BARLEY LEAF CHEMISTRY AND THE CEREAL LEAF BEETLE FEEDING RESPONSE

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ABSTRACT

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By

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Extracts of seedling barley, Hordeum vulgare L., a host for the cereal leaf beetle, Oulema melanopus (L.), and seedling pea plants, Pisum sativum L., a non-host, were fractionated and bioassayed in three per cent agar for beetle feeding responses. One response estimate was the count of beetles in contact with the bioassay medium after one, two, or three hours. The estimate considered most reliable was a visual examination of the bioassay medium after the test period. Feeding damage to the agar was graded from 0-6 in units of 0.1.

Greater sensitivity was demonstrated toward the hydrophobic compounds of barley than to the hydrophilic compounds. Numerical response to barley hydrophilic compounds was low from 20-300 ppm, but increased rapidly above this level to become equal with that produced by hydrophobic compounds. Statistically, the maximum numerical response to hydrophobic compounds occurred from about 300-2,000 ppm. Response was good from about 10-300 ppm. Determination of hydrophobic feeding stimulants became the prime objective of this study.

Pea extract was a repellent/deterrent in its crude form. The deterrence was found to reside partly with the surface wax, but was

strongest in the dewaxed apolar fraction of the hydrophobic compounds. Despite the deterrence of pea crude extract, incorporation of barley crude extract with it at a ratio of 1:1 or greater (barley:pea, wt/wt) renewed the beetle feeding behavior. Thus, a host-specific, chemical quality of barley overcame the effect of deterrents when the proper ratio between the two factors was achieved. These facts support the opinion that feeding deterrents and host-specific sign stimulants interact so that plant selection or rejection represents the net effect.

The cereal leaf beetle feeding response was based on a multicomponent stimulant system. The type of agar damage observed was, to a large extent, characteristic of the fraction being bioassayed and gave clues to their respective functions.

Primary alcohols were the only active fraction in the epicuticular wax. 1-Hexacosanol was the most effective alcohol bioassayed alone and was active at 1.0 ppm. Little response to concentration above the threshold level was seen with the alcohols, and the agar damage was dominated by biting and rashing which indicated that stimulation of the biting response was the function of these compounds. Some indication was seen that 1-hexacosanol combined with 1-docosanol at 20:1 (wt/wt) was more effective than 1-hexacosanol alone.

Apolar hydrophobic compounds of dewaxed barley seedlings were nonstimulative. Glycolipids and phospholipids were each active. Agar damage with these fractions contained a larger proportion of channels in the agar than found with the alcohols. It was concluded that these fractions reinforced the biting response and lowered the threshold to hydrophilic stimulants. The polar hydrophobic compounds, together, counteracted the deterrents in pea extract. The glycolipids and

phospholipids from pea seedlings did not stimulate feeding, but neither were they highly deterrent to the beetles.

Individual glycolipids of pea were also inactive. Barley monogalactosyldiglyceride and digalactosyldiglyceride were active in equal measure above 20 ppm, although there was little dose-dependent response. Barley sulfolipid was active at 1-2 ppm.

It was subsequently found that most of the activity of barley phospholipids was due to the neutral phospholipids. The acid phospholipids were stimulants of low effectiveness. The activity of the neutral phospholipids was, in turn, found to be due mostly to interaction with the alkaloid, gramine. Some indication was found, but not confirmed, that the fatty acid composition of phosphatidyl choline may have influenced the cereal leaf beetle response to that compound.

Gramine was a stimulant at the lowest level tested, 3 ppm. It converted formerly rejected glycolipids and phospholipids of pea seedlings into palatable substrates, and when mixed with barley hydrophilic compounds, counteracted the deterrents of pea apolar hydrophobic compounds. It was concluded that gramine acted as a sign stimulant in this study.

Little work was conducted with barley hydrophilic compounds. However, the cationic compounds were active. Sucrose evoked a low, consistent response at 0.002M (776 ppm). Agar damage with this complete fraction consisted primarily of channels which was interpreted to indicate highly directed efforts toward continued feeding. The only pea fraction to stimulate the feeding response was the hydrophilic fraction. It was concluded that these compounds acted beyond the sensory level of host recognition and served to forge the final link in the chain of

responses which result in continued feeding. A model of cereal leaf beetle host selection and feeding response was suggested from the results presented.

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By

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INTRODUCTION

The cereal leaf beetle, Oulema melanopus (L.), (CLB) is an introduced species to the United States (21). Castro et al. (22) have discussed the systematics and natural history of this chrysomelid pest of the plant family, Gramineae. In a study of its New-World hosts, Shade and Wilson (149) found that CLB developed best on wheat, Triticum vulgare Vill., oats, Avena sativa L., barley, Hordeum vulgare L., spelt, T. spelta L., and rye, Secale cereale L., all members of the subfamily, Festucoideae (130). As a result, this pest quickly became the object of extensive research aimed at limiting its potential damage to the small grain industry of the United States. Despite early quarantine efforts, the CLB now occupies a portion of the Province of Ontario, and several Northeastern and Mid-Western states (58).

Connin et al. (25) developed a system to rear the CLB in mass which enabled laboratory studies to become allied and coordinated with field studies. A published bibliography (184) and a Michigan State University Research Report (3), reveal how extensive the literature related to this pest had become up to 1970. These publications cover such areas as natural history and bionomics, population dynamics, economic impact and chemical control, biochemistry, physiology, and resistant crop varieties. The use of control by introduced egg and larval parasitoids has been inaugurated as a factor in an integrated control approach (95, 156, 157).

Efforts are underway to represent the pest-parasite-crop ecosystem by mathematical models which would be predictive of pest populations based on current environmental and biological data (59). Control recommendations will be based upon the predicted degree of interaction of all components of the ecosystem model. If it were possible to reduce the overall interaction between the plant and its pest, the resultant control costs would be reduced.

The most effective means of reducing host-plant interactions by any given amount is by an appropriate degree of plant resistance. Currently, the only well defined form of host resistance to the CLB is derived from a physical basis. Pubescent wheat leaves offer a physical deterrent both to oviposition and subsequent larval survival (138, 183), resulting in decreased damage to such protected seedlings (181). Closely spaced vascular bundles have been correlated with resistance to larval feeding on unfavored members of the Gramineae (150). Recently, it was reported that wheat lines having thin leaves were more resistant to the CLB than lines with thicker leaves (4).

Some varieties of barley seem to show a low level of resistance (52), but the actual basis is uncertain save for the designation "non-preference for oviposition" (180). An understanding of the chemical basis of CLB preference for a susceptible variety of barley might provide a means of developing a more effective varietal resistance by selecting against any feeding stimulants amenable to genetic manipulation. USDA researchers have screened several hundred compounds for CLB attractant properties in field tests with little success (unpublished data, ARS, Entomology and Small Grain Laboratory, East Lansing, Michigan). Haynes

et al. (59) pointed out that it is not known why the CLB feeds on wheat and oats (nor on any host for that matter).

Knowledge of CLB nutritional requirements should assist plant breeders toward directed selection of varieties possessing a nutritional imbalance and thus add another degree of resistance against this insect. An artificial diet has been under development, but has not yet allowed egg production and successive generations to be reared (182, personal communication). The addition of natural feeding stimulants to present diet mixtures might allow research in this area to proceed at a faster rate.

The work of Panella et al. (129) failed to demonstrate any significant olfactory response by the CLB to extracts of barley seedlings. However, strong responses were derived with crude extracts of susceptible barley seedlings incorporated into an agar medium as a bioassay. The objective of the present study is to increase the understanding of the chemical basis of host selection by the cereal leaf beetle once the plant is physically contacted by the insect. The method will be to isolate and identify from barley seedlings, a number of biochemicals which stimulate CLB feeding as determined by a modified form of the Panella et al. bioassay.

LITERATURE REVIEW

This review deals primarily with the chemical basis of host-plant selection or rejection by gustation once an insect has physically encountered a plant. Orientation to a plant from a distance by an insect often involves visual and olfactory perception. For consideration of visual orientation, see Prokopy and Haniotakis (131), Moericke et al. (116), Meyer (106) or Meyer and Raffensperger (107).

Many authors have reported on the olfactory responses of insects to plant volatiles. Papers by Trayiner (168), Kennedy and Moorhouse (89) and Schwinck (146) ought to be reviewed by anyone wishing to pursue such research. Dethier and Schoonhoven (35) reported an electrophysiological investigation of the neuronal basis of olfaction. Also, examples exist where the initial encounter of a host-plant appears to be a random happening (88, 119, 189).

I. Terminology

Normal feeding behavior by phytophagous insects has been divided into three components by Dethier (30): a) orientation to the food, b) a biting response, and c) continued feeding. Thorsteinson (165) viewed feeding broadly in terms of two antagonistic neuroregulatory systems in constant opposition. One system caused a settled state favoring feeding, the other, a dispersing drive when the thresholds for feeding became too high to hold the insect on a food source. Consequently, he proposed a fourth element for Dethier's framework, "dispersal."

To answer a need for a more descriptive terminology relating chemicals to aspects to insect feeding behavior, Dethier et al. (33) suggested the following terms and summarized definitions:

1. attractant - a chemical causing oriented movement toward the source.
2. arrestant - a chemical causing insects to aggregate in contact with it.
3. stimulant - a chemical eliciting feeding or oviposition.
4. repellent - a chemical causing oriented movement away from the source.
5. deterrent - a chemical which inhibits feeding or oviposition.

The term "phagostimulant" was proposed by Thorsteinson (163) for those chemicals to which insects respond by feeding. It has become part of the literature, although it was viewed by Kennedy (86) as an "etymological chimaera." Thorsteinson (164) classified those nutrients detected by an insect as "sapid nutrients."

It has been shown more clearly with larvae of the silkworm, Bombyx mori L., that each sequential step in feeding may be under the influence of different chemicals. Attractants were found to be citral, terpinyl acetate, linalyl acetate, and linalol. Biting required β-sitosterol and isoquercitrin. Cellulose was required for proper swallowing and sucrose, inositol, inorganic phosphate and silica were co-factors (53).

The chemicals responsible for such discrete compoments of feeding behavior would not be adequately described by the classification of Dethier et al. just presented. Beck (13) felt that a new system was needed to provide a versatile terminology for students of the insect feeding response. Table 1 represents his effort toward this end. Hsiao (66) used the term "sign stimulant" for botanically restricted substances releasing biting and feeding response.

Table 1. CLASSIFICATION OF RESPONSES AND STIMULI ASSOCIATED WITH THE FEEDING BEHAVIOR OF PHYTOPHAGOUS INSECTS - BECK (13).

Response	Evoking Stimulus	
	POSITIVE	NEGATIVE
Orientation	Attractant	Repellent
Orientation	Arrestant	Repellent
Biting or piercing	Incitant	Suppressant
Maintenance of feeding	Stimulant	Deterrent

II. Historical Period (1910-1953)

The Dutch botanist, Verschaffelt (175) was the first investigator to put the study of host preference by insects, particularly phytophagous insects, on a sound chemical basis. He observed that mustard oil glucosides [now termed glucosinolates (159)] were common to the plants (chiefly Crucifereae) forming the host range of Pieris rapae L. and P. Brassicae L., the lesser and the greater cabbage butterfly, respectively. Verschaffelt applied solutions of glucosinolate, sinigrin, to non-host leaves and only after such treatment were these leaves readily eaten by the Pieris larvae.

McIndoo (99, 100) demonstrated that odors emanating from a plant could attract a natural insect pest. He introduced the concept of an olfactometer. Using a Y-tube device, he found that 62.7% of the time, adult Colorado potato beetles, Leptinotarsa decemlineata (Say), would select the tube leading to the host odor. These insects failed to respond to odors from non-host plants.

Dethier (28) confirmed the principle of insect response to host odors. Larvae of the monarch butterfly, Danaus (=Anosia) plexippus (L.), recognized host leaves separated from them by a screen. Recognition was indicated by searching, turning movements when they were over host leaves. Rather straight movements occurred over non-host leaves.

Ten years later, Dethier (29) reviewed what had been learned regarding the chemical basis of host preference. From hindsight, it is clear that he saw host selection as a simple chemical phenomenon: "In every case, in the final analysis, odor is the organisms index, regardless of food." "Essentially, the problem of plant choice resolves itself into a study of attractants and repellents and vice versa." These odors originated with the essential oils, and plant selection could be "largely divorced from nutritional requirements."

A significant event in this field was the Insect/Plant Relationship Symposium in 1951 at the IXth International Congress of Entomology in Amsterdam. Only four papers were published, but they represented such polarized opinions that the resulting controversy inspired a great deal of research.

Dethier (30) continued to support the importance of plant volatiles as the ultimate determinant of a preferred host. These substances would cause biting and continued feeding by most monophagous and oligophagous species. Nevertheless, some insects seemed to require contact chemoreception to release their feeding behavior. For instance, Thorpe et al. (161) had shown that wireworms, Agriotes sp., were attracted by asparagine glutamine and amides of short chain fatty acids, but for biting, they required sugars, lipids and polypeptides.

Fraenkel (41), like Dethier, felt that the "odd" chemicals or secondary substances of plants were responsible for host selection among leaf feeding insects. He emphasized two points: a) among those insects studied, the nutritional requirements were very similar, and b) the chemical composition of the green leaves studied was similar. Therefore, he felt that good nutrition for any phytophagous insect could be achieved if a sufficient quantity of leaves of any non-toxic plant species were eaten; nutrition could not be a factor in the host specificity of insects.

Painter (128) considered a possible role for nutritional factors in host preference by insects. Previously, he had defined one mechanism of resistance as antibiosis (126,127) and suggested that required nutrients might be deficient or lacking.

Kennedy (84) restated his theory of "dual discrimination" (87), invoked to explain observations that Aphis fabae Scop. preferred growing and senescing leaves over mature leaves, and that the summer form of the aphid preferred to feed on potted, growing specimens of the winter host, spindle, Euonymus europaeus L., than on growing summer host, Beta vulgare L., under greenhouse conditions. One type of discrimination would fulfill the ecological need to distinguish between plants in a similar growth state and another would be associated with materials nutritionally good for the aphids. This latter sense allowed them to select among leaves on the same plant for their stage of physiological development.

III. The Current Period (1953-1975)

A. Insect Perception of Nutrient and Secondary Chemicals of Plants

To a great extent, research into host-plant selection since 1953 has related to these two positions, the "dual disrimination" theory and

selection based on the "odd" chemicals of plants. Kennedy had, of course, only a deduction based on circumstantial evidence that nutrients might affect the feeding behavior of phytophagous insects. Some direct evidence for subterranian insects had been provided by Thorpe et al. (161)

An agar medium was used by Thorsteinson (162) to show that neither nutrients nor sinigrin, alone, were very stimulating to larvae of the diamond-back moth, Plutella maculipennis (Curt.). Yet, as little as 2 ppm of sinigrin blended with the nutrients (2.0% pea leaf powder) evoked a marked response. He later found that larvae of P. maculipennis and L. decemlineata were stimulated to feed by ascorbic acid (163). Thorsteinson (163) also reported that thiamine stimulated a feeding response by L. decemlineata larvae. Feeding was evoked in a grasshopper, Chorthippus longicornus Lat., by sucrose, glucose, betaine, and monosodium glutamate. He concluded that nutrients could stimulate feeding in oligophagous and polyphagous insects. Elsewhere, he suggested that the perception of nutrients by plant feeding insects had not received due attention (164).

Mittler (110, 111) found that the sap exuded from stylets severed from aphids, Tuberolachnus salignus (Gemlin), feeding on potted willows, Salix sp., had a higher amino-nitrogen content when analyzed from growing leaves than they did on mature leaves. Kennedy (85) felt that this work better established the relationship between nutrition and host-plant selection.

Fraenkel (42) was unconvinced by these results and during this time he made a famous declaration that host-plant specificity depended entirely upon the presence of the secondary plant substances to which the insect would respond positively or negatively. Kennedy retained his conviction in the "dual discrimination" capabilities of insects, but was conservative

in his appraisal of results germane to the subject. He pointed out (86) that no direct evidence had yet been presented to link host selection to the overall quality of available nutrients acting as feeding stimulants.

