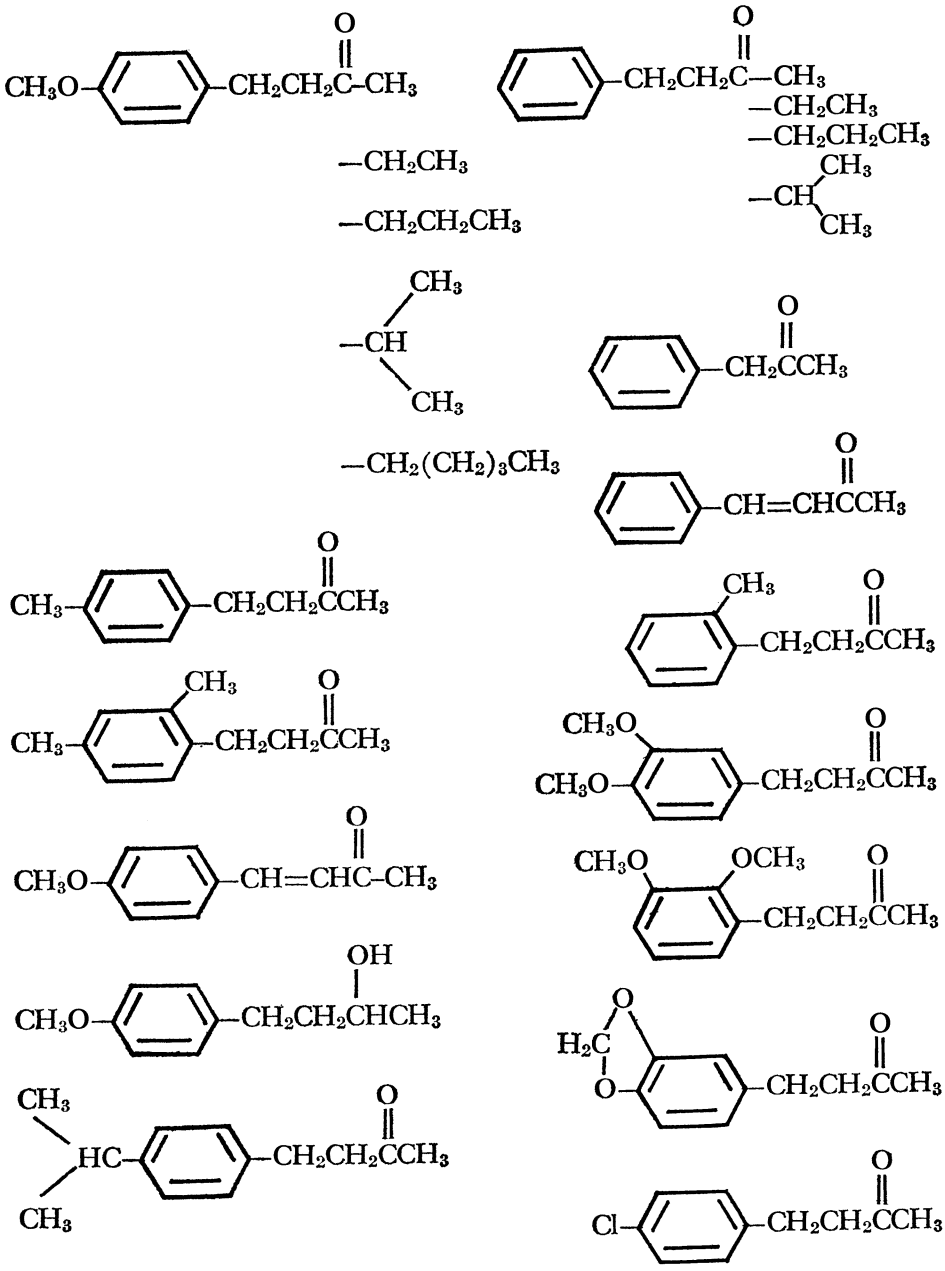


Figure 7. Benzylacetone and anisylacetone analogs tested as melon fly lures

Table III. Melon Flies Attracted by Cue-lure and Related Compounds (19)

Compound	*Flies Caught*
4-(*p*-Acetoxyphenyl)-2-butanone (cue-lure)	30,752
4-(*p*-Proprionoxyphenyl)-2-butanone	22,985
4-(*p*-Hydroxyphenyl)-2-butanone	15,574
4-(*p*-Butyryloxyphenyl)-2-butanone	12,508
4-(*p*-Isovaleryloxyphenyl)-2-butanone	6,894
Anisylacetone	2,408

All of the melon fly attractants have the same root—i.e., the 4-phenyl-2-butanone structure. Of the many derivatives prepared and tested by our group, the only ones that showed promise were *p*-substituted through an oxygen.

At Geneva, N. Y., several hundred compounds have been tested as candidate attractants for the European chafer [*Amphimallon majalis* (Raz.)], a serious pest of turf. The best compound was butyl sorbate; this was produced commercially and used for the first time in 1962. Although it is not much superior to the Java citronella oil-eugenol standard bait previously used (*96*), it has the advantage that it does not attract the Japanese beetle. With the old bait, Japanese beetles clogged the traps. Homologs of butyl sorbate are active.

Ammonia, amines, sulfides, and sometimes the fatty acids deserve special mention because many species, especially Diptera, have been reported to respond to these chemicals. They are products of decomposing organic matter and apparently represent a source of food to the insect. Many insects oviposit in the vicinity of these chemicals (*35*, *37*, *81*, *103*). Recent reports on ammoniacal lures include attraction of the cherry fruit fly [*Rhagoletis cingulata* (Loew)] (*45*), walnut husk fly (*R. completa* Cresson) (*12*), blow flies (*Calliphora* spp.) (*34*, *35*, *61*, *62*), olive fruit fly (*11*), and blueberry maggot [*R. pomonella* (Walsh)] (77). Sulfide-type odors attract blowflies (*44*, *47*) and eye gnats (*Hippelates* spp.) (*76*, *105*). Aliphatic fatty acids attract a variety of insects, including the oriental fruit moth (*46*), green June beetle [*Cotinis nitida* (L.)] (*79*), and several species of wireworms (*75*).

Lysine, reported to be a mosquito attractant (*23*, *24*), has shown attraction for the Mexican fruit fly [*Anestrepha ludens* (Loew)], the

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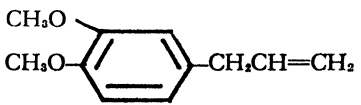
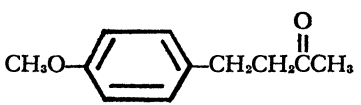
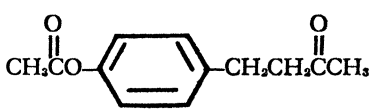
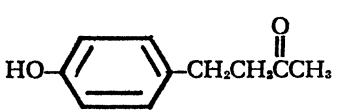
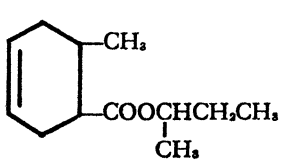
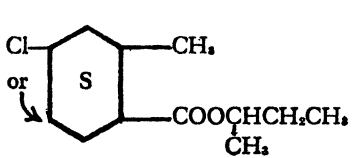
Table IV. Potent Attractants Made Synthetically

Common Name	*Structure*	*Species Attracted*	*Other Species Attracted*
Methyleugenol[a]		Oriental fruit fly (*Dacus dorsalis*) (*89*)	*Dacus umbrosus* (*33*)
Anisylacetone		Melon fly (*Dacus cucurbitae*) (*13*)	Queensland fruit fly (*D. tryoni*) (*104*) (*D. ochrosiae*) (*19*)
Cue-lure[a]		Melon fly (*Dacus cucurbitae*) (*19*)	Queensland fruit fly (*D. tryoni*) (*104*) (*D. ochrosiae*) (*19*)
——		Melon fly (*Dacus cucurbitae*) (*19*)	
Siglure		Mediterranean fruit fly (*Ceratitis capitata*) (*50*)	Walnut husk fly (*Rhagoletis completa*) (*12*)
Medlure		Mediterranean fruit fly (*Ceratitis capitata*) (*21*)	

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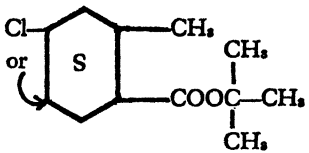
Trimedlure[a]		Mediterranean fruit fly (*Ceratitis capitata*) (*21*)	Natal fruit fly (*Pterandrus rosa*) (*48*)
Natural lure of gypsy moth[a]	*cis*— $CH_3(CH_2)_5CCH_2C{=}C(CH_3)_6CH_2OH$ (H, H, H; O; $CH_3{-}C{=}O$)	Gypsy moth (*Porthetria dispar*) (*70*)	— —
Gyplure	*cis*— $CH_3(CH_2)_5C{-}CH_2C{=}C(CH_3)_7CH_2OH$ (H, H, H; O; $CH_3{-}C{=}O$)	Gypsy moth (*Porthetria dispar*) (*30, 68*)	— —
Bombykol[a]	*trans*—10, *cis*—12— $CH_3CH_2CH_2CH{=}CH{-}CH{=}CH{-}(CH_2)_8CH_2OH$	Silkworm worth (*Bombyx mori*) (*28, 29*)	— —
Butyl sorbate[a]	$CH_3{-}CH{=}CH{-}CH{=}CH{-}COOC_4H_9$	European chafer (*Amphimallon majalis*)	— —
Methyl linolenate[a]	$CH_3{-}CH_2{-}CH{=}CH{-}CH_2{-}CH{=}CH{-}CH_2{-}CH{=}CH{-}(CH_2)_7{-}COO\,CH_3$	Bark beetles (*Ips typographus*) (*Hylurgops glabratus*) (*5, 6*)	— —

[a] Most effective lure for insect under "Species Attracted" column.

oriental fruit fly, the medfly, and the melon fly (*33*). Of the other amino acids tested, only glutamine was effective for the medfly. A recent report (*84*) states that certain mixtures of amino acids are attractive to mosquitoes but the individual amino acids are not. One would normally not expect amino acids to exhibit much attraction because of their low vapor pressure.

A summary of the most potent attractants that have been made synthetically is given in Table IV.

Conclusions

There is a real need for more and better insect attractants to increase the efficiency of detection and control operations. The need is great because increased international trade and traffic have increased the chances of introducing a foreign insect species into this country. A foreign pest in a new environment free of natural enemies could multiply explosively and menace our agricultural resources. The few available good attractants have already proved valuable and may be even more so when we learn how to use them better. Research directed toward finding and using good lures for injurious insects is definitely worthwhile.