A breakthrough in bioassay procedures for aphids occurred when Mittler and Dadd (113, 114) developed a means of supplying artificial test solutions to aphids via a parafilm sachet. Mittler (112) determined that growth and feeding rate of the aphid, Myzus persicae (Sulzer), could be influenced by the ratio of sucrose to a mixture of 20 amino acids. Sucrose ranged from 0 - 40% while amino acids were held at 2.4%. Then sucrose was held at 15% while amino acids varied from 0 - 4.8%. Uptake was poor on diets with less than 5% sucrose or when total amino acid was below 1%. Optimum concentration range for sucrose was 10 - 20% and for amino acids, the optimum level was 3%. Declines occurred above either optimum, emphasizing the "importance of the behavioral aspects of nutrition." A similar study was reported earlier by Auclair (7) which agrees with Mittler's conclusions.

van Emden (174) reported a complex study of M. persicae (polyphagous) and Brevicoryne brassicae (L.) (oligophagous on curcifers). These aphids were grown on two crucifers and two non-crucifers. The plant leaves were analyzed at different physiological ages for allyl isothiocyanate and total free amino acid content. Using multiple regression analysis, he concluded that both secondary substances and nutrients played a role in host susceptibility, but the oligophagous B. brassicae was less influenced by amino acids than the polyphagous M. persicae. Regression equations indicated that the amino acids correlating with good growth performance by B. brassicae would tend to remain relatively constant over age and growth condition differences and this aphid may not select for their

presence. Concentration changes for amino acids correlated with good performance by M. persicae were significant with changes in the host-plant leaves allowing the aphid to select for these plants on the basis of the physiological state of the host.

It appears that for aphids, the dual discrimination theory does have some basis in fact. Dethier (31) credited the theory with having "brought the nutritional aspects of the plant into a picture that had become unbalanced" at the time of its proposal.

Another attack on Fraenkel's hard-line "secondary substances" theory came, in part, from Waldbauer (176). It had been shown by Waldbauer and Fraenkel (178) that maxillectomized larvae would feed on normally rejected plants. Waldbauer analyzed the growth and reproduction of maxillectomized larvae of the tobacco hornworm, Manduca (=Protoparce) sexta (Johan.) fed upon a normal host, tomato, Lycopersicon esculentum Mill., and on four nonhosts. Success on dandelion, Taraxacum officinale Weber, was equal to tomato and reasonably good on burdock, Arctium minus (Hill). However, mullein, Verbascum thapsus L., was unsuitable. Mullein-fed hornworms had longer larval periods, 45% mortality, mean weight gain per day was less than one-half that on tomato, and females laid fewer eggs, all unviable. Catalpa, Catalpa speciosa Warder, was also a very poor growth medium. As a result, Waldbauer (176) stood in opposition to Fraenkel's view that all green leaves should satisfy the nutritional needs of all insect species. His belief was that nutritional considerations could, indeed, restrict the host-plant range of a phytophagous insect species.

In a further study of consumption, digestion and utilization of non-host leaves by maxillectomized M. sexta, Waldbauer (177) confirmed that

mullein was nutritionally inferior for this insect. Likewise, Mehta and Saxena (103) found that growth of larval cotton spotted bollworms, Earias fabia, was very poor on two non-hosts, Pisum sativum L. and Brassica oleraceae botrytis L., even though the index of consumption and adsorption was higher in each case than for plants providing better growth.

Additional studies correlating the nutritional composition of a diet with insect feeding behavior have been made. Auclair et al. (9) reported a lower concentration of amino acids and amides in three pea varieties susceptible to the aphid, Acyrthosiphon pisum (Harr.). Another report (6) revealed that A. pisum had a lower feeding rate on the resistant varieties than on the susceptible plants tested.

Sugars had been known to elicit a feeding response from insects (44). Feeding by polyphagous larval European corn borers, Ostrinia (=Pyrausta) nubilalis (Hübn.) was correlated with plant parts having the higher concentrations of sugars (12). A monophagous insect, the sweet clover weevil, Sitona cylindricollis Fahraeus, was highly influenced by the glucose, fructose and sucrose in its host plant (1). When the appeal of several sugars to phytophagous insects has been tested, sucrose has generally been preferred to any other (1, 49, 61, 70, 74, 129).

Larvae of Clerio euphorbiae L. showed a tendency to eat less and to gain less weight on an unbalanced diet compared to a balanced diet (64). When comparisons were made between diets diluted to 85, 70, and 50% of the nutrients in a control, weight gains were not significantly different, but the amount eaten definitely increased with dilution. In a later review, House (65) concluded that the quantitative aspects of nutrition, particularly the balance of nutrients, could affect insect food selection. Ma (95) tested P. brassicae larvae with an agar-cellulose medium and found

that when sucrose was held at 10^{-2}M while ascorbic acid was increased, feeding also increased until ascorbic acid reached 10^{-2}M, after which a deterrent reaction was observed. This deterrence was neutralized by increasing the sucrose to 10^{-1}M.

Auclair (8), too, felt that not only overall amino acid concentration, but also the relative concentrations of individual amino acids would weigh heavily on the susceptibility/resistance of plants to aphids. The pink bollworm, Pectinophora gossypiella (Saunders), was found by Vanderzant (172) to develop well on an artificial diet when the amino acid composition simulated cottonseed protein, but did not survive when the composition resembled that of casein.

Within a class of chemicals, some may stimulate positively, some not at all, and others may be deterrent. Beck and Hanec (14) found that L-alanine, L-serine, L-threonine, and L-methionine were stimulants for O. nubilalis larvae, while β-alanine, L-tryptophan, L-phenylalanine and L-arginine were deterrents. Larvae of L. decemlineata were highly stimulated by L-alanine, γ-aminobutyric acid and L-serine and moderately by other amino acids (70).

In a study where eight amino acids, considered essential to the aphid, A. fabae, were omitted from a synthetic diet, Leckstein and Llewellyn (93) concluded that alanine and proline were phagostimulants. Amino acids were non-stimulatory to larvae of the alfalfa weevil, Hypera postica (Gyll.) (66), but adenine and adenosine isolated from alfalfa, Medicago sativa L. were powerful stimulants (67). Other purines, pyrimidines and their nucleotides were inactive.

Phosphatilyl choline, phosphatidyl inositol, and to a lesser extent, phosphatidyl serine were good stimulants for the grasshopper,

Melanopus bivittatus (Say) and Camnula pellucida (Scudder) (166). These same phospholipids plus phosphatidyl ethanolamine were active stimulants for larvae of L. decemlineata (70). Hsiao (69) reported on the sensitivity of five species of Leptinotarsa to several nutrients. Only L. haldermani Rogers and L. decemlineata responded to vegetable lecithin. Phospholipids as a group evoked a greater response from Schistocerca gregaria Forsk. than from Locusta migratoria L. (102). Choline phospholipids were the only commercially obtained phospholipids which stimulated larval cabbage loopers, Trichoplusia ni (Hübn.) (49).

Other lipid nutrients have been determined to be feeding stimulants for plant feeding insects. Triglycerides and a mixture of free sterols and fatty acids from wheat germ oil elicited feeding from S. gregaria (102). β-Sitosterol was a stimulant for larvae of B. mori (54)

In spite of growing evidence that nutrients could strongly influence the feeding behavior of insects, Fraenkel (43) was willing to make only a small concession as to their importance in determining host specificity. He did recognize that all leaves would not equally well serve phytophagous insects as food, but if nutrients played any role in host selection, he felt that it was a minor one.

Indeed, there are many examples of "odd" chemicals which have been shown to be feeding stimulants for insects. Hsiao (68) and Schoonhoven (142), in particular have compiled extensive lists, not only of those that stimulated feeding, but also of many that were deterrent. So far, the latter outnumber the former. Table 2 is modified after Schoonhoven (142). It lists only coleopteran species and associated feeding stimulants.

Table 2. EXAMPLES OF SECONDARY PLANT SUBSTANCES KNOWN TO STIMULATE FE
ING IN SPECIES OF COLEOPTERA - modified from Schoonhoven (14

Stimulating Chemical	Insect Species	Refere
(+) Catechin-7-α-D-xylopyranoside*	Scolytus multistriatus (Marsham)	(37
Cucurbitacin	Aulacophora foveicollis Lucas	(152
Cucurbitacin	Diabrotica undecimpunctata Barb.**	(23
Gossypol	Anthonomus grandis Bohe.	(97
p-Hydroquinone	S. multistriatus	(125
Hypericin	Chrysolina brunsvicensis Grav.**	(133
Isoquercitrin	A. grandis	(60
Linamarin	Epilachna varivestis Mulsant	(121
Lotaustrin	E. varivestis	(121
Lupeyl cerotate	S. multistriatus	(36
Oxalic acid	Gastroidea viridula Deg.	(134
Phaseolutanin	E. varivestis	(121
Quercetin	A. grandis	(60
Quercitrin	A. grandis	(60
7-α-L-rhamnosyl-6-methoxyluteolin	Agasicles sp.**	(191
Salicin	Plagiodera versicolora (Laich.)	(96
Sinigrin*	Phyllotreta cruciferae (Goeze)**	(62
Sinigrin	Phaedon cochleariae Fab.	(158

* Additions by the present author.

**Chrysomelidae - indicated by the present author.

B. Interactions Between Chemicals

A highly significant phonomenon evident from the years of research is that many forms of interactions between plant chemicals occur in the sensory systems of phytophagous insects. One type of interaction is between nutrients. The effect may be additive or synergistic. Beck and Hanec (14) found that serine and glucose had an additive effect on the feeding response of larval European corn borers.

However, synergistic interactions seem to be more common. For the grasshopper, C. pellucida, Thorsteinson (165) reported that KH_2PO_4 alone was ineffective, but at 0.004M with sucrose at 0.02M, the response was appreciably enhanced over sucrose alone. He also reported that the amino acids, serine, alanine, and δ-aminobutyric acid, each at 0.008M, synergistically interacted and addition of 0.02M sucrose increased the response even more. The aphid, M. persicae, reacted in a synergistic way to a mixture of amino acids and sucrose compared to sucrose alone (115). Larvae of the spruce budworm, Choristoneura fumiferana (Clem.), showed a synergistic response to 0.03M each of glucose, fructuse and sucrose compared to 0.09M sucrose alone (61).

Gothlif and Beck (49) found synergism between K^+ salts (the anion had little or no effect) and the neutral lipids of wheat germ oil for T. ni. Ma (95), working with P. brassicae, found that vitamin C was ineffective alone, but synergized with sucrose. L-Proline also synergized the feeding response to sucrose by C. fumiferana larvae as did hydroxyproline and glutamate (61).

It is evident that sucrose was often involved in reported synergisms of the feeding response of insects. Thorsteinson (165) suggested that the gustatory effects of saccharides interacting with other plant

substances may influence food selection by phytophagous insects. Auclair (8) pointed out the apparent specific requirement for sucrose as a feeding stimulant for many aphids.

Another class of interactions involves nutrients with secondary substances. In fact, Thorsteinson (165) advised researchers to consider secondary substances as "synergizers." In that review, he reported that larvae of P. maculipennis responded very little to either sinigrin alone or to sucrose at any concentration, but the addition of 0.1% sinigrin to 0.2M sucrose was highly stimulatory. Regarding these "odd" chemicals which are known to stimulate insect feeding, Schoonhoven (142) indicated that they often require the presence of a sugar to be an effective stimulant. Heron (61) made a similar statement.

C. Electrophysiological Studies

Since host selection had been accepted as primarily a chemically based response, it was desirable to study as directly as possible, the chemoreceptors involved. Hodgson and Roeder (63) improved existing electrophysiological techniques for insect material and reported a study of the labellar setae of the blowfly, Phormia regina (Meigen). Since an electrolytic solution was required to be in contact with the insect's sensory apparatus, only water-soluble materials could be tested with this tip-recording method. Morita (118) introduced side-wall recording which enabled hydrophobic materials to be tested.

Torii and Morii (167), Ito et al. (73) [both according to Dethier, (31)] and Waldbauer and Fraenkel (178) demonstrated indirectly, by removal of the maxillae, that these structures were regions of gustatory sensory abilities. An olfactory ability of much less significance was found on

the maxillae (145). Ishikawa (72) was the first worker to demonstrate the gustatory function of the sensilla styloconica on the maxillae of B. mori larvae. Two such structures arise from each maxilla (one lateral, one medial) and each is innervated by four contact chemoreceptor cells. Schoonhoven (144) has reviewed the known sensory abilities of Lepidoptera sensilla styloconica. Each cell therein has a range of sensitivity, qualitatively and quantitatively somewhat unique to itself. In the lateral sensillum, for example, one cell is responsive to amino acids while another responds to sucrose. Broadly, the medial sensillum is often the location of deterrent recognition.

The most extensive study of chemoreceptor spectra to date seems to be that made by Dethier and Kuch (34), who studied ten species of lepidopterous larvae. Lateral and medial styloconica were exposed to 15-53 compounds from the following classes: salts, acids, sugars, amino acids, polyhydric alcohols, glucosides, sterols, PO_4^{-2} buffer, and quinine. A major purpose of this work was to counter an idea which had gotten into the literature that the receptor neurons of these structures had a narrow range of specificity. They were successful and confirmed a previous hypothesis (145) that "each of the eight cells is sensitive to a number of compounds."

Recent investigation of L. decemlineata larvae revealed amino acid receptors in the lateral sensillum of the galea and on the maxillary and labial palps (109). The medial sensillum did not respond to any chemical tested in a manner similar to the lateral sensillum, although low-frequency impulses were detected. The amino acids giving the greatest effect were those determined by Hsiao and Fraenkel (70) to be the most effective in behavioral studies. A similar correlation between behavior and

electrophysiological observations exists for M. sexta. The antennae of this insect responded less to odors from Nicotiana sp. than to odors from other hosts (145). Jermy et al. (80) found Manduca to be less inducable by Nicotiana than by other hosts. For discussions of the phenomenon of induced preference, also see Wicklund (187), Waldbauer and Fraenkel (178) and Schoonhoven (141).

D. The Physiological Basis of Insect Feeding Patterns

Another unsettled question relates to the basis for monophagous, oligophagous, and polyphagous food habits. The terms themselves are somewhat vague regarding the level of plant taxonomy at which they should be applied (165). Mechanistically, however, Dethier (29) believed that these patterns could be defined relative to the number of chemicals which were attractive to an insect. Monophagy resulted from attraction [this term is pre-Dethier et al., (33)] to one compound or to several confused as one by the insect. Oligophagous insects reacted to several distinct compounds while polyphagous insects did not require specific attractants, but fed on all plants not containing repellents. Thorsteinson (162) suggested that in oligophags, positive and negative influences might underlie host selection. He later assumed a broader position by stating that "a variety of mechanisms probably underlie oligophagy" (165).

Jermy (78) opted for oligophagy based more on avoiding deterrents than by responding to specific stimulants. Using single leaf-disc tests and sandwich tests, a disc of a non-host leaf between discs of a host leaf, he found that most non-host plants of nine insect species contained feeding deterrents. Both slightly and highly restricted feeders were very sensitive to the deterrents. A tendency existed for the highly restricted feeders to show the greater sensitivity. The author felt that

the importance of botanically restricted, specific stimulants would be reduced if substances more widely distributed could replace them by virtue of possessing a similar stereochemistry or configuration.

Noteworthy here is a paper by Meyer and Norris (105) where the molecular shapes of hydroquinone and p-hydroxybenzaldehyde were said to be similar. These two compounds were the best of six substances tested as feeding stimulants for the smaller European elm bark beetle, Scolytus multistriatus (Marsham). The topic of molecular structure and stimulating effectiveness was given some consideration by Schoonhoven (142).