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The Male Annihilation Technique in the Control of Fruit Flies

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In a test of the male annihilation technique involving all of the Bonin Islands in the Western Pacific, aerial distribution of Celotex wafers impregnated with methyleugenol containing 3% Dibrom at the rate of 70 or more per square mile along flight lines about 1/5 mile apart reduced the oriental fruit fly to only 28 males per 1000 trap days on Chichi Jima in 12 months. Indications are that use of the method on incipient infestations of the oriental fruit fly that may be found in continental United States will prevent their further development and spread, with eradication being a definite possibility. Male attractants for other tropical fruit flies are strong enough to warrant consideration as possible male annihilation agents.

The discovery since the turn of the century of a number of compounds strongly attractive to male fruit flies (Tephritidae) has suggested the possible utilitarian significance of male attractants. Ripley and Hepburn (*3*) considered that each male fly removed from the wild fly population by an attractant would represent one unmated female, since the two sexes were assumed to be present in approximately equal numbers and to mate only once. Use of the male attractant terpinyl

acetate in traps in citrus orchards in South Africa was advocated as a practical control measure. In 6 months, 120 glass traps baited with this attractant caught 58,500 male Natal fruit flies in a subtropical fruit orchard.

Methyleugenol

The outstanding attractiveness of methyleugenol to oriental fruit fly males (*Dacus dorsalis* Hendel) (*4*) suggested (*5*) that this lure might be useful in control of these flies. In a preliminary test in Opaeula Gulch in Hawaii 45 open-face box traps treated at 6-week intervals with a slurry of parathion-wettable powder and 4 to 8 ml. of methyleugenol removed 2,200,000 male oriental fruit flies during 13 months. Infestations in wild guavas averaged only 4.2 larvae per pound in the treated area compared with 31.6 to 39.6 in adjacent untreated gulches. This effect of removal of males was especially significant since a highly mobile fruit fly and limited isolation were involved as well as the adverse biological factor that each of the oriental fruit fly males had the capacity to supply sperm for fertilization of from two to five females.

In a 6-sq.-mile test of the male annihilation technique initiated in 1952 in Hawaii the methyleugenol, which oriental fruit flies consume avidly, was exposed on sugarcane fiberboard squares, distributed at the rate of about 40 per sq. mile. Pyrolan (3-methyl-1-phenyl-5-pyrazolyl dimethyl carbamate), a strong stomach poison, was added to the lure to kill the male flies. The poisoned feeding stations were retreated each month. Reductions in infestation averaged 74, 70, 82, and 60% at 700-, 1100-, 1500-, and 1900-foot elevations in the treated area, again with only partial isolation. Infestations rapidly increased after termination of the experiment.

A small experiment in a semi-isolated guava thicket near Kilauea volcano on Hawaii failed to give significant control because of immigrating fertile females.

The Hawaii tests permitted the conclusions (*5*) that the male annihilation technique is best adapted for use on whole or well-isolated populations. Its possible usefulness to combat incipient oriental fruit fly infestations whenever they may occur in continental United States was also suggested by this research.

Tests in Bonin Islands

Opportunity for a large-area test of the oriental fruit fly male annihilation method on an entire isolated infestation presented itself

in 1958 when the U. S. Navy reported excessive damage to tomatoes and other crops on Chichi Jima in the Bonin Islands. A cooperative eradication experiment involving aerial distribution of small, cane fiberboard squares impregnated with methyleugenol, with naled (1, 2-dibromo-2, 2-dichloroethyl dimethyl phosphate) as the toxicant, was promptly arranged under the direction of L. F. Steiner. Special poisoned methyleugenol feeding stations serviced from the ground were used in populated areas on Chichi Jima, to avoid possible injury to inhabitants from falling squares.

Aerial distribution of the treated squares was by a U. S. Navy flying boat. The squares were applied at the rate of 70 to 80 per square mile, or twice these numbers when unusually long intervals between treatments became necessary because of typhoons or other operational difficulties. Flight lines over the widely scattered islands totaled more than 800 miles, and approximately 5 hours of flying was required for each treatment.

Within a few months after treatments began in September 1960, a marked decline occurred in the wild fly population, as measured by traps. Before treatments catches on Chichi Jima averaged 47,400 oriental fruit flies per 1000 trap days in March 1958 and 13,700 in January 1960. Only 170 flies per 1000 trap days were caught in March and 28 flies per 1000 trap days in September 1961. In March, April, May, June, and July 1962, catches were 194, 114, 204, 81, and 34, respectively. In early 1962 harvested tomatoes on Chichi Jima were essentially free of infestation for the first time in several years.

Treatments were terminated in August 1962 because of further difficulties in maintaining treatment schedules.

Failure to eradicate the oriental fruit fly in the Bonin Islands was probably due to frequent interruptions in treatment schedules, but this explanation is not certain. Since the methyleugenol cane-fiberboard squares have considerable residual effectiveness, comparatively few of the intervals between treatments were long enough to relieve the flies entirely from treatment effect. There has been no evidence that the flies developed resistance to the attractiveness of methyleugenol during selection by this lure during more than a dozen generations. The possibility that a small portion of the males in any wild fly population may be nonresponsive to methyleugenol remains to be determined.

The high degree of effectiveness of the oriental fruit fly male annihilation technique in reducing the number of flies in the Bonin Islands to near extermination levels has provided assurance that use of this lure in areas in California where three oriental fruit flies were

caught in 1959 was a sound control procedure. With prompt elimination of males an incipient infestation would have had little, if any, opportunity to develop or spread.

Discovery of other strong male lures for the Mediterranean fruit fly [*Ceratitis capitata* (Weidemann)], such as angelica seed oil (*6*) and trimedlure (*2*), and for the melon fly (*Dacus cucurbitae* Coquillett) and Queensland fruit fly [*Dacus tryoni* (Froggatt)], such as cuelure (*1*), has stimulated interest in application of the male annihilation method to other fruit fly problems. Male annihilation experiments on the Mediterranean fruit fly in Central America, on the melon fly in Hawaii, and on the Queensland fruit fly in Australia are now in progress or under consideration. Even though the new lures do not possess the overwhelming attractiveness of methyleugenol to the oriental fruit fly, their liberal use may result in removal of enough of the total sperm mass to retard population growth, especially during seasons when adverse weather or host scarcity detracts heavily from normal reproductive potential.

An important prerequisite for successful application of the fruit fly male annihilation technique is an attractant compound or mixture powerful enough to reduce the male population to a level well below that required for efficient fertilization of the total egg mass. An appetite for the lure by the males after they are attracted to it is also essential for most effective application. With the overabundance of sperm production and mating capacity possessed by the oriental fruit fly, removal of from 50 to 80% of all males may be necessary before there will be an appreciable effect on egg hatch. Use of strong female lures in combination with the male elimination method would enhance effectiveness but, unfortunately, materials discovered thus far in intensive screening programs in Hawaii and Mexico have not possessed attractant qualities comparable to those of the male lures.

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4

Chemosterilants as a Potential Weapon for Insect Control

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Chemosterilization offers promise of inducing sterility in a high proportion of a natural population, without the necessity of rearing and releasing large numbers of insects, which might be injurious. Chemosterilization should be effective in species that spread a moderate distance from their breeding sites before mating. The chemosterilants now available are not highly stable, which might reduce residue problems, but compounds with greater stability are desirable. Although the available compounds are often toxic to mammals, the hazards connected with their use have not been fully established. Research to develop attractants and other highly selective methods of application will add greatly to the potential of these compounds for insect control.

Prospects for the control or eradication of insects through the use of chemosterilants are based on the success of programs for the eradication of the screwworm [*Cochliomyia hominivorax* (Coquerel)] by the release of males sterilized by gamma radiation and on the encouraging results obtained in exploratory studies with the chemosterilants themselves. The success in eradicating the screwworm, first on the island of Curacao (*1*) and then throughout its range in the

southeastern United States (*13*, *20*), established the practicability of Knipling's concept of the sterile-male technique (*10*). This concept involves the use of insects for the destruction of their own species through the induction of sterility in a large proportion of the males by utilization of their mating behavior to reach female insects that would not be affected by the usual insecticide techniques.

The success of the screwworm program also intensified interest in the possible use of chemicals in adaptations or extensions of the sterility approach to insect control. Knipling (*9*) discussed the possibilities of insect control through the use of sterile males and also (*12*) suggested the theoretical advantages of the use of chemicals to induce sterility in a large segment of the natural population. Shortly thereafter, the demonstration that both male and female houseflies (*Musca domestica* L.) could be sterilized by radiomimetic chemicals (*15*, *16*, *19*) directed attention to the possible advantages of chemosterilants over radiation in applying the sterile-male technique.

Perhaps it would be well, at this point, to define what we mean by the term chemosterilant, since this is a new word. A chemosterilant is a chemical capable of causing sexual sterility—that is, failure to reproduce—in insects or other organisms.

Insect chemosterilants may act in several ways. They may cause the insects to fail to produce ova or sperm; antimetabolites, when they are also chemosterilants, act in this way. Compounds that cause the death of sperm and ova after they have been produced would also be considered chemosterilants, but I do not know of any compounds of this type which are being considered for use in insect control. A third type of action, and the one in which we are most interested at the present time, is that shown by the radiomimetic compounds. These compounds apparently injure the chromatin, or genetic material, in the sperm and ova so severely that, although they remain alive and the sperm retain full motility, the zygotes, if formed, do not complete development into mature progeny. This type of action is desired because the males sterilized in this manner compete readily with normal males for the available females and transfer motile sperm to the spermathecae of the females, with the result that the mating requirements of the females are satisfied to the same extent as in a mating with a normal male.

Chemosterilants might be used in two basic ways—as a substitute for radiation to sterilize insects that had been reared for release in large numbers or as a means of inducing sterility in a large proportion of the natural population, thus avoiding the necessity for rearing and

releasing large numbers of insects. For the former purpose—rearing and release of overwhelming numbers of sterile insects—chemicals may prove to be more economical than radiation or to cause less general injury in treated males, and they almost certainly will permit greater mobility and dispersion of the rearing and sterilization facilities. However, there are many species of insects which could not readily be reared and released in overwhelming numbers, either because the species is not adaptable to laboratory rearing, since the numbers required to overwhelm the normal population would be too enormous, or because the released insects would themselves be dangerous, destructive, or annoying. To control or eradicate such species it would be highly advantageous to be able to induce sterility chemically in a large proportion of the natural population in such a way that the sterile males would mate with, and thus render infertile, the females that escaped the sterilizing chemical.