Gupta and Thorsteinson (51) applied larval P. maculipennis to 62 non-host plants (37 families) not containing the glucosinolate stimulants of normal hosts and found that nine species were fed upon, untreated, during an 18 hour period. Twelve species became acceptable when coated with sinigrin solution and 41 remained unacceptable following treatment with sinigrin. Because a normal host, black mustard, Brassica nigra Koch, was rejected when coated with aqueous extract of various fully rejected plants, feeding inhibitors were suggested as being potentially as significant as feeding stimulants in circumscribing an insect's host range. However, of the nine non-host plants acceptable to P. maculipennis, only pea supported successive generations from the egg stage. First instar larvae transferred from black mustard to sweet clover, Melilotus officinalis Lam., or to coumino clover, M. alba Boiss., produced some adults, but sinigrin applied to the two clovers did not increase survival. In these cases, some nutritional considerations would seem to be involved in host selection. In fact, botanically restricted compounds are not required to release the feeding behavior of many insects. None have yet been conclusively demonstrated for M. sexta or L. decemlineata (43). A

list of 15 insect species was prepared by Schoonhoven (142) of instances where one or more generations were reared on meridic diets containing no secondary substances.

The alkaloids in non-host solanaceous plants apparently prevent colonization of these plants by the Colorado potato beetle (78). Kogan and Goeden (90) compared behavior of larval Lema trilineata daturaphila (Oliv.) with published data for the tobacco hornworm and the Colorado potato beetle, all of which feed on Solanaceae. These species reacted differently to the various alkaloids contained in this family, and no positive stimulation has been attributed to these alkaloids, nor to alkaloids generally. Some steroidal alkaloids did not harm the Colorado potato beetle at high concentrations, while a tropane alkaloid, scopolamine, was toxic at 1%. Contrary to this fact, L. t. daturaphila avoided plants containing steroidal alkaloids, but suffered no ill effects from the tropane alkaloids of Datura sp. Injection of this insect with scopolamine at 12.5 mg/g of larval fresh weight did not prevent normal development. Kogan and Goeden (90) concluded that the range of these insects within the Solanaceae would be determined by the alkaloids deterrent to feeding activity, and that feeding excitants would exist generally throughout this family.

Regarding the negative influence of many "odd" chemicals on insect feeding preferences, Fraenkel (43) described their possible effects as feeding deterrents, toxins, and hormone-mimetic substances. Gordon (48) suggested that these protective secondary substances may sometimes act as antibiotics to microbial commensals which might help insects to overcome nutritional imbalances in their food.

It was once held that polyphagous insects did not require specific feeding stimulants (attractants), but would consume any leaf which did not contain repellent substances (28). However, Thorsteinson (164) stated that polyphagous insects did depend on specific gustatory stimulants for the expression of their feeding activities. Mehrotra and Rao (101) reported differences in phagostimulant requirements for L. migratoria and S. gregaria. Subsequently, Mehrotra and Rao (102) reported on the components of edible oils (six types) as phagostimulants for these two species. No feeding was induced by hydrocarbons, sterol esters, diglycerides, or monoglycerides. Active fractions were triglycerides, a free sterol-fatty acid mixture, and phospholipids. Triglycerides were stronger stimulants for Locusta than for Schistocerca.

The influence of deterrents in determining the feeding pattern of insects has been emphasized in many of the preceding examples, but Kennedy (86) seemed to feel that a more complete understanding of maxillary input to an insect's central nervous system was required. He referred to the suggestion of Waldbauer and Fraenkel (178) that maxillary palps may spontaneously provide inhibitory inputs which must be overcome be the presence of adequate positive stimuli.

In agricultural entomology, it is not entirely a matter of host selection between different plant species that demands our concern. The real consideration often is preference between varieties of a crop (86). In these instances, it seems very likely, based on the foregoing behavioral and electrophysiological studies and others, that selection would be a net-effect result, produced by both positive and negative stimuli. Several authors have subscribed to this opinion (11, 32, 73, 93, 95, 105, 144, 174.

MATERIALS AND METHODS

I. Beetles

The cereal leaf beetles used in this study were provided by the Entomology and Small Grains Laboratory of the USDA, ARS at East Lansing, Michigan. They were reared under the regime described by Connin et al. (25). Adults, newly emerged from the pupal cell, were collected at about 5:00 p.m. daily. They were maintained overnight under plastic refrigerator boxes (fewer than 200 per box) inverted over, but separated from a clean glass plate by a piece of nylon screen. An 11 cm circle of Whatman No. 2 paper was placed on the screen beneath the box and a slight excess of distilled-deionized water was applied to the box-paper interface at 5:30 p.m. and again at 9:45 a.m. the following day. A 75-watt incandescent bulb controlled by a 24-hour automatic timer from Sears, Roebuck and Co. (model 796.6445) was used to provide a 16 hour photophase when room lights were off overnight. Normal fluorescent lighting prevailed during the daytime.

The CLB were laboratory reared from about mid-October to about mid-July. The breeding stock was renewed with field-collected, pre-diapause adults each July. A number of bioassays were conducted with these field-collected beetles, and, when used, the results have been clearly distinguished from those employing cultured beetles.

II. Plants

A. Barley (Hordeum vulgare L., cultivar 'Lakeland')

All seeds and greenhouse facilities were supplied by the USDA, ARS, Entomology and Small Grains Laboratory. Approximately 60 barley seeds were sown per plastic pot (3.5 in. diam.) containing a mixed soil of three parts field soil, three parts peat, and one part sand, which had been sterilized for two hours by injected steam. In the greenhouse, the pots were held under a 16 hour daily photophase at 17°/21° day/night temperature. The seedlings were taken to the laboratory when the second leaf was ca. 1.0-1.5 cm in length. All harvests were made at 11:00 a.m. ± 5 min., just prior to extraction.

B. Pea (Pisum sativum L., cultivar 'Yellow Wonder')

Five seeds were planted per pot (3.5 in. diam.) and grown under the same conditions as the barley. The seedlings were removed when 6-8 cm in height and harvested just prior to extraction. Identical procedures were used to fractionate the barley and pea plants.

III. The Bioassay

A. Physical Considerations

The original report of a bioassay for CLB feeding responses was that by Panella et al. (129). The present bioassay was derived from that bioassay through various modifications. Each experiment in this study was composed of one or more treatments. A treatment consisted of an extracted plant fraction after it had been incorporated into an agar medium at a known concentration. Some fractions were too light for weight determination and have been recorded as weightless. Samples were taken

from these treatments to provide test units which became the immediate source of all bioassay data.

To prepare a treatment, 1.5 g of Bacto Agar (Difco Labs., Detroit, Mich.) was added to 52 ml of distilled-deionized water at about 90°. While on the hot plate, the test fraction was added in solution to the boiling agar. Two ml of water were assumed to vaporize during preparation and cooling of the agar medium so that the final weight, less the added fraction, was considered to be 51.5 g per treatment.

If the test solution were aqueous, an equivalent volume of water was deleted from the agar to maintain the treatment weight of 51.5 g. When organic solvents were used, intermittent heating and stirring was used until no solvent odor could be detected.

The hot treatments were poured into glass Petrie dishes (15x100 mm) and immediately covered with the top reversed to prevent condensation from collecting on the inside of the cover. After the agar had cooled, the covers were positioned normally and the dishes regrigerated at 4°-6°. Treatments generally were made up two or three hours before using, occasionally on the evening before. Control agar was prepared by adding the pure solvent equal to the greatest volume of test solvent used in that particular experiment.

A test unit consisted of a plastic Petrie dish (13x90 mm) inside of which two agar strips (ca. 6.5x0.15x0.6 cm) were positioned in the center to form a closed elliptical circle. Generally, one agar strip was a control and the other was a test strip, although two strips of the same treatment were sometimes used if deemed desirable. Three "X" marks were applied with a black marking pen to the lower exterior of the plastic dish

and the test strip was placed over these marks. This was done just before the bioassay was begun for the day.

The bioassay was started when 25 newly emerged, unfed, unsexed, adult CLB were placed in the center of the agar ellipse and the cover positioned. All test units were placed in a shallow cardboard box (capacity of 6 test units) and the box closed and sealed by a weight lengthwise along the cover seam. The lights of the windowless room were turned off and the doors closed. Room temperature was approximately 23°.

B. Temporal Considerations

The bioassays were started daily at 12:50 p.m. $\pm$ 5 min. At the end of each hour for three consecutive hours, a tally was made of beetles with at least the head in contact with the control or the test strip. Counts were recorded separately for the test and control strips.

The first count was made in the darkened room by light from a 25-watt bulb in a dark-room lamp equipped with a Kodak Safelight No. 2 filter which passed only light above 640 nm. The CLB is attracted by white light, and Wigglesworth (188) pointed out that such insects are less responsive to longer wavelengths than to those approaching the ultra-violet. The remaining two counts were quickly made in normal room light followed by a return of the room to darkness.

C. Experimental Design

Each experiment followed one of three bioassay formats: a) several concentrations of a single fraction, b) a comparison of different fractions at one or more concentrations, c) two or more fractions combined at various concentrations. At the outset of the study, data were collected from five or ten completely randomized test units (reps) per day from a single

concentration (treatment) of crude extract. Several concentrations of crude extract were tested in this manner. Subsequently, a randomized complete block design was employed where several different treatments were bioassayed with one test unit per day over a four day period (four reps). When possible, two control strips were included as a test unit in an experiment.

Due to daily fluctuations in the number of beetles available, portions of an experiment were now and then deleted after they were initiated. Occasionally, whole experiments had to be abandoned for that reason. Only treatments with three or more replicates were given serious consideration during data interpretation.

D. Treatment Composition

The composition of a treatment agar took one of three general forms:

a) The test fraction only was admixed with the agar.

b) The test fraction was agumented with sucrose in the agar.

c) The test fraction was combined with another fraction from barley or pea plants or commercially obtained biochemicals in the agar.

E. Scoring

Two methods were used to rate the response in each bioassay. To obtain a numerical response index for a treatment, the average hourly count of beetles on a test strip was calculated, based on the total number of hourly counts made over the duration of the experiment. If two identical test strips were used for an experiment, the sum of beetles responding to both strips was divided by twice the total number of hourly counts.

At the end of each day's test, a visual analysis of damage to the agar was made using a dissecting microscope to provide a second estimate

of feeding response. The strips were then rated as 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent). Four daily values for the control and the test strips were averaged by treatment to give each an activity index.

F. Validity of the Bioassay

To demonstrate the reliability of the bioassay, extracts of barley seedlings were compared to an extract of several non-host plants. The latter, except pea plants, were obtained from an organic gardener and had not been treated with any type of pesticide. The fresh leaves were frozen within 10 minutes of harvesting the entire plant. All extractions were in benzene:methanol (1:1, v/v) followed by a second extraction in 25 ml distilled-deionized water. Ten replicates of each test were bioassayed in completely randomized tests. No visual analysis was performed.

Under certain circumstances, deviations from the normal procedure detailed above were made. Departures such as incomplete experiments, altered number of beetles used, and other experimental details are noted in the RESULTS in appropriate tables or figures.

IV. Isolation of Plant Biochemicals

In the following procedures, all organic solvents used were analytical grade which had been glass-distilled. Methanol at 0.5% was added to redistilled chloroform as a stabilizer. All water was distilled, then deionized by passage through a bed of charcoal followed by a mixed resin bed (Barnstead, Boston, Mass.). Each fraction obtained was weighed until its series of bioassays had been completed after which weights were not taken to conserve material for subfraction bioassays. Extracts in

organic solvents were stored under nitrogen below 0° until analyzed. Aqueous samples were refrigerated at 4°-6°.

A. Crude Extract

A sample of seedling barley (10-12 g) was weighed to the third decimal place and then homogenized for two minutes in a blendor with isopropanol (120 ml) to deactivate lipolytic enzymes (82). The homogenate was filtered through Whatman No. 1 paper and 250 ug of butylated hydroxy-toluene added as an antioxidant (81). Successively, the residue plus filter paper were extracted with 120 ml of chloroform:methanol, 1:1 (v/v) for two minutes (83), then chloroform:methanol, 2:1 (v/v) for two minutes (24). The three combined extracts were filtered through a "c" sintered glass funnel and taken to near dryness in vacuo on a BÜCHI Rotovapor-R at 30°-35°. Compounds were dissolved in 25 ml of chloroform:methanol, 2:1 (v/v). This fraction was the crude extract.

B. The Hydrophobic Fraction

1. Isolating the Total Fraction

The above method did not provide for separation of the hydrophobic compounds from the hydrophilic compounds. A variation of procedure IV.A was therefore developed. At the third homogenization, the system, chloroform:methanol:water, 4:2:1 (v/v/v), was used (120 ml) and a fourth extraction with methanol:water, 1:1 (v/v) followed.

To these combined extractions in a 1 L. separatory funnel, ca. 150 ml of chloroform were added to develop two phases. The lower organic layer was removed to another separatory funnel where it was washed three times with 40 ml of water and these washes were added to the aqueous layer. Repeated chloroform washes of the aqueous were made and combined in a

third separatory funnel and backwashed with 2x20 ml water washes which were discarded. Both chloroform washes were then combined. The organic and aqueous phases were concentrated in vacuo at 30°-35° and 40°-45° respectively.

In addition to the procedure above, a column of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was used to separate the hydrophobic and hydrophilic compounds (190). Chloroform:methanol: water (200 ml, 100 ml, 75 ml, respectively) were mixed in a separatory funnel. Two phases were formed and collected separately into aluminum foil-wrapped Erlenmeyer flasks which were then stoppered. Part of the upper phase was used to imbibe the gel. The swollen gel was then pipetted into a column to make a bed of 1x5 cm. Approximately 50 m of the lower phase were used to displace the upper phase from around the gel beads in the column and to equilibrate with the upper phase within the beads before applying the crude extract.

Aliquots totalling 8 ml of the lower phase were used to take up the soluble materials from the concentrated extract in the near-dry state and to filter them through a "c" sintered glass funnel. This 8 ml of extract was applied to the Sephadex column and rinsed onto it with 2 ml followed by 30 ml of lower phase to remove the hydrophobic compounds. Hydrophilic materials had partitioned within the beads, giving a yellow color to the top of the bed, and were eluted with 30 ml of the upper phase.

2. Isolating Hydrophobic Subfractions

a. Epicuticular Wax

(1) Total Wax

This external group of hydrophobic compounds was isolated by a simple chloroform wash (92), but two approaches were used: (1) If waxes

were to be bioassayed, the seedlings were sealed in their pots with plaster of Paris. The pots were inverted and shaken to dislodge loose dirt and the seedlings were immersed and swirled in chloroform twice for periods of 3-5 seconds. The plants were discarded and the chloroform concentrated in vacuo at 30°-35° to produce a clean, white wax solution-suspension. (2) When hydrophobic compounds other than epicuticular waxes were to be bioassayed, the wax was removed from harvested seedlings by two 10 second washes in separate chloroform baths. The dewaxed seedlings were then extracted for further separations described later.

(2) Wax Fractions

von Wettstein-Knowles (185, 186) and Jackson (76) have reported on the composition of barley surface waxes. Tulloch and Weenink (171) and Tulloch and Hoffman (169, 170) have reported on wheat, Triticum compactum Host., oats, A. sativa, and rye, S. cereale. Surface waxes of these species showed qualitative similarity to barley, thus, isolation of barley wax components was first based on the preliminary silicic acid column separation described by Tulloch and Weenink (171).

Efficiency of the column separation was monitored by thin-layer chromatography (TLC) on 5x20 cm silica gel G F-254 plates (Merck) developed in chloroform and visualized with iodine vapor and then 50% H_2SO_4 at 110°. Modification of initial procedures was required which resulted in three different columns being used. The conditions of each column are presented below and the separations achieved are compared in Table 3 to those reported by Tulloch and Weenink (171) for T. compactum.

Column 1. A hexane slurry of 6 ml of unactivated Unisil (Clarkson Chemical Co., Williamsport, Pa.) was poured into a 1.8 cm (i.d.) glass column having a frittered glass drip tip. A wax sample of 55 mg was

applied as a hexane suspension. Elution was performed as described in Table 3.