Advantages of Chemosterilants

You may wonder why, if we are to treat an insect population with a chemical, we expect it to be more advantageous to sterilize the insects than to kill them with an insecticide. Knipling (*10*, *11*, *12*) has discussed in detail the mechanics by which the induction of sterility in a large proportion of the natural population would result in a constant reduction of the population greater than that which would be obtained by insecticidal action of similar intensity. The principal advantage of a chemosterilant over an insecticide may be explained briefly by assuming that we have a method of application that will reach 90% of the insects in the population. If we kill 90 males out of 100, and 90 females out of 100, the 10 females that escape the treatment will mate with the 10 males that escape, and there will be 10 fertile females to produce the next generation. However, if we reach the same 90% of the insects with a chemosterilant, 90 females out of 100 will be sterilized, and not reproduce, and in addition the 10 females that escaped treatment will be subjected to mating competition by 90 sterile males as well as 10 normal males. From this ratio we would expect nine of the normal females to mate with sterile males, and therefore fail to reproduce, and only one normal female to mate with a normal male and thus be available to produce the next generation.

In many species of insects the females mate only once, but even species with females that mate repeatedly might be susceptible to control with chemosterilants.

One would expect the chemosterilant technique to be effective in the control or eradication of most species in which the males and females that are produced over a sizable area mix thoroughly before mating takes place. Chemosterilants would lose much of their advantage over toxicants in the control of species in which both sexes remain near the site of emergence until after mating has taken place.

Chemosterilants may be effective when given in the food of insects, when applied topically or used as residues, and when added to the larval medium, although not every compound is effective by all the various methods of administration.

A wide variety of species has been shown to be susceptible to chemosterilization. Two reports on the sterilization of houseflies have been mentioned. Others include those by Konecky and Mitlin (*14*), Mitlin (*21*), Mitlin *et al.* (*21*, *22*), Borkovec (*2*), Plapp *et al.* (*27*), Kilgore and Painter (*8*), LaBrecque, Meifert, and Smith (*17*, *18*), Morgan and LaBrecque (*24*), and Piquett and Keller (*26*). Reports have also been published on the chemosterilization of mosquitoes (*27*, *29*, *30*), the screwworm fly (*3*, *4*), the Mexican fruit fly [*Anastrepha ludens* (Loew)] (*28*), and *Drosophila melanogaster* Meigen (*5*, *6*, 7, *25*). In addition, studies as yet unpublished by various investigators in the Entomology Research Division have shown that sterility can be induced by chemicals in other flies, moths, beetles, cockroaches, mites, and nematodes.

Two of the past uses of the compounds now being investigated as chemosterilants are of interest to us. Some of the compounds have been used in cancer chemotherapy to support or replace radiation. The experience gained in this way shows that intravenous injection or oral administration of very small quantities may affect the blood-producing systems. On the other hand, a few of the compounds have been used in sizable quantities in the fabric industry without disastrous effect among the workmen.

Toxicity

More information must be available on the toxicity of the promising compounds before the full range of possible methods of application for the control of various species can be determined. Chemosterilants can certainly be used in the control of some species to sterilize reared insects for release among natural populations. No doubt they will also prove acceptable for use with various baits and attractants, which would bring them into contact only with the species

concerned. The demonstration of a somewhat greater degree of safety would permit their use in more general, but still highly selective, methods of application. Their most efficient use will almost certainly require more detailed biological information regarding the species concerned than was required with insecticides.

Conclusions

As new compounds are developed and brought into use, we may anticipate a wider range of safety and a correspondingly greater number of practical uses. Greater chemical stability would also increase the possible methods of use, but the instability of some of the promising compounds might in itself be an advantage for methods of application in which durable residues might be hazardous.

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5

Chemosterilants for the Control of Houseflies

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The chemical induction of sterility in insects has been widely investigated, and has shown great promise in the control of species not particularly adapted to gamma irradiation techniques. Research conducted against houseflies (Musca domestica L.) has revealed that over 40 chemicals, principally alkylating agents and antimetabolites, can induce sterility. Many of these sterilized one or both sexes by ingestion with the food or penetration of the cuticle on contact with a treated surface. The sterility was usually irreversible and apparently accompanied by little damage to somatic tissue, since sterilized flies competed successfully with unsterilized flies in mating aggressiveness. In preliminary field tests, chemosterilants, periodically distributed as baits in dumps and poultry houses, meaningfully reduced housefly fertility, with a corresponding decrease in populations. However, flies from other breeding sites infiltrated throughout the tests and prevented elimination of the insect.

Housefly infestations have been controlled primarily by insecticidal measures, but with the development of resistance to the majority of available insecticides, new measures have been sought. Preliminary

investigations were directed primarily toward altering the insect's metabolic activity, genetic make-up, or reproductive potential, by any means other than insecticidal. Sexual sterilization was most promising and efforts were directed toward this approach.

Initially, sterilization by gamma irradiation was seriously considered, but the addition of vast numbers of an insect such as the fly to an existing population would definitely increase the disease and nuisance potential; moreover, the expense of rearing, sterilizing, and releasing enormous numbers of insects into an existing population would render the cost of a control program prohibitive. To circumvent these problems and yet utilize the sexual sterilization principle, research was initiated to find out whether chemicals might induce sterility in insects. It was reasoned that if a chemosterilant could be found and made available to the fly, sterility could be induced in a high proportion of the natural population, rendering the cost of a control program against this insect feasible.

Over the past few years more than 2000 chemicals have been screened at the Orlando, Fla., laboratory as potential chemosterilants for houseflies (*Musca domestica* L.). At present two groups of compounds—antimetabolites and alkylating agents—have shown the most promise, and comprise the majority of the 40 chemicals producing sterility in houseflies (*4*, *5*, 7).

These two groups of compounds have different mechanisms for inducing sterility. The antimetabolites have been considered as compounds wherein a metabolite essential to cell development has been changed in one or several ways and, when introduced into an animal, will elicit signs associated with a specific lack of the metabolite (*10*, *11*). Some of the more promising are methotrexate, aminopterin (*N*-{*p*-{[(2,4-diamino-6-pteridyl)methyl]amino}benzoyl}glutamic acid), 5-fluorouracil, and 5-fluoro-orotic acid.

The alkylating agents have been commonly referred to as radiomimetic compounds. These agents replace hydrogen in fundamental genetic material with an alkyl group that results in an effect similar to irradiation. They are highly effective in producing mitotic disturbances or nucleotoxic conditions, particularly in tissues where cell multiplication takes place at a high rate (*1*, *2*). Representative compounds of this group are tepa [tris(1-aziridinyl)phosphine oxide], metepa [tris(2-methyl-1-aziridinyl)phosphine oxide], and apholate [2, 2, 4, 4, 6, 6-hexahydro-2, 2, 4, 4, 6, 6-hexakis (1-aziridinyl)-1, 3, 5, 2, 4, 6-triazatriphosphorine].

Screening Program

In the screening program, the initial tests are made by offering the chemosterilant to a group of houseflies of mixed sexes in the food (1 part of powdered egg, 6 parts of nonfat dried milk, and 6 parts of sugar) at 0.1 and 1.0% concentrations. Seven to 9 days after this treated diet has been offered to newly emerged flies, oviposition medium (aged CSMA larval medium) is introduced into the cage for 24 hours. The medium is then checked for eggs and their viability determined 2 days later.

Aminopterin, methotrexate, 5-fluorouracil, 5-fluoro-orotic acid, tepa, metepa, and apholate all sterilized at the higher concentration, but only the antimetabolites sterilized at the lower concentration. Whenever a compound induces sterility, a second series of tests is run to determine the range of effective concentrations. Some antimetabolites sterilized at concentrations as low as 0.0025%, whereas the minimum for alkylating agents was 0.25%. In the third series of tests, the specific sex sterilized is determined. The chemosterilants are presented in the food at sterilizing concentrations to individual sexes of flies. These flies are then mated with normal flies of the opposite sex. All the chemicals mentioned above sterilized the females, but aminopterin and methotrexate failed to sterilize the male flies, and 5-fluorouracil produced male sterility in only a few tests. Tepa, 5-fluoro-orotic acid, metepa, and apholate, when included in the food, regularly sterilized both sexes. Because of the inability of aminopterin, methotrexate, and 5-fluorouracil to sterilize males, these compounds were not considered further.

Tepa, metepa, apholate, and 5-fluoro-orotic acid were then evaluated as contact sterilants. In these tests individual sexes of houseflies were exposed to residues of 10 to 250 mg. per sq. foot of the chemosterilant on glass. After exposure for 2 to 4 hours, the flies were mated with virgin flies. Sterility was obtained in both sexes with tepa and metepa, but apholate and 5-fluoro-orotic acid were ineffective as residues.

Tepa, metepa, and apholate were then further evaluated to determine the duration of the sterility induced when they were offered in the diet. When females were given food treated with apholate for the first 5 days of the adult stage, then given normal food and mated with fertile males, their sterility was irreversible, since all eggs laid for 1 month were nonviable. The effect was equally striking in the males. When males, upon eclosion, were fed the same diet for the

same period of time and then mated with new groups of virgin females at weekly intervals for 1 month, all eggs laid by these normal females were nonviable. Furthermore, upon dissection of the testes of the males, and the spermathecae of the females, it was noted that the sperm were motile in these organs throughout the test period.

Metepa, as well as apholate, when fed to the flies for intervals ranging from 3 to 5 days, apparently had little effect on longevity, since little mortality was noted during the 3- to 4-week test period. Further analysis of longevity, as determined by survivorship curves, has indicated that flies fed continuously on food treated with either 1 or 0.5% of metepa or apholate had a lifespan approximately half that of flies fed normal food. Fortunately, since the majority of mating occurs within the first week of the adult life cycle, sexual capabilites are not lost. This was further substantiated by experiments on mating competitiveness (*8*). Male flies, 5 to 6 days old, sterilized with apholate, and males fed normal food were introduced into cages and allowed to become oriented. Shortly afterward, virgin females were introduced into the cage. The effect of the sterile males in the population was measured by the per cent sterility among all the eggs laid by all females. When treated males, normal males, and normal females were present at ratios of 1:1:1, 1:1:2, 2:1:1, 3:1:1, 5:1:1, and 10:1:1, the proportion of sterile eggs equaled or exceeded that expected from the proportion of sterile males present.

Field Experiments

After these highly promising laboratory results were obtained, small field experiments were initiated to evaluate the potential for housefly control, and to develop techniques for judging the success of such experiments (*3, 6, 9*). Three fairly isolated sites were chosen: a dump on Bahia Honda Key (one of the Florida Keys); a dump on Pine Island, a few miles northwest of Fort Myers, Fla.; and a poultry house in the suburbs of Orlando, Fla. A bait of granulated corn meal, sugar, powdered milk, and powdered egg was the carrier used for application of the chemosterilants. On Bahia Honda, the bait included 0.5% of tepa and was broadcast at weekly intervals; on Pine Island it included 0.75% of apholate and initially was applied at weekly intervals, but because of heavy daily rains the gross weekly amount was divided by 5 and applied daily. At the poultry house 0.5% metepa baits were applied. Initially applications were made weekly; later, semiweekly.

In the first experiment (with 0.5% tepa) housefly populations, as evaluated by the number of flies landing on a grid (18 × 18 inches), decreased from 47 to 0 within 4 weeks after initial treatment, with a corresponding decrease in egg-mass viability from 100 to 10%. Further egg-viability evaluations could not be conducted because of the lack of flies.

In the second experiment with apholate at 0.75% in the fly food, grid counts were reduced from 68 to <1 within 10 weeks, whereas the untreated check counts ranged from 97 to >200 throughout the test period. Within 4 to 8 weeks egg fertility of females captured at the site of treatment was reduced from 81% to 3 to 10%, whereas females from the untreated area produced eggs with 80 to 99% viability. At the poultry house where the 0.5% metepa baits were applied, housefly abundance decreased sharply, but the most striking evidence of the activity of the chemosterilant was in the viability of eggs collected from the females, since the hatching rate was below 10% during most of the time that an effective bait was being used. The treated areas were subject to reinfestation, which to some degree adversely influenced the decline of housefly abundance.

The results of these experiments, although not conclusive in themselves, indicate that chemosterilants might be used to eliminate an insect from an extensive area.

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Aziridine Chemosterilants
Sulfur-Containing Aziridines

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The most important group of chemically related insect chemosterilants are the derivatives of aziridine. 1,1'-Sulfinyldiaziridine and its 2-methyl derivative were prepared by reaction of thionyl chloride with ethylenimine or 2-methylaziridine. Similarly substituted dithiodiaziridines were obtained from the reaction of sulfur monochloride with the appropriate aziridine. Permanganate oxidation of sulfinylaziridines gave the corresponding sulfonyldiaziridines. The comparison of insect-sterilizing activity of the sulfur-containing aziridines as well as several phosphorus-containing aziridines indicates that methyl substitution on the aziridine ring carbon lowers the activity of the compound.

In the past three years several thousand compounds have been tested for their insect-sterilizing properties by the Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture. The chemicals, obtained from industrial sources and academic and government laboratories, or synthesized by the Pesticide Chemicals Research Branch chemists, were tested by the entomologists in field stations at Orlando, Fla., toward houseflies (*Musca domestica* L.), at Kerrville, Tex., toward screwworm flies [*Cochliomyia hominivorax*

(Coquerel)], and at Mexico City, Mexico, toward Mexican fruit flies [*Anastrepha ludens* (Loew)]. In general, it was attempted to select compounds containing different functional groups, ring systems, and their combinations in order to cover the entire field of organic and inorganic structural types. It was obvious from the start that no complete coverage could be achieved and many attempts were made to discover some indications of structure-activity relationship, in order to minimize the number of tested compounds. The empirical correlation between antitumor or carcinostatic activity (7) and insect sterilization activity was, and still is, a most useful guide for the selection of candidate chemosterilants (*4*).

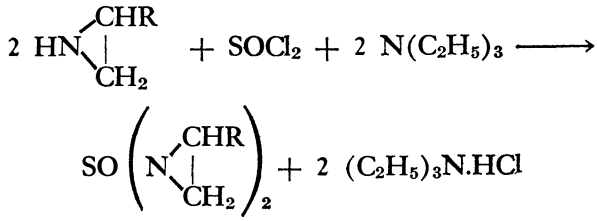
Although the most numerous group of chemosterilants are the derivatives of aziridines, a large number of miscellaneous compounds were found to have some activity at least in one insect species (*5*, *6*, *8*, *9*, *11*, *12*, *14*, *15*). Nevertheless, when chemosterilants are considered as possible insect-control agents, the aziridines are still on the top of the list, mainly because of their male-sterilizing activity which is often coupled with a female-sterilizing activity. Although in most instances the male sterilization is indispensable, the female sterilization is also of importance (*10*).

One of the distinguishing features of aziridines is their high reactivity with a variety of compounds, primarily with those containing an active hydrogen (*2*). Protonation of the aziridine nitrogen is critical in the ring opening reaction (alkylation) and it follows that under acidic conditions the decomposition and a consequent biological deactivation of aziridines proceed rather rapidly. Preliminary results of experiments conducted in our laboratory showed that tepa [tris(1-aziridinyl)phosphine oxide], which decomposed to a 50% extent within 3 to 4 weeks in aqueous solution at 25°, decomposed to the same extent within a few minutes in a solution buffered to pH 4. Similar results concerning metepa [tris(2-methyl-1-aziridinyl)phosphine oxide] were reported recently (*13*). This property has important practical consequences.

Honey, or dehydrated orange juice, which was used in some phases of our screening program as carrier for chemosterilants, yielded often inconsistent and contradictory results when aziridines were tested. The buffering capacity of honey being low, the errors were usually negligible, but the high acid content of orange juice led to misleading results with otherwise active compounds. Neutralization of the medium, or its replacement with a neutral carrier—e.g., sugar sirup—eliminated these discrepancies.

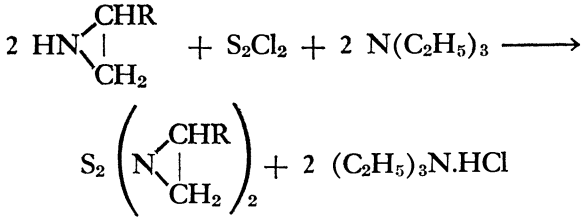
The biological activity and the reactivity of aziridines vary considerably with the number and properties of the ring substituents. This paper describes the synthesis and biological activity of some sulfur-containing aziridines which are somewhat similar to phosphorus-containing chemosterilants tepa and metepa.

The amides of sulfurous and thiosulfurous acids were readily obtained by a procedure similar to that used by Bestian to prepare tepa (*2*).



$R = H$ or CH_3

Thus thionyl chloride reacted with either ethylenimine or 2-methylaziridine in the presence of triethylamine, which neutralized the hydrochloric acid produced by the reaction. The resulting 1,1′-sulfinyldiaziridine and its 2-methyl analog were readily purified by distillation at reduced pressure. Similarly 1,1′-dithiodiaziridine and 1,1′-dithiobis(2-methylaziridine) were obtained by reaction of sulfur monochloride with the appropriate aziridine.

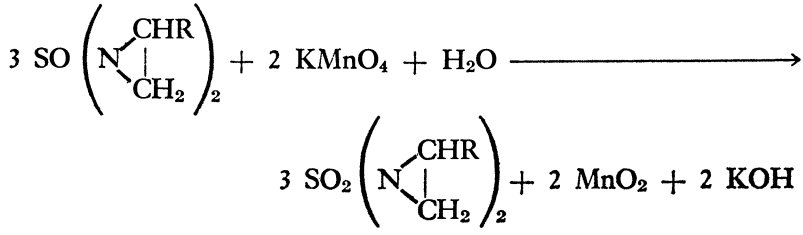


$R = H$ or CH_3

Yields in these four reactions ranged from 45 to 70%. Comparable results were obtained when a sodium hydroxide solution was used instead of triethylamine.

The sulfonyl aziridines could not be prepared by this method, even though the open-chain analog, tetramethyl sulfamide, is obtained on reaction of sulfuryl chloride with dimethylamine (*1*). Polymerization apparently occurs, since no distillable product was obtained from the

reaction of sulfuryl chloride with ethylenimine. The desired sulfonyl aziridines were readily obtained by the permanganate oxidation of the previously prepared sulfinyl aziridines.



$R = H$ or CH_3

However, only the 1,1′-sulfonylbis(2-methylaziridine) could be obtained for testing, because the sulfonyldiaziridine was too unstable to permit isolation or purification.

The aziridine monosulfides were obtained only in poor yield by the reaction of sulfur dichloride with an aziridine. This result was surprising, since dimethylamine reacts readily with sulfur dichloride to give about a 50% yield of bis(dimethylamine) sulfide (*3*). Only a small quantity of distillate was obtained and this appeared to consist of about half 1,1′-dithiodiaziridine. The presence of this material can be explained by the normal equilibrium that exists between sulfur dichloride and sulfur monochloride.

$$2\ SCl_2 \rightleftarrows S_2Cl_2 + Cl_2$$

The products could not be separated because of the small difference in boiling points and the sensitivity of the compounds to heat, which prevented the use of an efficient fractionating column.

Chemosterilant Results. The five aziridines were tested for chemosterilant activity against the housefly, the Mexican fruit fly, and the screwworm fly. For all tests the candidate chemosterilant was mixed with the insects' food and given to the newly emerged or young insects, and the eggs later laid by the insects were collected and given opportunity to hatch. The results are presented in Table I. The usual test feeding level for the housefly and the screwworm was 1% and for the Mexican fruit fly 2%, unless otherwise noted.

As has been observed when testing other chemosterilants, none of the compounds was highly active in all tests. The most effective compound was 1,1′-sulfinyldiaziridine, which completely inhibited hatch of screwworm and fruit fly eggs and caused considerable reduction in

Table I. Chemosterilant Activity

Compound	*Per Cent Hatch*		
	Screwworm	*Housefly*	*Mexican fruit fly*
1,1′-Sulfinyldiaziridine	0	20	0
1,1′-Sulfinylbis(2-methylaziridine)	2	66	8
1,1′-Dithiodiaziridine	0	8	100
	1 [a]		
1,1′-Dithiobis(2-methylaziridine)	Toxic	65	85
1,1′-Sulfonylbis(2-methylaziridine)	Toxic	0	Toxic
	No eggs [a]	25 [a]	

[a] 0.5% in food.

hatch of housefly eggs. 1,1′-Sulfinylbis(2-methylaziridine) was somewhat less effective in all tests. In contrast to these two compounds, 1,1′-dithiodiaziridine was completely ineffective in fruit fly tests but gave good results with the screwworm and housefly. 1,1′-Sulfonylbis(2-methylaziridine) was toxic to all insects except the housefly at the normal feeding levels but was effective at lower concentrations.