A yellow color appeared at the column top as development progressed. This colored portion was eluted and collected separately in the last 62 ml of the hexane:chloroform, 4:1 (v/v) eluent. The color was shown to be a contaminant of the distilled hexane as described under Column 2.

Column 2. A slurry of 6 ml of Unisil, activated at 130° for 5 hours was poured into a 1.8 cm (i.d.) glass column fitted with a fritted glass drip tip. A wax sample of 45 mg was applied in hexane. The elutropic series shown in Table 3 was employed.

To determine if the yellow color eluting from the first two columns was a contaminant of the hexane, 250 ml of the distilled hexane were concentrated to ca. 1-2 ml and subjected to TLC in chloroform. Several spots appeared in iodine vapor and with 50% H_2SO_4 spray. These spots corresponded with those found in the yellow fractions of columns 1 and 2. It was found that passing hexane through a short column of Unisil would remove these impurities. Silverstein et al. (151) confirm that aromatic impurities can be removed from hexane by silicic acid. Hexane purified in this manner was used in succeeding separations.

Column 3. A slurry of 12 ml of Unisil activated for 17 hours at 120° was poured in hexane into a 1.8 cm (i.d.) glass column fitted with a fritted glass drip tip. A wax sample of 112 mg was applied in hexane. The elution series shown in Table 3 was used.

The epicuticular alcohols were identified as a class from their blue color reaction after TLC when visualized with a vanillin-sulfuric acid spray at 110°. This chromogenic reagent was prepared by dissolving 1.0 g vanillin in 100 ml conc. H_2SO_4 (94). At a later time, co-chromatography with a standard alcohol, 1-tetracosanol, (Applied Science

Table 3. THIN-LAYER CHROMATOGRAPHIC COMPARISON OF ELUTION SERIES USED TO SEPARATE EPICUTICULAR WAXES OF BARLEY AND WHEAT LEAVES ON SILICIC ACID COLUMNS.

Eluents	Column Number and Type				
	Activated Biosil A[a]	Non-Activated Unisil		Activated Unisil	
	No. None	1	2	3	
	Rf Values and Composition[b]				
Hexane	0.74 (HC)	0.67 (HC, Es) 0.52 (Ca) 100 ml	0.72 (HC) 250 ml	0.72 (HC) 375 ml	
Hex:Chl.					Tube No.[c]
7:1	N.U.	N.U.	N.U.	Blank 0.72 (Es) 100 ml	1-3 4-5
5:1	N.U.	N.U.	0.67 (Es) 0.55 (Ca) 112 ml	0.70 (Es) 0.55 (Ca) 200 ml	1-3
4:1	N.U.	0.17 (Al)[d] 125 ml	0.16 (Al)[d] 120 ml	0.72 (tr.Es)[d,e] 0.55 (tr.Ca) 0.20 (Al) 300 ml	
4:1	N.U.	0.14 (Al)[f] 62 ml	0.16 (Al)[f] 120 ml	N.U.	
10-20% Chl. in Hex.	0.70 (Es)	N.U.	N.U.	N.U.	
1.5:1	N.U.	N.U.	N.U.	0.03-0.17 Streak 200 ml	
1:1	0.45 (β-Dik)	0.15 (Al) 100 ml	0.16 (Al) 0.00 (Ac) 160 ml	N.U.	
Chl.	0.15 (Al)	0.15 (Al) 0.00 (Ac) 100 ml	0.00 (Ac) 200 ml	0.21 (Al) 0.06 (?) 0.00 250 ml	
20% Ethanol in Chl.	0.06 (OH-β-Dik) 0.00 (Ac)	0.00-0.03 (?) 100 ml	0.00 (Ac) 100 ml	0.21 (Al) 0.00 (Ac) 150 ml	

Table 3. (Cont'd.)

[a]Initial procedure and results reported by Tullock and Weenink (171) for T. compactum.

[b]Identification of barley wax composition as described in MATERIALS AND METHODS and by comparison with data of Tulloch and Weenink (171). Development in chloroform on silica gel F-254 plates from Merck (Brinkman Instruments, Ins.)

[c]20 ml collected per tube.

[d]This fraction did not contain yellow impurities from hexane.

[e]Hexane impurities removed by silicic acid prior to use in column No. 3.

[f]This fraction contained the yellow impurities from hexane.

Hex. (hexane); Chl. (chloroform); HC (hydrocarbons); Es (esters); Al (alcohol); Dik (diketone); Ca (carbonyl); Ac (acids); tr (trace amount); N.U. (not used)

Laboratories, State College, Pa.) confirmed the identity.

To verify that the spot at Rf 0.67 in the hexane eluent of Column 1 contained esters along with hydrocarbons, a pair not always separated by TLC in chloroform (171), an aliquot of the hexane fraction was placed in a test tube with ca. 10 ml methanol and 10-15 mg of NaOH. This tube was placed into boiling water for 30 minutes and methanol was added to counter vaporization losses. TLC of this saponification milieu along with an untreated sample showed that the spot at 0.67 had largely disappeared from the treated sample. However, spots at Rf 0.18 and the origin appeared in the treated sample where none were found in the untreated sample. This indicated that alcohols and acids, respectively, had been freed from their esterified form.

Treatment with acetyl chloride (39) of a spot from the chloroform fraction of Column 1 on a TLC plate followed by development in chloroform along with an untreated spot, caused the spots at 0.16 and the origin to move to a higher Rf value. The alcohol had been esterified and the origin presumably contained free acids which migrated as acid anhydrides after the treatment. The Nilles and Schuetz (124) table of solvent properties indicated that an acid anhydride may be less polar than free acids, and, therefore, could rise off the origin in chloroform.

To establish the chromatographic behavior of the carbonyl reported by Jackson (76) for barley wax, a sample of whole wax was treated with Girard's "T" reagent (Fisher Chemical, Fairlawn, N. J.) according to Fieser and Fieser (40). Less than 10 mg of sample were combined with 0.5 g of the "T" reagent and 0.5 ml of conc. acetic acid in 5 ml of 95% ethanol. Following a 30 minute reflux, the mixture was transferred to a separatory funnel. Ethyl ether and saturated NaCl, 5 ml each, were

added. As revealed by TLC of the ether phase, the carbonyls had been derivatized and had partitioned into the water. The spot seen at Rf 0.52 remained in the untreated sample.

In some instances, whole waxes were separated into fractions by preparative TLC on silica gel H plates having a 500μ coating (Prekotes from Applied Science, Ann Arbor, Mich.). Approximately 20 mg of wax were applied in chloroform and the plates developed in chloroform. The desired fractions were: alcohols; hydrocarbons, esters and carbonyls; and acids plus other. The alcohol band was located by applying vanillin-sulfuric acid reagent along one edge of the developed plate and heating only that edge on a hot plate to produce a blue color in the alcohol zone. The respective zones were eluted from the silica gel with chloroform: methanol, 2:1 (v/v) in centrifuge tubes which were then spun at 1,000xg for five minutes to sediment the silica gel. Three such elutions were given to each fraction.

b. Hydrophobic Compounds Minus Wax, (H-W)

Before extraction, the surface wax was removed from barley seedlings as described in section IV.A.2.a.(1)(b). The crude extract was then passed through the Sephadex column described in section IV.B.1.

(1) Separation into Polar and Apolar Fractions

A slurry of Unisil in chloroform was degassed by water vacuum and poured into a 1.4 cm (i.d.) glass column fitted with a fritted glass drip tip. Approximately 1 ml of silicic acid was used per 10 mg of (H-W) applied and ca. 20 ml of chloroform were passed through the column before addition of the sample. The (H-W) apolar fraction was eluted with chloroform until the dark green pigments were eluted and a slower moving yellow band neared the bottom of the column. Methanol, 100 ml, was then used to elute the polar materials.

(2) Separation of the Polar Fraction

The polar lipids of plants are comprised chiefly of glycolipids and phospholipids (122). The system used to separate these two major groups was that described by Rouser et al. (136). A 10 g portion of Unisil (200-325 mesh) and later, Adsorbosil-CAB (200-250 mesh) from Applied Science, was slurried in chloroform into a 2.0 cm (i.d.) column fitted with a teflon stopcock. Elution was by chloroform, 80-100 ml, to remove apolar materials; acetone, 300 ml, to elute glycolipids, and methanol, 200 ml, to remove phospholipids. It was noted that the phospholipids contained an unknown compound later identified as gramine (see RESULTS, section IV.F.) which reacted with the vanillin-sulfuric acid reagent at room temperature to produce a light pink color and at 120 to produce a purple color. The phospholipids were bioassayed with this compound until a method could be devised to remove it.

Separations were monitored by TLC on 5x20 cm Merck Pre-Coated silica gel F-254 plates (250μcoating) (Brinkman Instruments, Inc., Westbury N. Y.) developed in chloroform:methanol:7N ammonium hydroxide, 60:35:5, (v/v/v) (Skidmore and Enteman, 1962). Phospholipids were identified by reaction with a phosphomolybdic acid spray (Applied Science) to produce a blue color at room temperature. Glycolipids, mono- and di-galactosyldiglycerides and sulfolipid, were visualized by the vanillin-sulfuric acid spray with which they produced a light red color at room temperature.

(a) Isolation of Individual Glycolipids

Preparative TLC was used to obtain purified glycolipids which were identified by co-chromatography with standards from Applied Science. The TLC plates were 20x20 cm^2, silica gel H Prekotes (500μ coating). Approximately 20 mg of glycolipid were applied to each plate followed by

development in the system of Skidmore and Enteman (153). A 1.5 cm strip of coating was isolated along one edge of the developed plate with a pencil point and the vanillin-sulfuric acid reagent applied with a Pasteur pipett. Selective heating of this strip with a hot plate disclosed the location of the desired glycolipid classes. These bands were carefully scraped and eluted as described for wax fractions (section IV.B.2.a(2).).

(b) Isolation of Neutral and Acidic Phospholipids

Cellex D, a diethylaminoethyl cellulose anion exchanger (DEAE) from Bio-Rad Laboratories (Richmond, Calif.) was prepared according to Rouser et al. (135). The fine particles were first decanted several times from an aqueous suspension. The cellulose was washed 3 times in a Büchner funnel. Each wash consisted of 1N HCl (100 ml), deionized water until neutral, 0.1N KOH, and deionized water until neutral. Following further washes of acetone, methanol and then ethanol, the cellulose was oven dried at 100° to a constant weight.

A 7.5 g portion of cleaned and dried Cellex D was mixed with excess conc. acetic acid and left overnight with stirring by a magnetic rod to remove the clumps. The resultant slurry was carefully poured down a glass rod into a 2.0 cm (i.d.) glass column fitted with a teflon stopcock. The final bed was ca. 20-22 cm high.

After eluting the excess acetic acid with 100 ml of methanol at 3 ml per minute, mixtures of chloroform/methanol were passed through the column with gradual enrichment in chloroform until the solvent ratio was reached in which the phospholipid sample would be applied. To separate the neutral from the acidic phospholipids, the column was stabilized in chloroform:ethanol, 2:1 (v/v).

Following the scheme of Rouser et al. (135), all of the neutral phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, lysophosphatidyl choline and lysophosphatidyl ethanolamine) were eluted with 200 ml of chloroform:methanol, 2:1. Next followed 100 ml of methanol to remove acetates and other salts in the sample. TLC of this fraction showed only traces of phospholipid and it was routinely discarded. Acidic phospholipids were generally not identified individually, although there was some evidence for the presence of phosphatidyl serine. As a group, the acidic phospholipids were eluted with 200 ml of chloroform:methanol, 4:1 (v/v) that was 0.05M in ammonium acetate. Another 100 ml of methanol were passed through the column and discarded, thereby removing ammonium acetate from the column. The column was then reactivated with 60 ml of conc. acetic acid. Excess acetic acid was eluted with 100 ml methanol which then served to maintain the column until it was prepared for the next separation. Fractions were concentrated in vacuo at 30°-35° and taken up in 2.0, 5.0, or 10.0 ml of chloroform.

To prepare the ammonium acetate solution for acidic phospholipid elution, 4 ml of 28% ammonia (concentrated NH_4OH) were added to the chloroform:methanol, 4:1 followed by 0.6 g conc. acetic acid. The NH_4OH was first filtered with a Millipore filtering system (Gellman filter pad, Metricel, Type VM-1, 5.0) to remove crystalline impurities.

With the possible exception of phosphatidyl serine, discussed later in this section, the acidic phospholipids were not eluted individually from the DEAE column, nor were they further isolated by preparative TLC. Four of the first silicic acid columns used to separate glycolipids from phospholipids were smaller than those described in section IV.B.2.b. (2), with the result that a glycolipid having the chromatographic (TLC and

DEAE anion exchange) and the chromogenic reaction (vanillin-sulfuric acid) of the sulfolipid was a contaminant of the acid phospholipids derived from those columns. These phospholipids were bioassayed with the contaminant present and the results interpreted with this fact in mind.

The ammonium acetate was removed from the acid phospholipids partly by means of the Sephadex G-25 column of section IV.B.1. Further salt removal was effected during in vacuo concentration, since ammonium acetate is slightly volatile (135). Repeated addition of chloroform:methanol, 2:1, to the sample aided in salt removal. In some instances, the acid phospholipids were partitioned between chloroform and the upper phase used for the Sephadex G-25 column.

Since an exchange of positive metal ions occurs between acid phospholipids and silicic acid (123), the possible influence of these ions on CLB behavior in the bioassay was investigated. The acid phospholipids from the DEAE column were partitioned against a saturated Na_2-EDTA solution to exchange sodium ions for those cations present with the phospholipids. In another case, acid phospholipids from two barley samples were combined and one-third remained untreated while two-thirds were washed with a saturated $CaCl_2$ solution to impart a heavy calcium concentration (19). From this two-thirds portion, one-half was further treated with Na_2-EDTA to replace the calcium with sodium. Three different classes of ion composition were thus available for bioassay.

It is not known for certain whether phosphatidyl serine was found in this study. After TLC in the chloroform:methanol:7N ammonium hydroxide system [section IV.B.2.b.(1)(a)], a ninhydrin positive spot was observed. This same compound eluted from the DEAE column with conc. acetic acid as

phosphatidyl serine did according to Rouser et al. (135). The Rf of that spot in this study (0.14-0.17) was similar to that for phosphatidyl serine reported by Skidmore and Enteman (153) for the same TLC system (0.19). In the RESULTS, this fraction has been called phosphatidyl serine, but is accompanied by a question mark (?). Benson and Mauro (15) did not find phosphatidyl serine in barley seedlings.

The neutral phospholipids were easily eluted from the DEAE column by chloroform:methanol, 2:1. It was in this fraction that gramine also eluted.

(c) Isolation of Individual Neutral Phospholipids

The elution scheme presented by Rouser et al. (137) to isolate individual neutral phospholipids from a DEAE column was seldom totally successful in this study. Even after considerable modification, some degree of fraction overlap usually resulted. Since several different ratios of chloroform:methanol were used from one DEAE column to the next, and given that eight columns were developed in the attempt to isolate individual neutral phospholipids, the qualitative results of these separations are presented only with the tabulation of results of their bioassay.

As a last resort, preparative TLC was twice used to obtain an individual class of neutral phospholipid. The concern for oxidation of the lipids as a likely result of TLC prevented a more extensive use of this technique in this area.

Another question which seemed open to bioassay was that of the influence of the fatty acid composition in a particular class of phospholipid. Three species of phosphatidyl choline were obtained from Applied Science Laboratories. These species were L-α-1-stearoyl-2-oleoyl lecithin, L-α-dilinoleoyl lecithin, and L-α-distearoyl lecithin.

Identification of neutral phospholipids was made through co-chromatography with standards from Applied Science (phosphatidyl choline and phosphatidyl ethanolamine). A ninhydrin reagent (0.3 g ninhydrin in 100 ml of ethanol) aided in identification of the ethanolamine phospholipids and Dragendorf's reagent (obtained from Applied Science) was used to visualize the choline phospholipids.