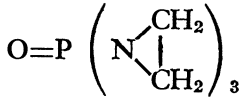
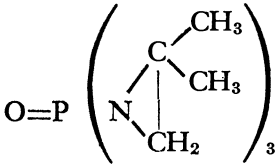
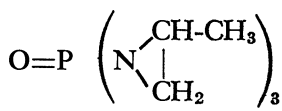
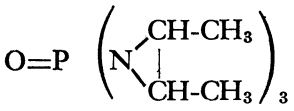
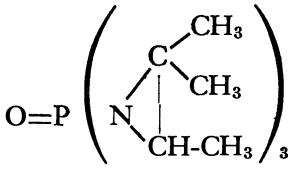
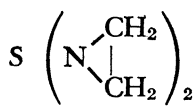
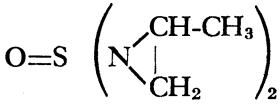
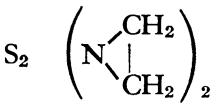
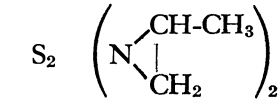
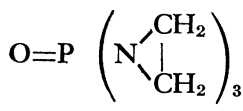
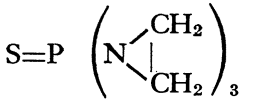
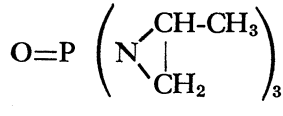
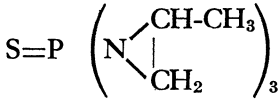
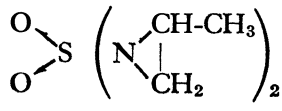
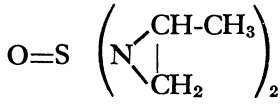
Comparison of the sulfinyldiaziridine and dithiodiaziridine with their 2-methyl homologs discloses a marked difference in activity.

This effect is in agreement with a previously reported observation (*4*) that *C*-alkyl aziridines were generally less active as insect sterilants than the unsubstituted aziridines. Several methyl homologs of tepa and thiotepa [tris(1-aziridinyl)phosphine sulfide] were tested on houseflies (Table II).

The first group of compounds in Table II shows clearly the deactivating effect of the methyl substituents. The introduction of one methyl group in a highly active compound (tepa) did not decrease the activity significantly, but two or even three methyl groups produced inactive or only slightly active compounds. In the second part of Table II the difference between the two columns lies in the changes in electropositivity of the central atom to which the aziridinyl groups are attached. The results indicate that the compounds with a more electropositive central atom (left column) are more active than analogous compounds with a less electropositive central atom (right column).

Mammalian Toxicity. Acute toxicities of these compounds to mice were obtained by intravenous injection of an aqueous solution. These tests were of a preliminary nature and the values given are only to indicate a range rather than an absolute value. 1,1′-Dithiodiaziridine

Table II. Relative Sterilizing Activity of Substituted Aziridines

Active	*Activity Lowered or Absent*
	 [a]
 [a]	 [a]
	 [a]
	
	
	
 [a]	 [a]
	

[a] Samples kindly supplied by the Interchemical Corp., New York, N. Y.

was the most toxic, with an LD_{50} of less than 6 mg. per kg., and 1,1′-sulfinyldiaziridine next, with a value of 20 mg. per kg. The 2-methyl-substituted homologs were both roughly one tenth as toxic and 1,1′-sulfonylbis(2-methylaziridine) had an LD_{50} of 200 mg. per kg. These compounds are thus all considerably more toxic than tepa or metepa, whose LD_{50}'s by this method were greater than 300 mg. per kg., but nothing is known of their chronic toxicity effects.

Experimental

1,1′-Sulfinyldiaziridine. To a cold solution of 51.6 grams (1.2 moles) of ethylenimine and 106 grams (1.5 moles) of triethylamine in 500 ml. of dry benzene was added dropwise 59.6 grams (0.5 mole) of thionyl chloride in 300 ml. of benzene. The temperature was kept at 5° during the addition; the reaction mixture was then brought to room temperature and allowed to stand overnight. After the precipitated triethylamine hydrochloride was removed by filtration, distillation of the filtrate gave 32 grams (48%) of 1,1′-sulfinyldiaziridine, b.p. 49-53° (0.1 mm.); n_D^{25} 1.5130.

Calculated for $C_4H_8N_2OS$: C, 36.34; H, 6.10; S, 24.15. Found: C, 36.46; H, 5.95; S, 24.25.

In an alternate procedure 59.5 grams (0.5 mole) of thionyl chloride in 200 ml. of methylene chloride was added to a cooled mixture of 64.4 grams (1.5 moles) of ethylenimine in 200 ml. of methylene chloride and 44.0 grams (1.1 moles) of sodium hydroxide in 100 ml. of water. Distillation of the dried organic phase gave 41 grams (62%) of 1,1′-sulfinyldiaziridine, b.p. 50-55° (0.2 mm.); n_D^{25} 1.5136.

1,1′-Sulfinylbis(2-methylaziridine). The procedure was the same as for sulfinyldiaziridine except that 2-methylaziridine reacted with the thionyl chloride. From 59.6 grams (0.5 mole) of thionyl chloride, 54 grams (68%) of 1,1′-sulfinylbis(2-methylaziridine), b.p. 52-6° (0.1 mm.), was obtained; n_D^{25} 1.4854.

Calculated for $C_6H_{12}N_2OS$: C, 44.97; H, 7.55; S, 20.01. Found: C, 44.96; H, 7.54; S, 19.93.

1,1′-Dithiodiaziridine. A solution of 67.5 grams (0.5 mole) of sulfur monochloride in 200 ml. of anhydrous benzene was added dropwise to a cooled and stirred solution of 47.4 grams (1.1 moles) of ethylenimine and 111 grams (1.1 moles) of triethylamine in 800 ml. of anhydrous benzene. The reaction mixture was kept at 5° during the addition and allowed to stand at room temperature overnight. Distillation of the filtered reaction mixture gave 44 grams (60%) of 1,1′-dithiodiaziridine, b.p. 44-6° (0.1 mm.); n_D^{25} 1.5776.

Calculated for $C_4H_8N_2S_2$: C, 32.41; H, 5.44; S, 43.25. Found: C, 32.58; H, 5.28; S, 43.19.

1,1′-Dithiobis(2-methylaziridine). This procedure was the same as for dithiodiaziridine except that 2-methylaziridine reacted with the sulfur monochloride. From 67.5 grams (0.5 mole) of sulfur monochloride, 37 grams (42%) of 1,1′-dithiobis(2-methylaziridine) was obtained, b.p. 52-6° (0.2 mm.); n_D^{25} 1.5368.

Calculated for $C_6H_{12}N_2S_2$: C, 40.87; H, 6.86; S, 36.37. Found: C, 41.05; H, 6.71; S, 36.50.

1,1′-Sulfonylbis(2-methylaziridine). To a solution of 35 grams (0.22 mole) of sulfinylbis(2-methyl-1-aziridine) in 1 liter of acetone was added 35 grams (0.22 mole) of finely ground potassium permanganate in 4-gram quantities. The permanganate color was not discharged after the last addition. The precipitated manganese dioxide was removed by filtration and the filtrate gave 22 grams (58%) of 1,1′-sulfonylbis(2-methylaziridine) which distilled at 77-82° (0.15 mm.); n_D^{25} 1.4700.

Calculated for $C_6H_{12}N_2O_2S$: C, 40.89; H, 6.86; S, 18.19. Found: C, 41.07; H, 6.83; S, 18.35.

1,1′-Sulfonyldiaziridine. Sulfinyldiaziridine was oxidized with an equimolar quantity of permanganate in the same manner as 1,1′-sulfinylbis(2-methylaziridine). A colorless acetone solution was obtained upon filtration from manganese dioxide and the evaporation of the acetone under vacuum at a bath temperature of 25° gave a colorless oil which immediately polymerized. The polymerization was not inhibited by addition of alkaline materials such as potassium carbonate, potassium hydroxide, or triethylamine.

Bis(2-methyl-1-aziridine) Sulfide. A solution of 25.8 grams (0.25 mole) of sulfur dichloride in 100 ml. of ether was added dropwise to a cooled and stirred mixture of 42.7 grams (0.75 mole) of 2-methylaziridine in 300 ml. of ether and 40 grams (1.0 mole) of sodium hydroxide in 40 ml. of water. The temperature was maintained at 5° during the addition and the mixture was then slowly brought to room temperature over a period of 2 hours. The organic phase was separated, dried with magnesium sulfate, and distilled to give 7.0 grams of product boiling at 35-53° (0.1 mm.). Fractionation of this material into three fractions of equal portions gave a first fraction boiling at 32-5° (0.1 mm.); n_D^{25} 1.4932.

Calculated for $C_6H_{12}N_2S$: S, 22.23. Found: 24.04.

The last fraction boiled at 45-55° (0.1 mm.); n_D^{25} 1.5268.

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7

Antifeeding Compounds for Insect Control

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While antifeeding compounds are not new in the pest control field, having been used as mothproofing materials and animal repellents for many years, their use for the protection of crops is a recent innovation. Beneficial insects are not harmed, feeding damage is more limited than with conventional methods, the mammalian toxicity is generally lower, and the approach is compatible with biological or chemical control. On the other hand, good coverage is required, new growth is not protected as it expands, and piercing-sucking or penetrating insects are not usually controlled. The advantages and limitations of the antifeeding approach are illustrated by laboratory and field tests with antifeeding compound 24,055, 4′-(dimethyltriazeno)acetanilide.

An antifeeding compound can be defined as a compound which will prevent the feeding of pests on a treated material, without necessarily killing or repelling them. It is not a repellent, for insects are neither driven away nor kept away by such compounds, nor is it an anorexient, since the appetite of the pest is not affected. "Gustatory repellent" comes close to the above meaning, but the word "repellent" has been so used and misused in the past as to render it ambiguous, even when modified. Dethier (*1*) recognized this problem, and subsequently proposed a number of terms to cover the different types of

action commonly embraced by the word "repellent." Under his terminology, antifeeding compounds would be called "feeding deterrents."

In principle, antifeeding compounds have been known for a number of years. Although there has been no prior use for the protection of crops from insects, such compounds have been used elsewhere, primarily in mothproofing. As early as 1928, I. G. Farbenindustrie was using Eulan New, sodium salt of bis-[α,α-(3,4-dichloro-2-hydroxyphenyl)]-*o*-toluenesulfonic acid, one of a series of chlorinated triphenylmethanes, as well as a number of triaryl phosphines, stibines, arsines, and tins for mothproofing woolens. Two years later, it was also using triphenylphosphonium salts for the same purpose, and Geigy in 1939 introduced Mitin FF, 5-chloro-2-[4-chloro-2-(3,4-dichlorophenylureido)phenoxy]benzenesulfonic acid. These have all been described (*5*) as preventing the feeding of the moth larvae, rather than killing or repelling them.

The only past usage in the field of agriculture has been the use of Z. I. P., the zinc salt of dimethyldithiocarbamic acid compound with cyclohexylamine, to keep deer, rabbits, and other rodents from feeding on the bark and twigs of dormant trees in the winter. It cannot be used on the foliage because of phytotoxicity.

While much work has been done by innumerable workers trying to extract, purify, and identify various components of plants known to be relatively free from attack by insects, most of these materials have fallen in the category of true repellents rather than antifeeding compounds, and none have shown promise for practical production and usage.

Compound 24,055

In the course of screening materials as insecticides, an occasional compound was observed to inhibit the feeding of southern armyworm caterpillars, although not otherwise affecting them. These materials were more or less a curiosity until compound 24,055, 4′-(dimethyltriazeno)acetanilide, entered the testing program. It inhibited feeding of the armyworms not only at the initial dosage, but at lower concentrations. Since it protected the leaves at rates in the range of many commercial insecticides, interest was aroused in its possible utility, and a search stimulated for other compounds showing this type of action. The result was a considerable number of materials, which included several chemical types. From this array 24,055 and its relatives were selected as the most promising from the point of high

activity, patentability, and ease and cost of manufacture. Although more than 30 compounds in this series were synthesized, none surpassed 24,055 in effectiveness.

Initial screening had shown that the compound was not effective against aphids or mites, but was effective against armyworm caterpillars and Mexican bean beetle larvae. Subsequent tests in the laboratory, the greenhouse, and a small garden plot showed that 24,055 was effective against chewing insects, but not against piercing or sucking insects.

One of the first garden tests was against a heavy population of Japanese beetles. When grape vines were treated with ½ to 4 pounds per hundred gallons, the effect was apparent from a considerable distance. The untreated vines were riddled; the vines receiving the recommended insecticides looked fairly good, but the 24,055-treated vines (at 2 and 4 pounds) had no sign of damage, except for leaves or parts of leaves that had not been covered. Up to seven days' control was obtained, depending on the dosage applied. A subsequent treatment of roses controlled these beetles as long as the blossoms, buds, and leaves were adequately covered.

Surface Protection

The material was also tried on a number of crops and pests in a vegetable plot, with varying results, depending again on the method of feeding of the particular pest. As before, insects such as cabbage worms, asparagus beetles, and bean beetles, which fed on the surface, were inhibited, while those that fed by piercing or penetrating the treated surface, such as mites, aphids, leafhoppers, and squash bugs, were not.

Further tests in the laboratory showed that 24,055 was effective when applied to surfaces other than growing plants. Wool impregnated with the compound was not attacked by the larvae of the black carpet beetle, and lesser grain borers would not penetrate treated paper bags containing grain.

At this point, 24,055 showed promise for control of a variety of insects at practical rates. On the other hand, this control seemed to be limited to certain types of insects, and even those that were controlled were not killed. It was decided to give 24,055 limited field testing, hoping that it might prove to be a marketable compound, or failing that, that we would get a good idea of the effectiveness and acceptability of the antifeeding concept as a method of insect control.

Results of the first season of testing were promising. Most of the leaf-feeding caterpillars and beetles were controlled by the material, but against chewing insects which fed inside the fruit, such as the plum curculia, corn earworm, and cornborer, the compound was ineffective.

The initial laboratory reports of tests on the cotton insects were promising and showed reduction of feeding of the boll weevil, bollworm, and leafworm. Unfortunately, the compound did not perform well in the field. The general picture throughout the cotton belt seemed to be that the early season control under low population pressure was satisfactory, but under heavy pressure from increasing population, the compound was not effective.

As expected, 24,055 had no effect on the predator complex in the field. Since the residues of the material were nontoxic, the predators were not harmed, and contact with treated foliage did not seem to affect their appetites. The material was not highly toxic to honey bees, and surprisingly, did not inhibit their feeding on treated sugar solutions.

Field tests also substantiated preliminary findings that coverage was important. In one of the few cases where there was a side-by-side comparison, the dust gave better coverage and protection than the wettable powder spray. In cases where low gallonage sprays containing high concentrations of 24,055 (4 to 8 pounds in 16 gallons of water per acre) were applied, we found poor control. Closer inspection revealed that distribution of the material on the plants was poor and much of the leaf area was not covered or protected. With conventional insecticides this is not important, since any insect walking over a treated leaf or eating it would die, but with an antifeeding compound the insect would avoid treated areas and continue to feed.

On the other hand, laboratory tests showed that it was not necessary to cover both sides of the leaf. There was no difference between tests in which only one surface of the leaves was dusted, and those in which leaves had been thoroughly treated on both sides. Since the caterpillars ate the entire thickness of the leaf, this was not surprising; however, the same results were found with the Mexican bean beetle larvae, an insect which feeds primarily by rasping the surface of the leaf. Either the larvae were eating a trace of the opposite, treated side of the leaf and being affected by that small amount, or the compound was being absorbed, or penetrated, through the thickness of the leaf.

In light of the above, it is interesting that 24,055 has shown limited systemic action in laboratory tests both by cut-stem immersion tests and by foliar application with translocation up the plant, unfortunately, at rates too high to be of practical importance in the field.

Mode of Action

Considerable work has been done in an attempt to discover the mode of action of antifeeding compounds. While the exact mode of action has not been pinpointed, certain theories have been ruled out. All antifeeding compounds tested to date have been effective only by ingestion, and not by contact. Perhaps "by taste" might be more exact, since the effect is immediate. When offered a treated leaf, a southern armyworm caterpillar will approach it normally and begin to feed. After making a tiny hole, the worm will stop feeding, move to another spot on the leaf, and begin to feed again. Following many hesitant bites of the leaf here and there, it will cease foraging and remain in one spot, although appearing in no way sluggish or affected. When confined to a treated leaf, the insect will not feed, and eventually starves to death. Insects will walk over treated surfaces and feed, and will even stand on treated surfaces while they feed on clean leaves.

Armyworm caterpillars that have been dipped in a solution of the compound will feed normally when offered untreated leaves. Using microinjection techniques, a small amount of solution of 24,055 was placed inside the mouth cavity of the armyworms; they fed normally. Injection of the material into the body cavity of the caterpillars also had no effect on their feeding. Thus, feeding seems to be affected only if the insect actually bites and/or tastes the material on its food.

While an effect on the gut, possibly paralysis, was considered, the foraging activity of the insect on a treated leaf, and the immediate return of normal feeding when offered an untreated leaf, seem to rule this out.

It has been proposed (*2*) that 24,055 is acting as an antimetabolite, but work in our laboratory and subsequent work by Lange (*3*) fail to substantiate this. A nutritional defect would also not appear in a matter of seconds, as does the antifeeding effect.

We are thus led to some sort of effect on the sensory receptors of the mouth which causes the insect to stop feeding on the compound, perhaps through inhibition of the biting or swallowing responses. The latter would seem to be the predominant effect at first, since the insect continues to take small bites for some time, but eventually stops.

Crude efforts were made to assess the effect of the removal of the maxillary palps and maxillae, both accessory mouth parts with many receptors assumed to be connected with the senses of taste or smell. The results were inconclusive, since insects so operated on refused to feed on either treated or untreated leaves, although this could also be interpreted to indicate that 24,055 had the same effect as removal of the sensory receptors—that is, it inhibited the response of the receptors so that the insect failed to recognize the treated material as food.

Advantages

Several seasons of field testing have shown a number of advantages in the use of the antifeeding approach to insect control. First, it is selective. Antifeeding compounds affect only pests which feed on the crop protected. Parasites and predators which walk over the treated foliage or feed on the affected insects are not killed, as with conventional insecticides. Honey bees and other pollinators are not affected by toxic deposits.

Another advantage of antifeeding compounds is their low toxicity in comparison with many of today's insecticides. 24,055 itself has an acute rat oral LD_{50} of 510 mg. per kg., and 36-day chronic feeding studies showed no effect on rats resulting from daily ingestion of up to 1250 p.p.m. in the diet (*4*).

Limitations

As with other methods of pest control, there are limitations in the use of the antifeeding method. Foremost is that, in general, only surface-feeding insects are controlled. This is not unexpected, since antifeeding compounds apparently work by taste, and insects which do not feed on the surface, with its residue of material, are not affected.

While many chewing insects are controlled by 24,055, even here there are limitations. If the insect attacks by penetrating the surface and feeding inside, control is not obtained. Pests which feed inside the fruit, such as the cotton bollworm, the corn borer, and the plum curculio—all chewing insects—take such a small amount of the treated surface before eating their way into the interior of the fruit, that control is difficult with an antifeeding compound.

Control is adequate, however, where the insect is small and the hole to be made is relatively large. Thus, the lesser grain borer will not penetrate paper bags treated with an antifeeding material. In this case the borer must chew a hole through the bag large enough to admit the insect. Paper or other bagging material is also absorptive

and will take up the treatment throughout much of its thickness, whereas most fruits or foliage are waxy and will not absorb the material as readily. The process of penetration in the case of the grain borer takes many bites and many hours, whereas the other penetrators mentioned can enter the skin of the fruit with relatively few bites in a matter of minutes.

The housefly presents an unusual case. Here is an insect which feeds on the surface of foods with a sucking mouthpart, rasping bits of food loose, liquefying them with regurgitated juices, and sucking up the resulting liquid. Surprisingly, antifeeding compounds do not work against this insect.

Another limitation is that plant growth forming after application of the material is not protected, and the insects, not having been killed by the treated portions, are still alive and present when the succulent new growth appears. This too is the part of the plant on which most insects prefer to feed. This points to the need for a systemic antifeeding material that will be translocated to the new growth as it emerges.

Somewhat of a limitation is that the insects are not killed by antifeeding compounds. Conventional insecticides kill by contact in most cases, and the insect does not have to ingest the material. In the case of 24,055 the insect can walk over a residue indefinitely without effect. The insects are free to attack the new growth or any poorly covered older growth. However, in field tests where there was good control, this was not a problem. Rapidly moving insects soon left the area of the treated plants. Slower moving insects or those on larger plots either died of starvation or were small enough to be not noticeable or objectionable.

This, incidentally, was one problem involved in field testing antifeeding compound 24,055—namely, getting data appropriate to the concept. The number of living insects in a plot does not indicate the effectiveness of the test material, yet the per cent kill or the number of live insects per some unit is the conventional measure of effectiveness. The best measure in the field was found to be an evaluation of the amount of feeding damage. In laboratory tests various workers used measurements of body weight loss, fecal deposits, size of feeding scars, or amount of oviposition as indications of effectiveness.