(d) Isolation of Gramine

Through observation of the chromatographic behavior on silicic acid of the phospholipid contaminant, it was found that much of it trailed the entire phospholipid fraction off silicic acid with the methanol eluent. It could then be collected pure in the last 50 ml of the 200 ml used. It was also found that this unknown could be removed from neutral phospholipids on the DEAE column with 75 ml of chloroform: methanol, 35:1 (v/v), although some phosphatidyl choline tended to elute with it.

Infrared data on the unknown was obtained in micropellet form (KBr), using the material eluted pure from the silicic acid column. A Perkin-Elmer 337 Grating Infrared Spectrometer was used. The IR data were compared to those contained in the Spec Finder volume of the Stadtler Index System. The best fitting compound was an indole, 5-amino-3-(dimethyl-aminomethyl)-indole.

Mass spectral data were obtained by a direct probe on a Bell and Howell 21-490 mass spectrometer which was interfaced to a Digital PDP 12 computer to provide summarized data for mass and mass intensity. The results indicated a molecular weight of 178 for the unknown. Since an alkaloid seemed a possibility, a check of library references on that topic led to Raffauf's work (132) on alkaloids in plants. Barley,

indeed, possessed an alkaloid called gramine, 3-(dimethylaminomethyl)-indole, a derivative of tryptophan (16). A quantity of gramine was obtained from Sigma Chemical Co. and its mass spectrum was found to be identical to that for the unknown isolated from barley. Similar congruence was found between the two IR spectrums; also, the TLC behavior and chromogenic reaction with vanillin-sulfuric acid reagent were alike for commercial and isolated gramine.

C. The Hydrophilic Fraction

1. Extraction of the Hydrophilic Compounds

When bioassays of the complete hydrophilic fraction were conducted, these compounds were obtained by the methods described in section IV.B.1. Another method, outlined in the next section, was used when subfractions of this fraction were bioassayed.

2. Fractionation of the Hydrophilic Compounds

Work by Seikel and Geissman (148) demonstrated the presence in barley leaves of a glycoflavone, saponarin. Harborne (55) stated that the leaves of wheat, oats, and barley contained glycoglavones, compounds otherwise rarely found in monocots and therefore, a characteristic chemical feature of the Gramineae. Thus, saponarin seemed a likely compound to bioassay as a botanically restricted secondary plant compound. Another barley glycoflavone, lutonarin, was only present in plants grown out-of-doors (147) and was not investigated in this study.

Gross extraction of saponarin followed the procedure of Harborne and Hall (56). Approximately 25 g of freshly cut barley leaves were refluxed in 400 ml of methanol for 2 hours. The extract was concentrated to ca. 2 ml *in vacuo* at 50°-55°, then transferred with deionized water and petroleum ether, 8:1 (v/v) to a 50 ml centrifuge tube fitted with a

glass stopper. Hydrophobic materials were partitioned into the pet ether followed by centrifugation at 1,000xg for 3 minutes. The resulting aqueous solution was taken to dryness *in vacuo* at 45°-50° and taken up in 5 ml of 10% methanol in water.

It was found rather serendipitously that a small column of Sephadex G-10 (fine grade) packed in water separated the saponarin quickly from most other hydrophilic materials present due to an adsorption effect which caused it to "hang up" on the column after most other substances had been eluted with water. To construct the column, ca. 10 g of Sephadex G-10 beads were swollen in water and poured into a 2.0 cm (i.d.) glass column having a teflon stopcock. The void volume as measured by a run of dextran blue dye was 9.5 ml and total volume (V_T) was 25 ml.

A 2 ml volume of extract was found to be satisfactory for application to the column. The first effort was collected as fractions in the volumes: 9.5 ml (void), 1.5 ml, 4.0 ml, 2x5.0 ml, 3x10.0 ml, 7.5 ml, and 62.5 ml. Initial characterization was by paper chromatography as described by Seikel and Geissman (148). Aliquots from each fraction were spotted onto Whatman No. 3 paper and developed in a mixture of equal volumes of n-butanol and 27% acetic acid in water. Under UV light, (254 nm), patterns of fluorescence were marked on the paper by pencil and the chromatogram exposed to ammonia vapors to visualize the saponarin which turns bright yellow under alkaline conditions (Seikel and Geissman, 1957).

Results from this column showed that the second column should be collected in fractions of 25.0 ml (including void volume), 37.5 ml and 75.0 ml. A diagramatic representation of these developed fractions is shown in Figure 1. Later columns may have had different elution volumes,

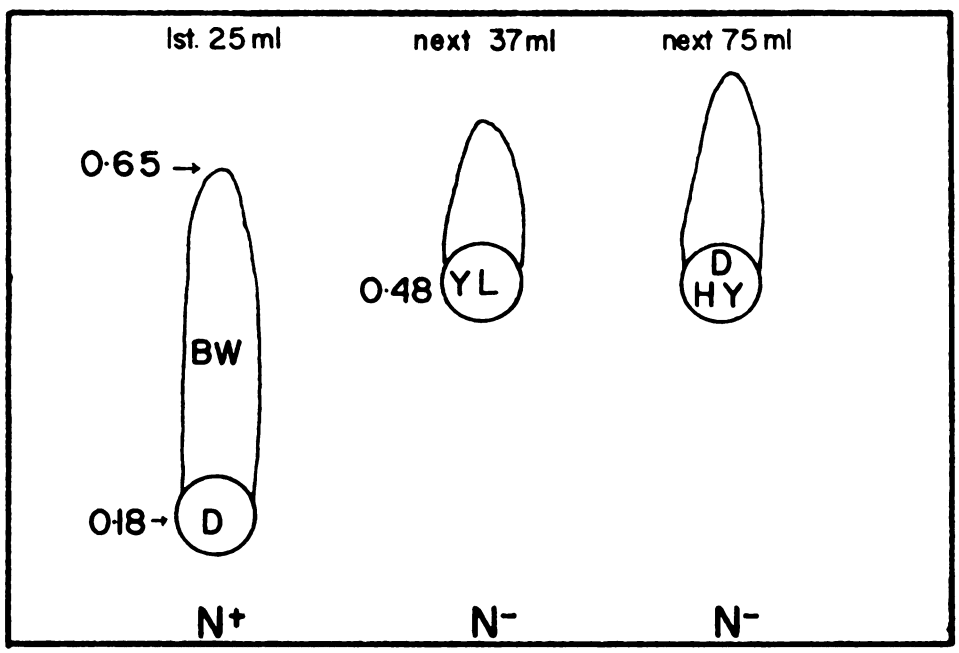

Figure 1. Diagram of a paper chromatogram of barley seedling hydrophilic compounds eluted from a Sephadex G-10 column.

System: Whatman No.3 paper developed in n-butanol:27% acetic acid, 1:1 (v/v).

D (dark appearance in UV light - 254 nm)

BW (blue-white fluoresence in UV light - 254 nm)

YL (light yellow in ammonia vapors)

HY (deep yellow in ammonia vapors)

N± (ninhydrin positive, ninhydrin negative)

but the qualitative patterns were similar. All of the ninhydrin reaction due to amino acids was found in the first 25 ml (V_T = 25 ml). The 2nd and 3rd fractions contained a substance which had an Rf of 0.48 and appeared dark under the UV lamp and bright yellow in ammonia vapor. Above these spots were areas which fluoresced blue-white under UV light. Concentrated samples were dissolved in 10% methanol in water and refrigerated at 4°-6°.

In the 2nd and 3rd fractions, material was seen to precipitate after a few days of refrigeration, an event not seen in the "whole" sample. Rinsing the precipitate in its volumetric flask several times with deionized water removed non-precipitates. The addition of 95% ethanol followed by sonication yielded a sample for UV analysis. The UV scan in 95% ethanol was performed on a Beckman DB-G spectrophotometer using a 1 cm quartz cell. The peaks obtained at 334 nm and 272 nm agreed with those reported by Seikel and Geissman (148) for saponarin. The precipitate also gave the bright yellow reaction with ammonia vapor on paper. This precitatate was bioassayed as indicated in RESULTS.

Analysis of an aliquot of the total methanol extract for gramine was accomplished by the method of Audette et al. (10). The extract was adjusted to pH 10 with KOH (2N), then extracted with chloroform into which gramine would partition. The test for gramine was positive. Subsequent TLC of the fractions from Sephadex G-10 on silica gel G in the system of Skidmore and Enteman (153) showed that gramine was predominantly in fraction No. 2, light in No. 3 and absent from No. 1. A similar examination of preserved hydrophilic materials obtained as in section IV.B.1 also revealed gramine to be present and, thus, probably present in the hydrophilic fractions bioassayed from other extracted samples.

An aniline-phosphoric reagent (20), was used to locate sugars in the fractions. TLC of these along with a sucrose standard showed that sugars were present only in fraction No. 1. The elution volumes and general composition of each fraction in the bioassays conducted have been included in the tabulated bioassay results.

To fractionate the first 25 ml sample from the Sephadex G-10 column, a Dowex 50-X12 cation exchange resin, 40-80 mesh, was used (Bio. Rad). A column of the resin (1.4x6.0 cm) was converted to the H^+ form by 10 ml of 2N HCl, then washed to neutrality with deionized water. The concentrated sample (5 ml) from the Sephadex column was applied to the Dowex-50 column and 30 ml of deionized water was used to elute neutral and anionic compounds. A 50 ml volume of 10% NH_4OH was used next to elute cations. Paper chromatography in the respective systems described, showed nearly all of the ninhydrin reaction to be with the basic fraction, but a faint ninhydrin reaction was seen in the neutral-anionic fraction. This latter ninhydrin positive zone was also positive to a sugar detecting reagent (aniline-phosphoric acid). The identity of the compound(s) was not established.

3. Non-Extracted Hydrophilic Compounds

Another approach to the elucidation of CLB feeding stimulants involved the bioassay of nutrient type chemicals available as shelf chemicals in the laboratory. Such compounds as sucrose, and various amino acids were tested in several concentrations and combinations. Synthetic mixtures of amino acids corresponding to those reported by Fauconneau (38) for a CLB host, orchard grass, Dactylis glomerata L. and a non-host, alfalfa, Medicago sativa L., were bioassayed at different concentrations. Though not a nutrient, indole-3-acetic acid was also bioassayed.

RESULTS

I. Validity of the Bioassay

The CLB did not respond to crude extracts of non-host plants when an extract of barley was present. Nor were these non-host extracts effective stimulants in the presence of a blank agar strip (Table 4).

II. Crude Extract

The crude extract was bioassayed to gain experience with the bioassay and to otbain an estimate of the range of subfraction concentrations to be used in later experiments. Figure 2 summarizes the data from the completely randomized experiments where one concentration at a time was studied. Figure 3 represents subsequent randomized complete block experiments where several concentrations were bioassayed at once, one test unit per day over four days.

Computer analysis of the data produced a series of coefficients for polynomial equations up through the 5th degree. A 4th degree and a 5th degree equation provided the best fit for the data in Figure 2 and Figure 3 respectively. The responses to all controls for analysis of the crude extract have been summarized in the lowermost curve of Figure 3. This curve was fitted by eye, and the responses were typical of those obtained throughout the remainder of the study for the control strips. Consequently, no other count data for controls has been presented graphically.

Table 4. RESPONSE OF FIELD-COLLECTED, ADULT CEREAL LEAF BEETLES TO CRUDE EXTRACT OF BARLEY AND NON-HOST PLANTS INCORPORATED INTO THREE PER CENT AGAR*.

Test Unit Combination	PPM	Counts[a]
Barley (*H. vulgare*)	7969.0	6.3
vs		
Pepper (*Capsicum annum* L.)	9211.0	0.5
Blank	0.0	1.8
vs		
C. annum	9211.0	1.5
H. vulgare	7969.0	5.4
vs		
Broccoli (*Brassica oleraceae italica* L.)	5903.0	1.0
Blank	0.0	0.8
vs		
B. o. italica	5903.0	1.3
H. vulgare	6289.0	3.3
vs		
P. sativum	7082.0	1.0
H. vulgare	6289.0	3.0
vs		
Kohlrabi (*B. o. caulo-rapa* L.)	5777.0	1.3
H. vulgare	6289.0	3.8
vs		
Sunflower (*Helianthus annuus* L.)	6233.0	0.3
H. vulgare	4469.0	3.1
vs		
Tomato (*Lycopersicon esculentum* Mill.)	5648.0	0.1

*25 beetles (fasted 24 hours) per test unit.

[a]Average number of beetles responding to each strip after one hour. Ten replicates.

Figure 2. Numerical response of newly emerged, unfed, adult cereal leaf beetles to the concentration of barley seedling crude extract in three per cent agar - I.

Data were collected from a series of completely randomized experiments of 5 replicates (•) or 3 replicates (o): 25 laboratory reared beetles per test unit.

Counts were averaged from two hourly observations of beetles for each experiment.

The curve of best fit was described by a 4th degree polynomial equation:

Coefficients	Index of Determination: 0.20
-215.7	Standard error of estimate for Y: 2.02
370.7	
-228.9	
61.7	
-6.1	

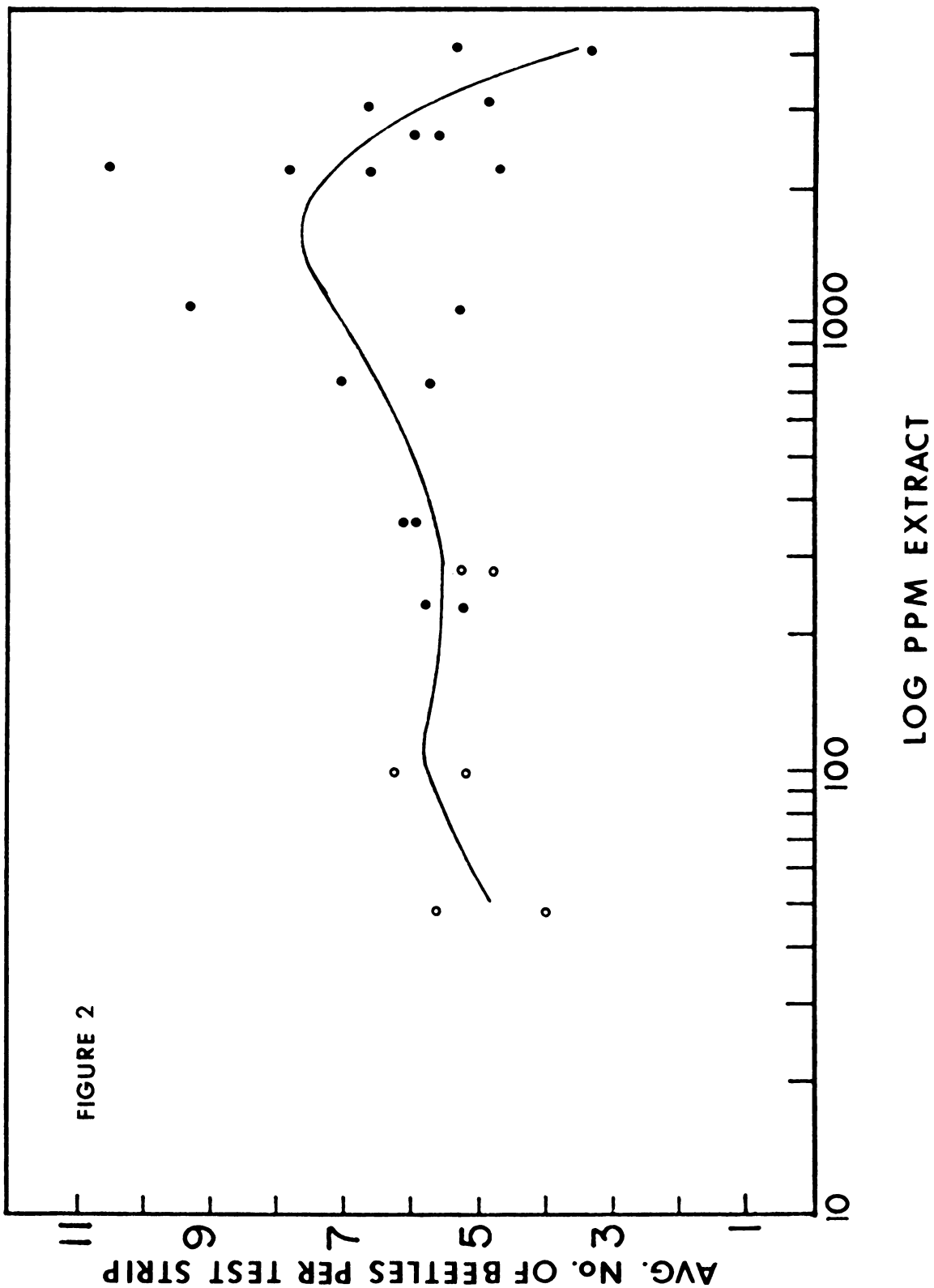
AVG. No. OF BEETLES PER TEST STRIP
11
9
7
5
3
1
10
100
1000
LOG PPM EXTRACT

FIGURE 2

Figure 3. Numerical response of newly emerged, unfed, adult cereal leaf beetles to the concentration of barley seedling crude extract in three per cent agar - II.

Data were collected from a series of randomized complete block experiments: 25 laboratory reared beetles per test unit; one test unit per concentration per day.

Counts were averaged from two hourly observations of beetles per day over four days.

Control data for Figure 2 and Figure 3 are shown together in Figure 3.

The curve of best fit for the extract data was described by a 5th degree polynomial equation:

Coefficients	Index of Determination: 0.43
286.2	Standard error of estimate for Y: 2.03
-665.1	
607.3	
-269.9	
58.8	
5.0	

The curve for the control data was simply fitted by eye.

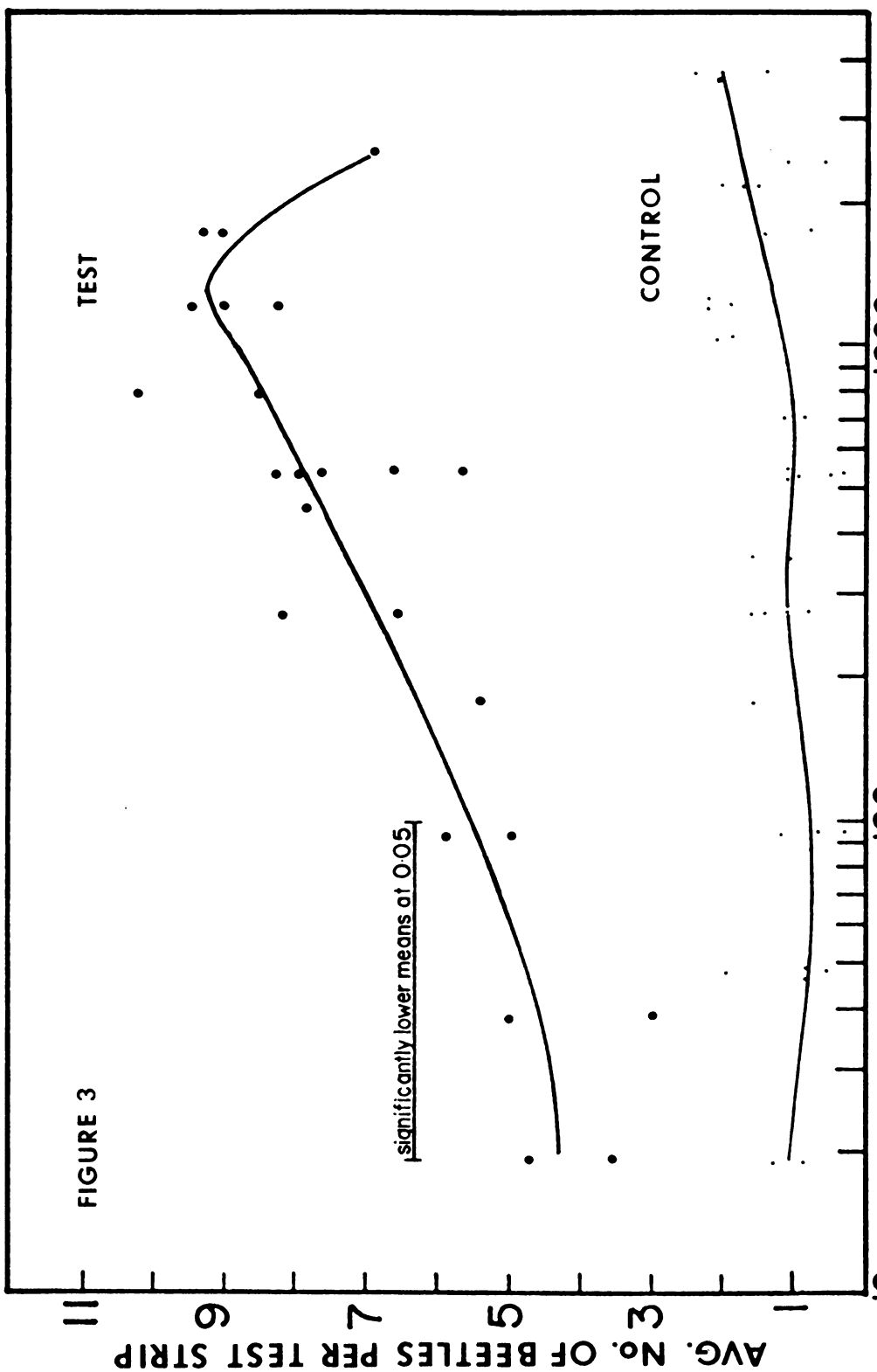
AVG. No. OF BEETLES PER TEST STRIP
11
9
7
5
3
1
10
100
1000
LOG PPM EXTRACT
TEST
CONTROL
significantly lower means at 0.05

FIGURE 3

No visual analysis was made on some of the early bioassays. Thus, a statistical treatment was performed on the count data for the test strips of two randomized complete block experiments where the same concentration levels were involved. The analysis of variance is presented in Table 5. It shows that the experimental effect was found to be insignificant at the 5% level. Both treatment effect and daily variability (reps) were significant at the 5% level. No treatment X experiment interaction was found. Since daily variability could be a significant factor in subsequent bioassays, the randomized complete block design was adopted to average out this variability.

Two groups of significantly different means (5% level) were found by Duncan's Multiple Range Test. The significantly lower range, 19.9 to 97.0 ppm, is under the horizontal line in Figure 3. Statistically, the range for the maximum response is very wide, ca. 300 to 1938 ppm. Figure 2 and Figure 3 were derived from two hourly counts of beetles per day.

Similar experiments were made with pea seedling crude extract. This extract was bioassayed alone at various concentrations and then combined with barley crude extract which was varied in concentration while the pea extract was held constant. The results of two separate experiments are shown in Figure 4.

There was a significant deterrent quality to the pea extract which prevented feeding on the pea extract-agar mixture (curve a). This effect was overcome, in part, by the presence of barley extract (curves a-1, b-1). Activity scores for curve a-1 indicated renewed feeding at a barley:pea ratio of ca. 1:1, while those for curve b-1 did not show renewed feeding until at a ratio of 2.3:1. In both cases, however, the

Table 5. ANOVA TABLE FOR NUMERICAL RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO CONCENTRATIONS OF BARLEY CRUDE EXTRACT IN THREE PER CENT AGAR†

Source	D. F.	Mean Square
Exp.	1	1.14282
Rep.	3	9.48812*
Error (A)	3	0.369059
Tmt.	6	47.0982**
Exp X Tmt	6	4.24703
Error (B)	36	2.52579

†25 beetles per test unit; two randomized complete block experiments analyzed together.

*Significance at the 5% level.

**Significance at the 1% level.

Figure 4. Response of newly emerged, adult cereal leaf beetles to the concentration of barley and pea seedling crude extract, separate and combined, in three per cent agar.

Data obtained from two randomized complete block experiments: 25 laboratory reared beetles per test unit; one test unit per concentration per day.

Counts were averaged from three hourly observations per day over four days.

Activity (in parentheses; control left, test right) was averaged from two daily agar damage scores for experiment a, a-1, and from four daily scores for experiment b, b-1: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

a - pea seedling extract

b - barley seedling extract

a-1, b-1 - 30 mg pea seedling crude extract in barley crude extract. PPM based on barley extract only.

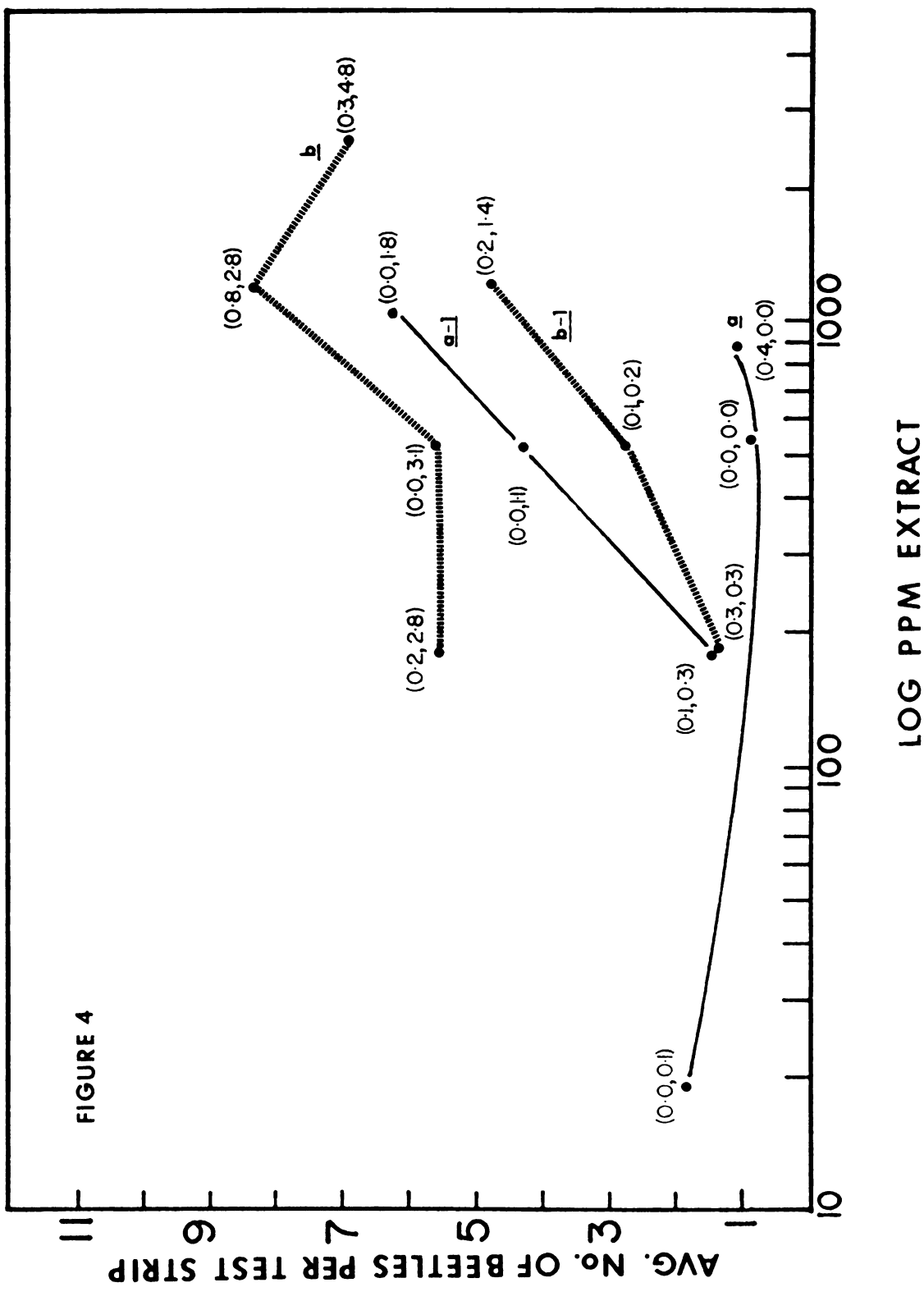
AVG. No. OF BEETLES PER TEST STRIP
11
9
7
5
3
1
10
100
1000
LOG PPM EXTRACT
b
(0·8,2·8)
(0·3,4·8)
g-1
(0·0,1·8)
(0·0,3·1)
(0·2,2·8)
b-1
(0·2,1·4)
(0·1,0·2)
(0·0,1·1)
(0·3,0·3)
(0·1,0·3)
g
(0·0,0·0)
(0·4,0·0)
(0·0,0·1)

FIGURE 4

attractive power of the combined extracts was enhanced at a 1:1 ratio over that of the pea extract alone, based on the higher CLB counts on the mixtures.

III. Hydrophobic Compounds Vs. Hydrophilic Compounds

Several bioassays of the hydrophobic and hydrophilic fraction each were performed. The data were analyzed by computer to derive coefficients for the polynomial equation of best fit (Figure 5). Greater sensitivity was shown by the beetles to the hydrophobic materials at the lower concentrations (up to 300 ppm), but the average count and activity relating to the hydrophilic compounds increased rapidly beyond this point to approximate equality with the hydrophobic fraction. Selected activity scores for control and test strips are given in parentheses. Based on the data of Figure 5, it was decided to emphasize the determination of hydrophobic feeding stimulants.

A similar bioassay for pea plants was performed for only the hydrophilic compounds (Table 6) because bioassays, to be reported later, indicated that the major deterrence of the pea extract came with the hydrophobic materials. The values for both count and feeding activity were lower for pea hydrophilic compounds than for similar concentrations of barley hydrophilic materials, but it was clear that the beetles fed upon this fraction from pea plants.

To determine the relative deterrent influence of the hydrophobic and hydrophilic fractions of pea seedlings to CLB feeding, a test was made. A 485 ppm portion of barley crude extract was added to 388 ppm of pea crude extract, to 227 ppm of pea hydrophobic compounds, to 161 ppm of pea hydrophilic compounds (each equivalent to 333 ppm of whole crude

Figure 5. Response of newly emerged, unfed, adult cereal leaf beetles to concentration of total hydrophobic and hydrophilic compounds of barley incorporated into three per cent agar.

Data summarized from randomized complete and incomplete block experiments: 25 laboratory reared beetles per test unit; one test unit per concentration per day.

Counts averaged from three hourly observations of beetles per day over four days.