Conclusion

Antifeeding compound 24,055 was successful against chewing insects which fed on a surface that could be covered with the com-

pound, but not effective against piercing-sucking insects which fed on the juices, or against penetrating, chewing insects which fed on the untreated interior portions of the plants. With this limited spectrum of activity, together with the relatively high dosage required (compared with conventional insecticides), it was concluded that 24,055 did not warrant the costs of development and registration involved in commercialization of a compound, and further work with it has been terminated.

The search is being continued for further compounds of this type which will have a broader range of activity, especially one that would control the piercing-sucking insects, and might have systemic properties enabling it to be translocated to new growth.

Our experience did show, however, that the antifeeding approach to insect control appears both practical and acceptable. The compound was harmless to all but the pests being controlled, and it fitted in well with the integrated control type of program in which parasites and predators are disturbed as little as possible.

Acknowledgment

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8

The Status of Bacillus thuringiensis

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During the past ten years, a great deal of attention has been given to a group of spore-forming, crystalliferous bacteria related to Bacillus thuringiensis **var.** thuringiensis **Berliner. These bacteria are produced in large quantities for insect control in several countries, including the United States of America, France, Germany, Czechoslovakia, and Russia. Work on the crystalliferous spore formers is reviewed, including the results of field tests. Recent investigations indicate that as many as four distinct agents, toxic for insects, are produced by these bacteria. Their characterization, chemistry, and mode of action are discussed in detail. Standardization of commercial bacterial preparations has proved to be very difficult, because of the large number and variety of susceptible species, and their individual reactions to the multiple toxins. Recent work on this aspect is critically reviewed.**

Five years ago it would have been possible to live up to such an ambitious title in a 30-minute talk. Today it is virtually impossible. Accordingly, I am going to discuss the results of mode of action

studies and their practical implication in the field and on standardization of commercial *Bacillus thuringiensis* preparations.

Review of Past Work

Bacillus thuringiensis var. *thuringiensis* Berliner, isolated from diseased larvae of the Mediterranean flour moth [*Anagasta kuehniella* (Zeller)] about 50 years ago, is the type species of a group of spore-forming bacteria. This bacterium represents two other species in the group—*Bacillus entomocidus* var. *entomocidus* Heimpel and Angus and *Bacillus finitimus* Heimpel and Angus.

These aerobic spore formers resemble the common soil organism, *Bacillus cereus* Fr. and Fr., very closely indeed.

B. thuringiensis has been used periodically in insect control experiments during the past three to four decades.

In North America, Steinhaus (*25*) tested *B. thuringiensis* against the alfalfa caterpillar, *Colias philodice eurytheme* Boisduval. He found it to be an effective pathogen capable of controlling this insect. At this time only approximately ten Lepidoptera species were known to be susceptible.

Today, the total number of susceptible species stands at approximately 110 Lepidoptera and eight Diptera. This list is only at its beginning. We hear of successful tests with new species, conducted all over the world, at almost monthly intervals.

This tremendous range of activity is brought about by the ability of *B. thuringiensis* and its varieties to produce not one, but at least five, substances toxic to insects. The possibility that a sixth toxin exists is matter for further investigation.

In 1953 Hannay (*12*) discovered that *B. thuringiensis* produced a diamond-shaped parasporal body in each cell at time of sporulation. He pointed out that this "crystal" was alkali-soluble and suggested that it might be connected with the pathogenicity of *B. thuringiensis*.

After the rediscovery of the crystal [Berliner had described it previously (*3*)], it was not long before crystal-forming bacteria were isolated all over the world. Table I lists the isolates now in existence, which brings the total to 27 separate strains, varieties, and species. Wherever insect pathologists have investigated diseased insects, crystalliferous bacteria have been found. Apparently these bacteria enjoy a natural, worldwide distribution.

Much taxonomic confusion still exists. In 1958, Heimpel and Angus (*14*) proposed a key to the nomenclature of the crystal formers. This key was extended in 1960 and enlarged by Krieg (*17*) in 1961.

Table I. Crystalliferous Bacteria Related to Bacillus thuringiensis

Strain of Bacillus	*Original Host*	*Reference and Country Where Work Done*
thuringiensis var. thuringiensis Berliner	*Anagasta kuehniella* (*Zeller*)	Berliner (*3*), Germany
thuringiensis var. thuringiensis	*Pristiphora erichsonii* (*Hartig*)	Smirnoff and Heimpel (*24*), Canada
thuringiensis var. sotto Ishiwata	*Bombyx mori* (L.)	Ishiwata, Japan
thuringiensis var. alesti Toumanoff and Vago	*Bombyx mori* (L.)	Toumanoff and Vago (*30*), France
thuringiensis var. alesti (Anduze) Vago	*Bombyx mori* (L.)	Vago, France
finitimus Heimpel and Angus	*Malacosoma disstria* (Hübner)	Heimpel and Angus (*14*), Canada
Bacillus sp. 1P-BT 06.58	*Thaumetopoea pityocampa* (Denis & Schiffermüller)	Beguin 1958, France
Bacillus sp. 77-BT 06.58	*Hypomeuta* sp.	Beguin 1958
dendrolimus Talalaev	*Dendrolimus sibericus* Tschetverikov	Talalaev (*27*), Russia
cereus var. *galleriae* (Russian strain)	*Galleria mellonella* (L.)	Shvetsova, Russia
cereus var. *galleriae* (Rumania)	*Galleria mellonella* (L.)	From Bucharest, Rumania
cereus var. *galleriae* (Germany)	*Galleria mellonella* (L.)	Krieg (*17*), Germany
Bacillus sp. G 1	*Galleria mellonella* (L.)	Norris (1961) England
Bacillus sp. G 2	*Galleria mellonella* (L.)	Norris (1961) England
cereus var. *euxoae* Krieg	*Euxoa segetum* (Denis and Schiffermüller)	Krieg (*17*), Germany
Bacillus sp. 058	?	France
Bacillus sp. BT 2-12-58	?	France
Bacillus sp. BT 20-09-60	?	France
Bacillus sp. BT 17-03-59	?	France
Bacillus sp. BT 7-01-60	?	France
Bacillus sp. BT 12-06-59	?	France

TABLE I (Continued)

Bacillus sp. Epe-2000	*Ephestia elutella* (Hübner)	Dutky, U. S. A.
entomocidus var. *entomocidus* Heimpel and Angus	*Plodia interpunctella* (Hübner)	Steinhaus (*26*), U. S. A.
entomocidus var. *subtoxicus* Heimpel and Angus	*Aphomia gularis* (Zeller)	Steinhaus (*26*), U. S. A.

These bacteria are separated from *B. cereus* by their ability to form a crystal. They are again subdivided on the basis of their ability to form acetylmethylcarbinol and to produce phospholipase C. Further breakdown is then accomplished by reactions to sugars and on the basis of pathogenicity for insects, and other specific tests.

Heimpel and Angus (*14*, *16*) and Krieg(*17*) defined five groups with the crystal formers:

1. *B. thuringiensis* var. *thuringiensis*
2. *B. thuringiensis* var. *alesti*
3. *B. thuringiensis* var. *sotto*
4. *B. entomocidus*
5. *B. finitimus*

This classification still did not permit entirely satisfactory separation of the new isolates. However, De Barjac and Bonnefoi (*9*) have recently reported a biochemical and serological study of 24 strains. These investigators came to the same conclusions as the previous authors, but they created a sixth group, *B. thuringiensis* var. *galleriae*. More important, their serological study based on H antigen identified the same six groups within the 24 strains. Obviously a standard source of sera must be established; however, in future the literature on this subject will probably cite names such as *Bacillus thuringiensis*, serotype 1 (*thuringiensis*), or *Bacillus thuringiensis*, serotype 4 (*sotto*). The establishment of a serological basis for nomenclature is much to be desired.

At the time that Hannay discovered the crystal, Angus (*1*), working with another crystalliferous strain, *Bacillus sotto* Ishiwata, reported that a sterile alkaline extract of sporulating cultures of *B. sotto* killed silkworm larvae. He went on to show that *B. sotto* produced crystals at sporulation and by separating the spores and crystals managed to demonstrate that the crystals, and an alkaline extract of crystals, were both capable of inducing a lethal paralysis in

these larvae after 60 to 80 minutes. He later showed that the crystal was capable of killing other Lepidoptera larvae.

Angus (*1*) demonstrated that the crystal was a protein, with approximately 17% nitrogen and no phosphorus. The amino acid analysis of the crystal reveals nothing unusual (Table II).

Table II. Amino Acid Composition of Bacillus sotto **Toxin and Crystalline Inclusions** (1)

Amino Acid[a]	*Crystalline Inclusions*	*Toxin*	
		Average	*Range*
Arginine	9.4	9.6	9.5 — 9.7
Lysine	4.2	3.6	3.6 — 3.9
Cysteine and/or cystein	1.1	1.2	1.2 — 1.3
Histidine	1.7	2.7	2.7 — 2.8
Aspartic acid	9.5	9.6	9.3 — 10.2
Glutamic acid	12.9	11.8	11.6 — 12.0
Glycine	2.7	3.2	3.1 — 3.3
Serine	5.6	4.8	4.7 — 4.9
Alanine	3.2	2.8	2.7 — 2.9
Proline	6.7	7.5	7.4 — 7.6
Tyrosine	3.9	6.8	6.6 — 7.0
Threonine	5.2	4.5	4.3 — 4.7
Methionine	0.6	1.3	1.3 — 1.4
Phenylalanine	7.4	8.6	8.5 — 8.7
Valine	5.0	5.3	5.2 — 5.4
Leucine and/or isoleucine	10.4	11.2	11.2 — 11.3
Tryptophan[b]	2.1	2.6	2.5 — 2.7
Total	91.6	97.1	

[a] Estimated by paper chromatography of acid hydrolyzates, and expressed as grams of amino acid residues per 100 grams of protein analyzed.
[b] Determined separately.

The crystal protein from *Bacillus sotto* dissolves at pH 10.2 in 0.05*N* NaOH and the *B. thuringiensis* toxic protein at approximately pH 11.8; recently formed crystals are soluble at a lower pH. Addition of a reducing agent, such as sodium thioglycollate, reduces the solubility point to about pH 9.0 or less, suggesting that S-S bonds bind the molecules. It is significant that many Lepidoptera have alkaline midguts and in many of these insects the midgut contents are strongly reducing. There is little doubt that the crystal is dissolved in the gut of susceptible insects.

Heimpel and Angus (*15*) showed that there were three responses to the crystal toxin among susceptible Lepidoptera tested. They divided these insects into three types on the basis of these responses.

Type I, represented by the silkworm (*Bombyx mori*), the Chinese oak silkworm (*Antherea pernyi* Guérin), the tobacco hornworm [*Protoparce sexta* (Johannson)], and the tomato hornworm [*Protoparce quinquemaculata* (Hawthorn)], responds to the toxin by exhibiting a total paralysis within 1 to 7 hours. Later it was shown that the guts of these insects become paralyzed within 20 to 30 minutes after consuming crystal toxin.

Apparently the toxin affects the permeability of the midgut in Type I insects, since the complete paralysis sets in later, brought about by leakage of highly buffered, alkaline midgut contents into the blood of the insect. The blood pH is raised from 1 to 1.5 pH units, and this sudden change invariably brings on paralysis and death.

In the silkworm the food and toxin reach the midgut about 5 minutes after feeding begins. Five minutes later an upward trend can be detected in the blood pH. Thus, the toxin acts on and changes some part of the midgut within 5 minutes. Histological evidence from several susceptible insects suggests that the cell-cementing substances are the sensitive sites of the toxin, since the cells come free from one another and from the basement membrane. This result is very striking in the silkworm, in which relaxation of the midgut and disorganization of the midgut epithelium are obvious 45 minutes after ingestion of the toxin. Very little is known of cell-cementing substances in insects, although it is suspected that they are mucopolysaccharide-protein complexes, as in vertebrates. Studies of these materials have been undertaken by Z. E. Estes, Entomology Research Division, U. S. Department of Agriculture.

The Type II insect reaction is represented by the bulk of susceptible insects. The fed toxin brings about gut paralysis within a few hours. The insects cease feeding and wander about until they die 48 hours to 4 days later. The gut contents do not leak into the blood, and no general paralysis takes place. The spores ingested with the crystals germinate and multiply; the resulting toxic metabolites produced probably hasten the demise of the infested larvae.

The Type III insect represented by *A. kuehniella* was baffling to the early investigators. Neither spores nor crystals by themselves, nor any other ratio of both agents (other than the natural combination of one spore to one crystal), killed all the insects. When equal weights were fed, spores alone caused 4 to 8% mortality; crystals caused 12 to 13% mortality. Original culture spore-crystal ratio 1 to 1 caused 80 to 92% mortality. A previously undetected soluble toxic material, known as the McConnel-Richards toxic complex, is produced in

soluble form in the growth medium. This toxin, dried with spores and crystals, is probably the agent responsible for the singular results obtained with *A. kuehniella.* Recent reports by Burgerjon and Yamvrias (7) and Martouret(*23*) support this theory.

Finally the French workers have added a fourth reaction to the crystal toxin: the negative. The insects–the black cutworm [*Agrotis ipsilon* (Hufnagel)], *Euxoa segetum* Schiff, *Mamestra brassicae* (L.), and *Peridroma* (*Lycophotia*) *saucia* Hubner–are not susceptible to the *B. thuringiensis* preparation (*6*).

In 1958 Heimpel and Angus suggested that the crystal toxin must be in solution before the toxin can act. In 1959 they pointed out that the foregut contents of the silkworm contained enzymes that could break down dissolved crystal protein to amino acids in 10 minutes at room temperature. This breakdown was the basis for their suggestion that the crystal might be a protoxin and that the true toxin might be a subunit of the protein. Martouret (*23*) states that the crystal is dissolved in the gut of *A. ipsilon* and *E. segetum* without forming a toxin from the protoxin. In *M. brassicae* and *L. saucia* the crystal is not dissolved, no toxin is formed, and these insects are not susceptible.

Lecadet and Martouret (*20*) extracted enzymes from the pierid gut, and reacted these against dissolved crystals in vitro; they produced a lysate that was toxic by injection into larvae of the imported cabbageworm [*Pieris rapae* (L.)]. They suggested that this toxicity was brought about by a breakdown of the crystal protein by the gut proteinase, yielding a smaller toxic molecule which they thought acted in the body cavity. However, there is some doubt now that this sequence of events actually occurs. Benz (*2*) has shown that the pierids have a toxic principle in their gut, which when injected into the body cavity causes paralysis and death. Apparently the French workers were extracting a toxic material from the gut rather than causing the breakdown of the crystal protoxin to a toxin.

Obviously, the standardization of this toxin is going to be complicated. Several factors are involved, including the strain of bacteria producing the crystal, the manner in which it is grown, and the host directly involved.

The crystal is only one of the toxins produced by these bacteria. In 1953 Toumanoff (*28*) reported on a study of the genus *Bacillus* and their phospholipase C activity. In 1954 he found (*29*) that the precipitate of this enzyme from broth filtrates caused death when injected or fed to larvae of the greater wax moth [*Galleria mellonella* (L.)] In 1955 Heimpel (*13*) showed that strains of *B. cereus* and *B. thuringiensis*

that produced a large amount of phospholipase C caused higher mortality when fed to the larch sawfly [*Pristiphona erichsonii* (Hartig)] than did low phospholipase C–producing *B. cereus* strains. This observation was later confirmed by Kushner and Heimpel (*19*) and Bonnefoi and Beguin (*4*).

Histological studies of infected larch sawfly larvae suggest that the damage to midgut cells and other tissues in the body involves parts of the cells that are known to be partially made up of phospholipides.

The enzyme in question is produced only by the multiplying vegetative cell. When lecithin is incorporated in nutrient agar and bacteria producing phospholipase C are plated thereon, the soluble enzyme diffuses into the medium and breaks down the phospolipide, leaving soluble phosphorylcholine and a diglyceride which, in the form of oily droplets, produces turbidity around the colony. Several of the crystal formers—e.g., *B. entomocidus* varieties, *Bacillus* sp. G_1 and G_2, and *B. thuringiensis* var. *galleriae* strains from Russia and Rumania—do not produce phospholipase C. However, the latter bacteria affect lecithin in egg-yolk agar plates in yet another way. The effect is to clear the lecithin, implying that it is broken down into two soluble materials. If these bacteria produce phospholipases A or B, or both, fatty acids would be formed, leaving glycerolphosphorylcholine, which in effect would cause the clearing on lecithin agar. Work on this problem is currently being carried out by the Insect Pathology Laboratory at Beltsville, but regardless of the outcome, strains that do not produce phospholipase C apparently do break down lecithin.

The bacteria must grow in the gut of the insect to produce these enzymes and therefore they cannot be produced or act in insects with a pH of the midgut above pH 9. However, growth is often possible, since stresses or metabolic pressures may be exerted on the high-pH-gut insect, changing the activity or reaction of the gut. This change occurs when crystal toxin is consumed by Type II insects. To complicate the problem, some insects that are normally susceptible to *B. cereus* or *B. thuringiensis* may feed on foliage containing bacteriostatic materials. Since the growth of the bacteria in the gut is impeded, the insect survives unharmed (*18*).

It is very likely that the phospholipases are effective in killing insects in the field. It is therefore essential that a high count of viable spores be available on the leaves after the *B. thuringiensis* preparations are sprayed.

Finally, in 1959, McConnell and Richards (*22*) reported on a water-soluble, dialyzable, heat-stable substance produced in growing cultures of *B. thuringiensis* and a culture of *B. cereus*. They found that the toxin was not effective against the greater wax moth *per os*, but was toxic when injected.

Hall and Arakawa (*11*) and Dunn (*10*) showed that the housefly (*Musca domestica* L.) was susceptible to *B. thuringiensis* preparations, and Briggs (*5*) suggested that a material in the supernatant was responsible for the toxicity. Liles and Dunn (*21*) reported on the action of this toxin on larvae of *Aedes aegypti* L. and Chao and Wisterich (*8*) on the response by larvae of *Culex pipiens quinquefasciatus* Say.

In 1960 Burgerjon and de Barjac (*6*) tested six of the available strains and varieties of crystalliferous bacteria, two strains of *B. cereus*, and one of *Bacillus laterosporus*. They found that only *B. thuringiensis* var. *thuringiensis* produced the thermostable toxin in appreciable quantities.

Martouret (*23*) reports that this toxic material kills greater wax moth larvae 5 to 15 days after injection. If injected in low dosage, it retards or interferes with pupation and emergence of adults.

Krieg, in 1961 (*17*), stated that in his opinion the so-called McConnell and Richards "exotoxin" was a heterogeneous mixture of growth-metabolite end products and not a defined chemical substance. Feeding tests on housefly larvae, currently being conducted on the exotoxin by George Cantwell in our laboratory at Beltsville, indicate that there are at least two toxic fractions in the supernatant. One of these fractions is heat-stable. Contrary to Krieg's opinion, strong evidence exists that clearly defined toxic substances are produced by the growing vegetative cell of *B. thuringiensis*. The production of this toxic complex is apparently dependent upon the manner in which the bacterium is grown. Since the toxins are innocuous for vertebrates (they actually pass through the gut of vertebrates without losing activity), they might represent a new group of insecticides. The study of the pure toxins and their action on the insect is of prime importance to insect control.

Use in Insect Control

What has all this got to do with the standardization of the toxin and the knowledgeable use of the bacterium in the field? This relation is immediately obvious.

Here we have a tremendously effective tool to use against a wide spectrum of insects. The versatility of these bacteria has not yet been

fully realized. At present, only one of these bacteria has been commercially produced in the United States of America—*Bacillus thuringiensis* var. *thuringiensis*. Other strains and varieties have been grown on a limited scale by various government agencies throughout the world and some of these are more effective against specific insects than var. *thuringiensis*. The decision as to what strain is the most effective depends on a complete knowledge of the special attributes of the bacterial variety and the specific susceptibility of the host; it is therefore absolutely necessary to study each insect so as to detect its Achilles heel.

Standardization. Standardization of *B. thuringiensis* is very difficult. The method commonly practiced is to estimate a spore count by microscopic examination and plating and then conduct a biological assay on a standard insect. The spore count is perhaps the more significant step, since the number of spores is a means of predicting the enzyme potential of the preparation, and at the same time indicates the amount of multiplication that has taken place. Efforts are being made by the Insect Pathology Laboratory of the Entomology Research Division, with the full cooperation of the fermentation industry, to develop accurate counting methods.

The biological test is less dependable, because no one species of insect can be used to estimate the relative amount of all toxins present. Such a result is only an indication of the titer of those toxins present to which the specific animal is the most susceptible.

Conclusion

The work in developing the crystal-forming bacteria as control agents is well worth the effort involved. They are superb control agents. *Bacillus thuringiensis* has failed infrequently, and only when misused. I am confident that it will eventually be of great benefit in the control of some noxious insect species.

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