Activity (selected values in parentheses; control left, test right) was averaged from four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

The curve of best fit for the hydrophobic compounds was described by a 3rd degree polynomial equation:

Coefficients	Index of Determination: 0.40
2.5	Standard Error of Estimate of Y: 1.99
-0.8	
2.9	
-0.7	

The curve of best fit for the hydrophilic compounds was described by a 3rd degree polynomial equation:

Coefficients	Index of Determination: 0.52
-0.2	Standard Error of Estimate for Y: 1.63
4.6	
-3.0	
0.7	

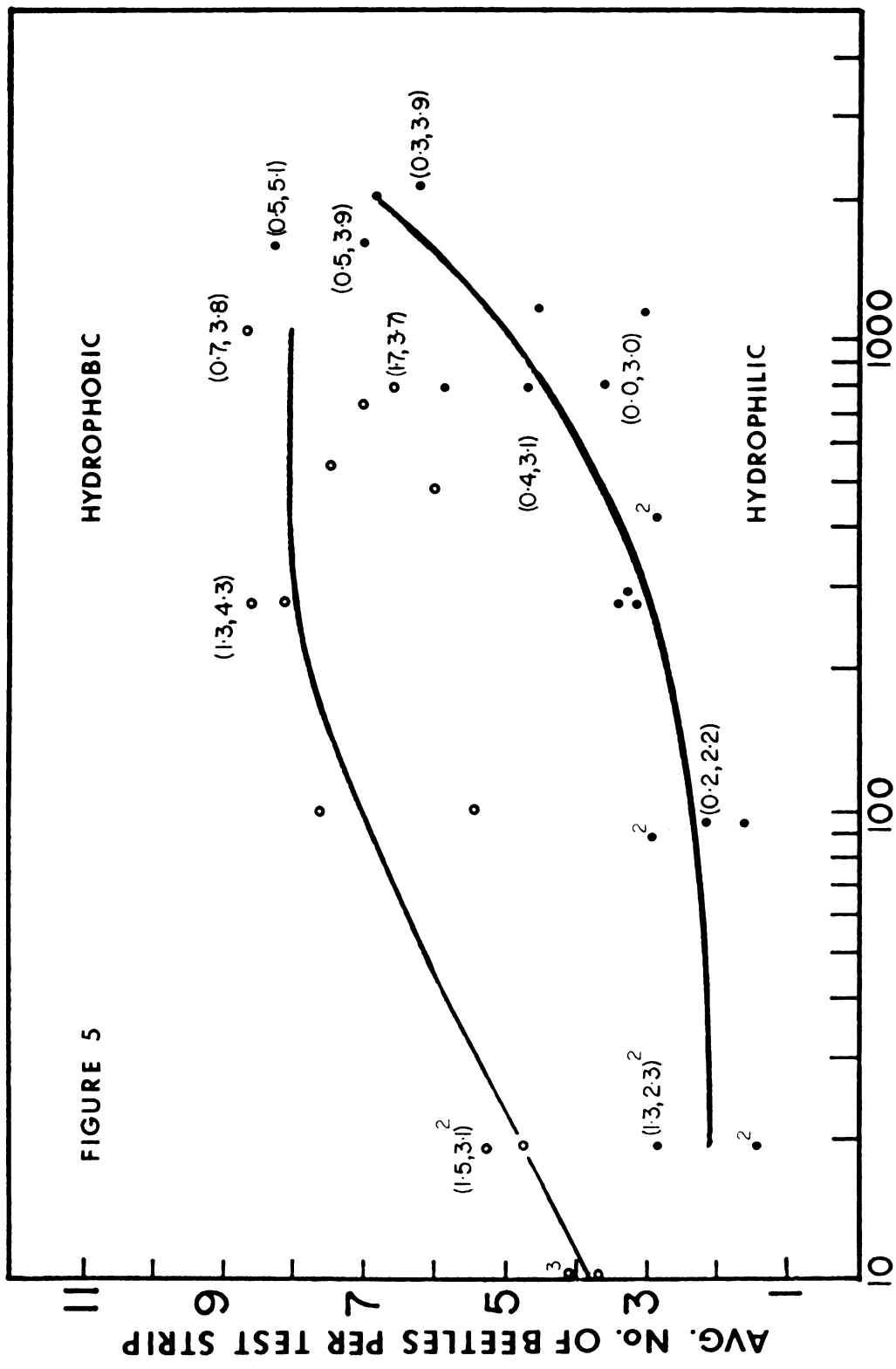
HYDROPHOBIC
HYDROPHILIC
(0·7, 3·8)
(0·5, 5·1)
(0·5, 3·9)
(0·3, 3·9)
(1·7, 3·7)
(1·3, 4·3)
(0·4, 3·1)
(0·0, 3·0)
(0·2, 2·2)
(1·5, 3·1)
(1·3, 2·3)
AVG. No. OF BEETLES PER TEST STRIP
LOG PPM EXTRACT
11
9
7
5
3
1
10
100
1000

FIGURE 5

Table 6. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO PEA SEEDLING HYDROPHILIC COMPOUNDS IN THREE PER CENT AGAR*.

Concentration of Extract - ppm	Average Counts[a]		Average Activity[b]	
	Control	Test	Control	Test
0.0	0.7	1.3	0.7	0.9
0.0	1.8	0.8	1.5	0.6
291.0	1.2	1.7	0.4	1.8
1164.0	1.0	2.5	0.2	1.3
1164.0	1.0	3.4	0.5	3.6
1746.0	0.7	2.2	0.2	2.0
1746	0.6	4.4	0.6	2.6

*25 laboratory reared beetles per test unit; one test unit per treatment per day; one control and one test agar strip per test unit.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

extract), and to both fractions recombined. Activity was compared to a blank test and to 485 ppm of barley crude extract in agar. The results (Table 7), confirmed that the hydrophilic materials were not the major source of deterrents detected in pea extract by the CLB. Yet, there was some indication that they did possess a small degree of deterrence, although this matter was not investigated further. Again, it was found that barley crude extract partially overcame the effect of pea crude extract when both were combined at approximately a 1:1 ratio.

IV. Hydrophobic Compounds

A. Complete Epicuticular Wax

Data from five experiments, some incomplete due to a lack of beetles, are presented in Table 8. These data revealed that feeding activity was stimulated by barley epitucular wax, but no consistent dose response was found when either average counts or average activity were considered.

Further evidence that the barley epicuticular wax was involved in the CLB feeding response is presented in Figure 6. After removal of the wax, the resulting hydrophobic compounds minus wax, (H-W), were extracted and bioassayed with the wax readded at 0.0, 10.0 or 16.0%. The epicuticular wax amounted to 15.3% of the total barley hydrophobic compounds.

Both epicuticular wax and other hydrophobic factors clearly were CLB feeding stimulants. Response to the recombined (H-W) and wax fractions was consistently better than that for corresponding (H-W) concentrations alone.

Investigation of pea epicuticular wax was also made. Simultaneously, the influence of pea wax upon CLB response to barley crude extract was

Table 7. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY CRUDE EXTRACT ADDED WITH EXTRACTS OF PEA SEEDLINGS TO THREE PER CENT AGAR*.

Barley Extract	Pea Extract	Average Count[a]		Average Activity[b]	
(ppm)	(ppm)	Control	Test[c]	Control	Test
0.0	0.0	1.3	1.1 (K)	0.4	0.7
485.0	0.0	1.2	8.0 (O)	0.6	4.6
485.0	R, 161.0	1.1	7.0 (N)	0.1	3.5
485.0	S, 388.0	1.1	3.3 (M)	0.1	1.9
485.0	T, 227.0	0.8	3.8 (M)	0.3	2.8
485.0	R+T=388.0	1.5	2.6 (L)	0.2	1.7

*25 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[c]Means sorted by Duncan's MRT. Values opposite the same letter were not significantly different at the 5% level. Transformation: $(Y)^{\frac{1}{2}}$

R (pea hydrophilic compounds), S (pea crude extract), T (pea hydrophobic compounds).

Table 8. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY EPICUTICULAR WAX INCORPORATED INTO THREE PER CENT AGAR.*

Wax Concentration (ppm)	Experimental Type 1[a] Average Count[c] Test	2[b] Average Count[c] Control	2[b] Average Count[c] Test	2[b] Average Activity[d] Control	2[b] Average Activity[d] Test
0.0	1.9[7]	1.8	1.2[12]	0.9	0.7[8]
1.0	1.5[5]	---	---	---	---
4.8	---	1.6	2.5	0.9	2.4
9.7	2.7[7]	2.3	4.7[4]	1.6	5.0[2]
19.4	3.3[3]	1.9	2.9	1.1	3.1[6]
38.8	---	1.4	3.9[8]	0.8	3.4[6]
58.0	2.2[1]	---	---	---	---
87.0	---	1.9	3.1	1.0	1.0[2]
97.0	2.4	---	---	---	---
116.0	---	1.5	3.7	1.0	4.7
126.0	---	3.2	3.8	1.7	3.5[2]
155.0	---	2.9	2.2	1.4	3.1

*25 beetles per test unit; one test unit per concentration per day.

[a]Type 1 test units contained two agar strips from the same treatment per test unit; average is for two hourly counts per day; no visual analysis.

[b]Type 2 test units contained one control and one test agar strip per test unit.

[c]Average of three hourly counts of beetles per strip per day over four days.

[d]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

1-12 Superscript applies to all values to its left until superseded indicating the number of days in the computed average if not four days.

Figure 6. Numerical response of newly emerged, unfed, adult cereal leaf beetles to the concentration of dewaxed-barley hydrophobic compounds with and without the readdition of epicuticular waxes.

Summarized data from randomized complete and imcomplete block experiments: 25 laboratory reared beetles per test unit; one test unit per concentration per day.

Counts were averaged from three hourly observations per day over four days. No visual analysis was performed.

The curve of best fit for "minus wax" data was described by a 3rd degree polynomial equation:

Coefficients	Index of determination: 0.26
12.4	Standard error of estimate for Y: 1.61
-21.6	
14.2	
-2.7	

The curve of best fit for "wax readded" data was described by a 2nd degree polynomial equation:

Coefficients	Index of determination: 0.20
-0.7	Standard error of estimate for Y: 2.21
5.6	
-1.0	

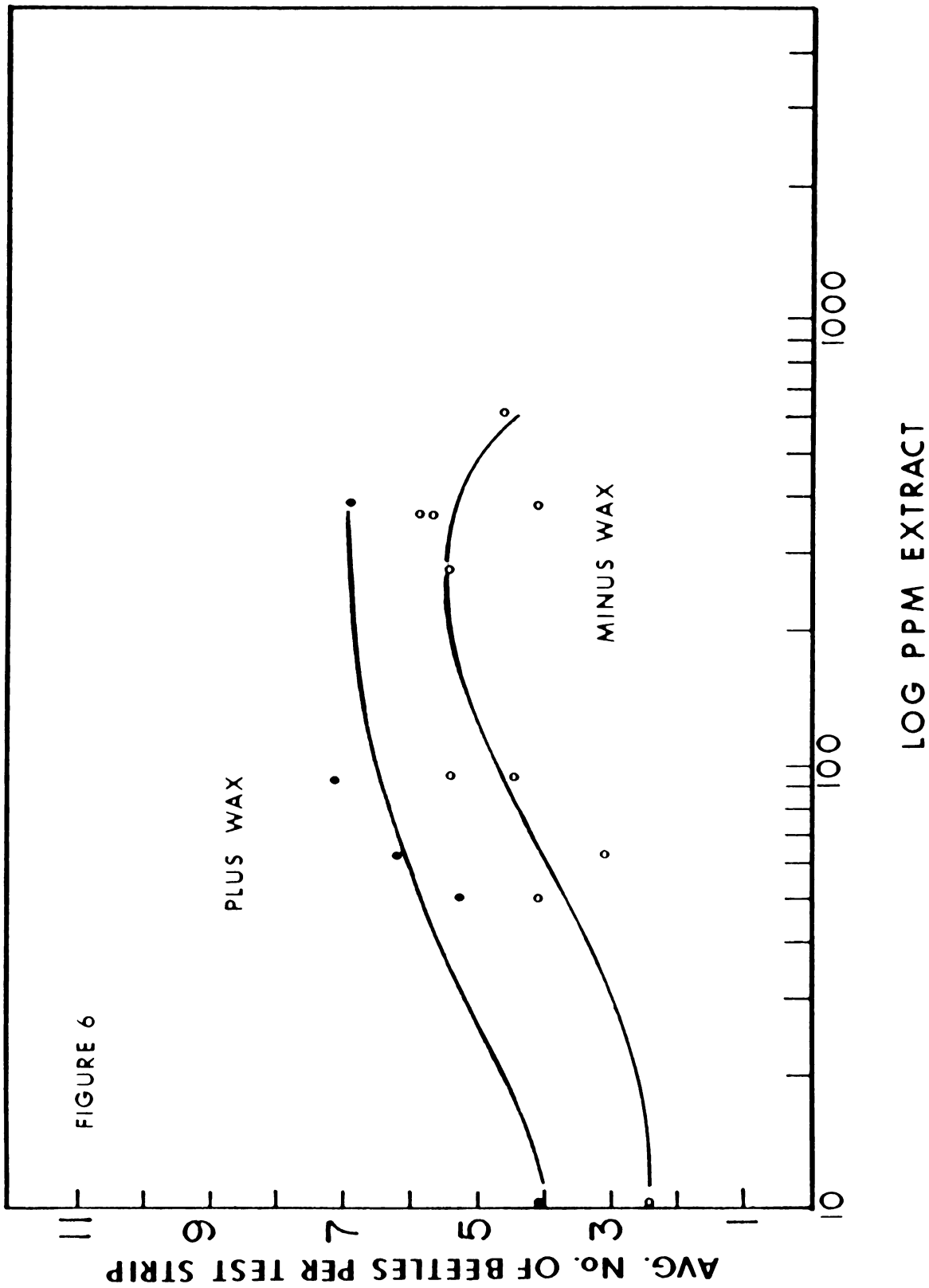
AVG. No. OF BEETLES PER TEST STRIP
11
9
7
5
3
1
10
100
1000
LOG PPM EXTRACT
PLUS WAX
MINUS WAX

FIGURE 6

tested. The results of both experiments are presented in Table 9. Response to pea wax was very low when barley extract was absent from the agar. These waxes also significantly reduced the numerical response and feeding activity of the CLB to crude barley extract which indicated a deterrent effect for the pea wax. However, there was no increased deterrence with increased pea wax content. The deterrent quality of pea wax was confirmed by adding it to 40 mg of sucrose (0.002M) (Table 10), which preliminary work had shown would stimulate a consistent, low level of activity. One ppm of pea wax significantly reduced the CLB response to the sucrose.

B. Epicuticular Wax Fractions

To locate the activity of the barley epicuticular wax, silicic acid columns were first used to separate the wax. Various degrees of fraction purity resulted. It was apparent (Table 11) that the major portion, perhaps all, of the activity found was due to the alcohols. The effect was seen with fractions bioassayed alone or in combination with sucrose.

The three fractions obtained by preparative TLC, the alcohols; the hydrocarbons, esters, and carbonyls; and the acids plus other, were bioassayed (Table 12). To avoid induced activity on the control strip by the test strip, only strips from the same treatment were used in a test unit. The experiment was designed to determine whether any interaction of wax fractions might produce increased activity. No interaction of non-alcohol fractions was observed, while any combination containing the alcohols was an effective stimulant. A 38.8 ppm sample of silica gel H from a preparative TLC plate was added to a whole wax fraction (38.8 ppm) and it reduced the response to the wax.

Table 9. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO EPICUTICULAR WAX OF PEA SEEDLINGS, ALONE AND WITH BARLEY CRUDE EXTRACT IN THREE PER CENT AGAR*.

Treatment Mixture		Average Count[a]		Average Activity[b]	
Barley - ppm	Pea wax - ppm	Control	Test	Control	Test
0.0	0.0	1.4	2.8	0.7	0.9
0.0	9.7	1.0	2.0	0.1	0.2
0.0	19.4	0.8	1.7	0.4	0.2^3
0.0	48.5	1.3	0.9	0.3	0.1
291.0	0.0	1.0	7.6x	0.6	4.7
291.0	29.0	0.1	4.1x	0.3	2.6
291.0	58.0	0.9	5.6x	0.6	3.1
291.0	87.3	1.4	4.9x	0.3	3.2

*25 laboratory reared beetles, one control and one test agar strip per test unit; one test unit per treatment per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

x [no significant difference at the 5% level among those means of count data analyzed. Transformation: $(Y)^{\frac{1}{2}}$]

[3]Superscript applies to all values to its left, indicating the number of days in computed averages if not four days.

Table 10. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO SUCROSE IN THE PRESENCE OF PEA SEEDLING EPICUTICULAR WAX IN THREE PER CENT AGAR*.

Treatment Mixture		Average Count[a]		Average Activity[b]	
Sucrose (ppm)	Pea wax (ppm)	Control	Test	Control	Test
0.0	0.0	0.3	1.7	0.7	1.8[1]
776.0	0.0	0.4	3.1	0.6	2.4
776.0	1.0	0.0	2.0	0.0	0.6[1]
776.0	1.9	0.3	2.3	0.4	1.2[1]
776.0	9.7	0.5	1.5	0.0	0.9[2]
776.0	27.2	0.8	3.4	0.3	0.6

*25 laboratory reared beetles, one control and one test agar strip per test unit; one test unit per treatment per day.

[a]Average of three hourly count of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

1-2 Superscript applies to all values to its left, indicating the number of days in the computed averages if not four days.

Table 11. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY EPICUTICULAR WAX FRACTIONS ELUTED FROM SILICIC ACID COLUMNS AND INCORPORATED INTO THREE PER CENT AGAR WITH OR WITHOUT SUCROSE*.

Wax Fraction With or Without Sucrose						Sucrose Control[s]	
Content	PPM	Average Count[a] Control	Test	Average Activity[b] Control	Test	Count[a] Test	Activity[b] Test
n	7.8	1.2	1.3	3.0	1.8[2]	---	---
n,o,p	5.4	1.5	0.9	2.1	0.9[3]	---	---
n,o,p	10.9	0.9	1.3[i]	1.4	1.5[i]	---[i]	---
n,s	0.8	---	3.4[i,4]	---	---	2.1[i,1]	---
n,s	0.8	---	1.7[i]	---	---[2]	1.3[i]	---
o,p	4.7	1.7	1.0[i]	1.0	1.9[i]	---[i]	---[i]
o,s	8.5	---	0.7[i]	---	1.0[1,2]	1.5[i]	1.1[i]
p,s	1.9	---	1.0[i]	---	1.6[i]	1.4[i]	2.0[i]
q	0.8	1.6	1.6	0.9	3.4	---	---
q	12.4	0.5	2.0[i]	1.1	3.0[i]	---[i]	---
q,s	17.5	---	3.1[i]	---	2.6[i]	1.4[i]	1.1[i]
q,s	33.0	---	3.5[i]	---	4.4[1,2]	1.5[i]	1.1[i]
q,s	34.9	---	5.3[i]	---	3.9[i]	1.4[i]	1.1[i]
ql	2.3	1.5	2.7[i]	2.1	3.2[i,3]	---	---
ql,s	23.3	---	4.3[i]	---	4.3[i,2]	---[i]	---[i]
ql,s	46.6	---	3.9[i]	---	3.5[i]	1.4[i]	1.1[i]
q,r	0.4	0.9	2.5[i]	0.8	3.6[i]	---[i]	---[i]
q,r,s	11.6	---	3.6[i]	---	3.8[i]	1.9[i]	1.9[i]
r	1.0	1.4	1.3[i]	0.9	0.7[i,2]	---[i]	---[i]
r,s	X	---	2.0[i]	---	1.1[i]	1.6[i]	0.9[i]
r,s	2X	---	2.3[i]	---	1.5[i]	1.6[i]	0.9[i]

*25 laboratory reared beetles, one control, one test agar strip per test unit, except "i" units; one test per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[i]Test units contain two strips from the same treatment.

n (hydrocarbons); o (esters); p (carbonyl); q (alcohol); ql (alcohol plus yellow contaminants from hexane); r (acid, other); s (776 ppm sucrose).

X (weightless sample).

[1-3]Superscript applies to all values to its left until superseded, indicating the number of days in the computed average if not four days.

Table 12. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY EPICUTICULAR WAX FRACTIONS OBTAINED BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY AND INCORPORATED INTO THREE PER CENT AGAR*.

Wax Fraction	PPM	Average Counts[a,1]	Average Activity[b]
Control	0.0	1.8 (D)	1.1
U	7.0	1.3 (C)	0.9
R	25.0	3.4 (H)	3.9
L^r	1.2	2.0 (DE)	1.1
U, R	7.0, 25.0	2.7 (EF)	2.4
U, L^r	7.0, 1.2	1.8 (D)	0.9
R, L	25.0, 1.2	2.4 (DE)	1.9
U, R, L	7.0, 25.0, 1.2	3.3 (H)	2.8
Whole wax	38.4	2.9 (G)	3.3
Whole wax, gel H	38.4, 38.4	2.7 (EF)	2.1
L^s	1.2	2.0	0.9
R, L^s	25.0, 1.2	3.4	2.6

*25 laboratory reared beetles, two agar strips from the same treatment per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per strip over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[r]Slight indication of alcohols by TLC.

[s]After removal of trace alcohol from (L) by preparative TLC.

U (hydrocarbons, esters, carbonyl), R (alcohols), L (acids, other).

[1]Means sorted by Duncan's MRT. Values opposite the same letter were not significantly different at the 5% level. Transformation: $(Y)^{\frac{1}{2}}$

When only field-collected beetles were available, several bioassays of wax fractions were also performed with samples obtained using either silicic acid columns or preparative TLC. These beetles had fed on barley or native grasses as adults and were of unknown age and they were less responsive to the bioassay than the newly emerged, unfed adults produced in the laboratory. Two similar agar strips from one treatment were provided in each test unit and either a blank test unit or a sucrose test unit provided the control. Activity was rated as to how much above the respective control each test unit scored. The column of activity values in both Table 13 and Table 14 showed again that only the alcohol fraction stimulated a significant response, with or without the presence of sucrose.

Samples of the primary alcohols present in barley epicuticular wax were obtained commercially and bioassayed (Table 15). When comparable concentrations were compared in the absence of sucrose, the progression was from low or no activity for 1-docosanol (C-22), to better activity for 1-tetracosanol (C-24), to still better activity for 1-hexacosanol (C-26). The differences were considered significant. Those tests containing sucrose were too incomplete to be conclusive.

The field-collected beetles were tested with the commercially obtained alcohols, both with and without sucrose (Table 16). Without sucrose, the response beyond a blank control was very light for each alcohol bioassayed alone. When the C-26 and C-22 alcohols were mixed in a ratio of 20:1 there was a significant increase in activity beyond the blank control.

Field-collected beetles did not seem to prefer any sucrose-alcohol mixture to the sucrose control (Table 16), but mixtures of the C-26 and C-22 alcohols at ratios from 20:1 to 1:1 (C-26:C-22, wt/wt), showed

Table 13. RESPONSE OF FIELD-COLLECTED, ADULT CEREAL LEAF BEETLES TO FRACTIONS OF BARLEY EPICUTICULAR WAX ELUTED FROM SILICIC ACID COLUMNS AND INCORPORATED INTO THREE PER CENT AGAR*.

Wax Fraction				Sucrose	Blank
Component	PPM	Count[a]	Activity[b]	Count[a]	Count[a]
n,s	0.4	2.4	0.0	2.4	---
n,s	1.2	2.2	0.0	2.4	---
n,s	2.9	2.8	0.0	2.4	---
n,o,p,s	11.2	1.0	0.0	1.2	---
n,o,p,s	22.5	1.4	0.0	1.2	---
n,o,p,s	33.7	1.0	0.0	1.2	---
o,s	8.5	2.0	0.8	1.8	---
o,s	17.0	1.8	0.3	1.8	---
p	9.3	1.0	0.0	---	1.1
p,s	3.9	2.3	0.3	2.3	---
p,s	7.8	2.8	1.3	2.3	---
p,s	9.3	1.6	1.0	1.4	---
q	38.8	1.4	2.7	---	1.5
q,s	19.4	1.5	2.5	0.9	---
q,s	29.0	1.4	1.8	0.9	---
q,s	38.8	1.7	2.5	0.9	---
q,s	38.8	2.3	1.8	1.8	---
q,s	38.8	3.9	2.0	2.4	---
q,s	48.0	1.7	3.3	0.9	---
q,r,s	1.2	2.7	1.3	2.3	---
q,r,s	3.5	2.8	2.5	2.3	---
r	1.7	1.1	0.0	---	1.5
r,s	1.2	2.7	0.8	2.3	---
r,s	3.5	2.0	0.3	2.3	---

*25 beetles, fasted, but watered for two days prior to test, and two agar strips from the same treatment per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily scores of agar damage relative to control: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

n (hydrocarbons), o (esters), p (carbonyl), q (alcohol), r (acid), s (776 ppm sucrose).

Table 14. RESPONSE OF FIELD-COLLECTED, ADULT CEREAL LEAF BEETLES TO EPICUTICULAR WAX FRACTIONS OF BARLEY OBTAINED BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY AND INCORPORATED INTO THREE PER CENT AGAR*.

Component	PPM	Average Count[a]	Average Activity[b]
None	0.0	1.3	0.0 (The base score)
Whole Wax	38.0	0.6	1.5
U	Q	1.2	0.3
R	38.8	0.5	2.0
L	Q	0.5	0.8
U, R	Q, 38.8	1.3	2.3
U, L	Q, Q	1.0	0.8
R, L	38.8, Q	1.6	2.8

*25 beetles, fasted, but watered for two days prior to test, and two agar strips from the same treatment per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily scores of agar damage relative to control: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

Q equivalent to 38.8 ppm alcohol (equal volumes taken from equal volumes).

U (hydrocarbons, esters, carbonyl), R (alcohols), L (acids, other).

Table 15. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO COMMERCIALLY OBTAINED PRIMARY ALCOHOLS IN THREE PER CENT AGAR WITH AND WITHOUT SUCROSE*.

Alcohol				Sucrose[f]	
Chain length	PPM	Count[a]	Activity[b]	Count[a]	Activity[b]
C-22	19.4	0.9	0.6	---	---
	38.8	1.3	0.7	---	---
C-24f	1.9	2.7	1.4^{2}_{4}	1.0	0.5^{1}_{2}
	9.7	2.2	3.3^{4}	2.0	2.8^{2}
C-24	19.4	1.7	1.1	---	---
C-24f	19.4	2.8	3.1^{4}	0.8	2.0^{1}_{2}
	29.0	2.5	1.9	2.0	2.8^{2}
	38.0	2.0	2.0	---	---
C-26	0.2	1.3	1.3	---	---
	19.4	1.8	2.8	---	---
	19.4	2.5	2.1	---	---
	38.8	2.9	3.0	---	---

*25 laboratory reared beetles, two agar strips from the same treatment per test unit; one test unit per concentration per day.

[a]Averages of three hourly counts of beetles per strip per day over four days.

[b]Average of four daily scores of agar damage: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[f](776 ppm sucrose added to test agar).

[1,2]Superscript applies to all values to its left until superseded, indicating the number of days in computed averages if not four days.

Table 16. RESPONSE OF FIELD-COLLECTED, ADULT CEREAL LEAF BEETLES TO COMMERCIALLY OBTAINED PRIMARY ALCOHOLS INCORPORATED INTO THREE PER CENT AGAR WITH AND WITHOUT SUCROSE*.

Alcohol				Sucrose[f]
Chain length	PPM	Count[a]	Activity[b]	Count[a]
C-22f	1.9	1.2	0.0	1.8
	1.9	2.4	0.0	2.8
C-22	1.9	2.0	0.0	---
C-22f	3.8	1.2	0.0	1.8
C-22	28.1	1.4	0.3	---
C-22f	38.8	1.5	0.3	1.8
	38.8	2.1	0.0	2.8
C-24f	1.9	2.3	0.0	2.8
C-24	28.1	1.5	0.0	---
C-24f	38.8	2.6	1.3	2.8
C-26	28.1	1.7	0.8	---
C-26f	38.8	1.8	0.5	1.8
	38.8	1.8	0.0	2.8
	38.8	3.0	0.7	2.8
C-22, 26	1.9, 38.8	1.8	1.5	---
C-22, 26f	1.9, 38.8	1.8	2.3	1.8
	1.9, 38.8	2.6	0.3	2.8
	3.8, 38.8	2.7	2.3	1.8
	38.8, 38.8	2.0	2.5	1.8
C-24, 26f	1.9, 38.8	2.3	0.0	2.8
C-22, 24, 26f	1.9, 1.9, 38.8	2.6	1.3	2.8

*25 beetles, fasted, but watered, for two days prior to test, and two agar strips from the same treatment per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[f](776 ppm sucrose added to the treatment).

significant activity relative to the sucrose control. This result was in agreement with the similar test without sucrose. The combination of C-26 and C-24 alcohols showed no activity over control, but was tested only once. The three alcohols together did not show any greater response than the C-22 and C-26 combinations.

C. Hydrophobic Compounds Minus Wax, (H-W)

The results of bioassay of the hydrophobic compounds minus wax, (H-W), from barley is presented in Figure 6 where barley epicuticular wax data have been presented also. The (H-W) fraction possesses stimulant qualities by itself.

To determine whether the pea epicuticular wax was the sole deterrent source to the CLB seen in Table 9 and Figure 4, pea seedlings were dewaxed before extraction. The resulting (H-W) fraction was bioassayed alone and at two levels in combination with 482 ppm of barley crude extract (Table 17). The pea (H-W) fraction caused overall activity to be less within the test units (control plus test) at both concentrations bioassayed alone than was found for the blank test unit. This fraction, in combination at 169 ppm with 482 ppm of barley crude extract, significantly reduced the CLB feeding response toward the barley agar. As the amount of pea (H-W) added was increased and then supplemented with 33 ppm of pea surface wax, there was a significant trend to greater reduction in feeding response to the barley extract.

D. (H-W) Apolar Fraction Vs. (H-W) Polar Fraction

Having determined that the barley (H-W) fraction was an effective stimulant (Figure 6), this fraction was further separated to more closely isolate the active principles. Initially, the (H-W) apolar compounds

Table 17. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO DEWAXED PEA SEEDLING HYDROPHOBIC COMPOUNDS INCORPORATED WITH AND WITHOUT BARLEY CRUDE EXTRACT INTO THREE PER CENT AGAR*.

Treatment Mixture		Average Count[a]		Average Activity[b]	
Barley (ppm)	Pea (ppm)	Control	Test[c]	Control	Test
0.0	0.0	0.8	1.1	0.7	0.9
0.0	291.0	0.1	1.1	0.2	0.0
0.0	482.0	1.0	0.5	0.2	0.1
482.0	0.0	0.6	8.2 (L)	0.7	4.8
482.0	169.0	0.2	4.3 (K)	0.4	2.1
482.0	337.0	0.5	2.4 (K)	0.3	1.8
482.0	337.0, 33.0[d]	0.6	3.3 (K)	0.3	1.8

*25 laboratory reared beetles, one control and one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[c]Means sorted by Duncan's MRT. Values opposite the same letter were not significantly different at the 5% level. Transformation: $(Y+1)^{\frac{1}{2}}$

[d]Pea epicuticular wax.

were separated from the (H-W) polar compounds by silicic acid columns and each fraction was bioassayed at concentrations which made each equivalent to the same amount of total (H-W). A positive dose-response was found with the (H-W) polar, but none was found among the (H-W) apolar bioassays (Figure 7).

The (H-W) apolar and (H-W) polar fractions of pea seedlings were bioassayed alone and combined with barley crude extract (Table 18). Neither class of compounds was able to stimulate feeding behavior by itself. The (H-W) apolar group was strongly deterrent at the levels tested and significantly reduced response toward the barley extract. Pea (H-W) polar compounds also reduced the activity (but not the counts) toward barley extract, but not nearly as much as did (H-W) apolars and then only at much greater concentrations than required of (H-W) apolar compounds.

To answer the reciprocal question for barley, location of fractions effective in overcoming the deterrence of pea extract was determined. Two experiments were directed to this question (Table 19). The pea extract significantly reduced the response to both hydrophobic and to hydrophilic compounds of barley. Yet, the hydrophobic compounds slightly, but significantly, more effectively counteracted the deterrence of the pea extract despite being at half the concentration of the hydrophilic compounds.

Since barley crude extract at a 1:1 ratio with pea crude extract was able to overcome the deterrence of pea extract (Figure 4), various hydrophobic fractions of barley were readded to the barley hydrophilic materials in the presence of 582 ppm of pea crude extract (Table 19). Each was readded in such amounts that it was equivalent to its level in

Figure 7. Response of newly emerged, unfed, adult cereal leaf beetles to the concentration of dewaxed-barley seedling hydrophobic polar and apolar compounds in three per cent agar.

Data summarized from randomized complete and incomplete block experiments: 25 laboratory reared beetles per test unit; one test unit per concentration per day.

Counts were averaged from three hourly observations of beetles per day over four days.

Activity (in parentheses; control left, test right) was averaged from four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

The curve of best fit for polar compounds data was described by a 4th degree polynomial equation:

Coefficients	Index of determination: 0.56
2.2	Standard error of estimate for Y: 1.36
6.3	
-14.4	
9.2	
1.7	

The curve of best fit for apolar compounds was described by a 3rd degree polynomial equation:

Coefficients	Index of determination: 0.16
42.0	Standard error of estimate for Y: 1.26
-59.4	
28.9	
-4.6	

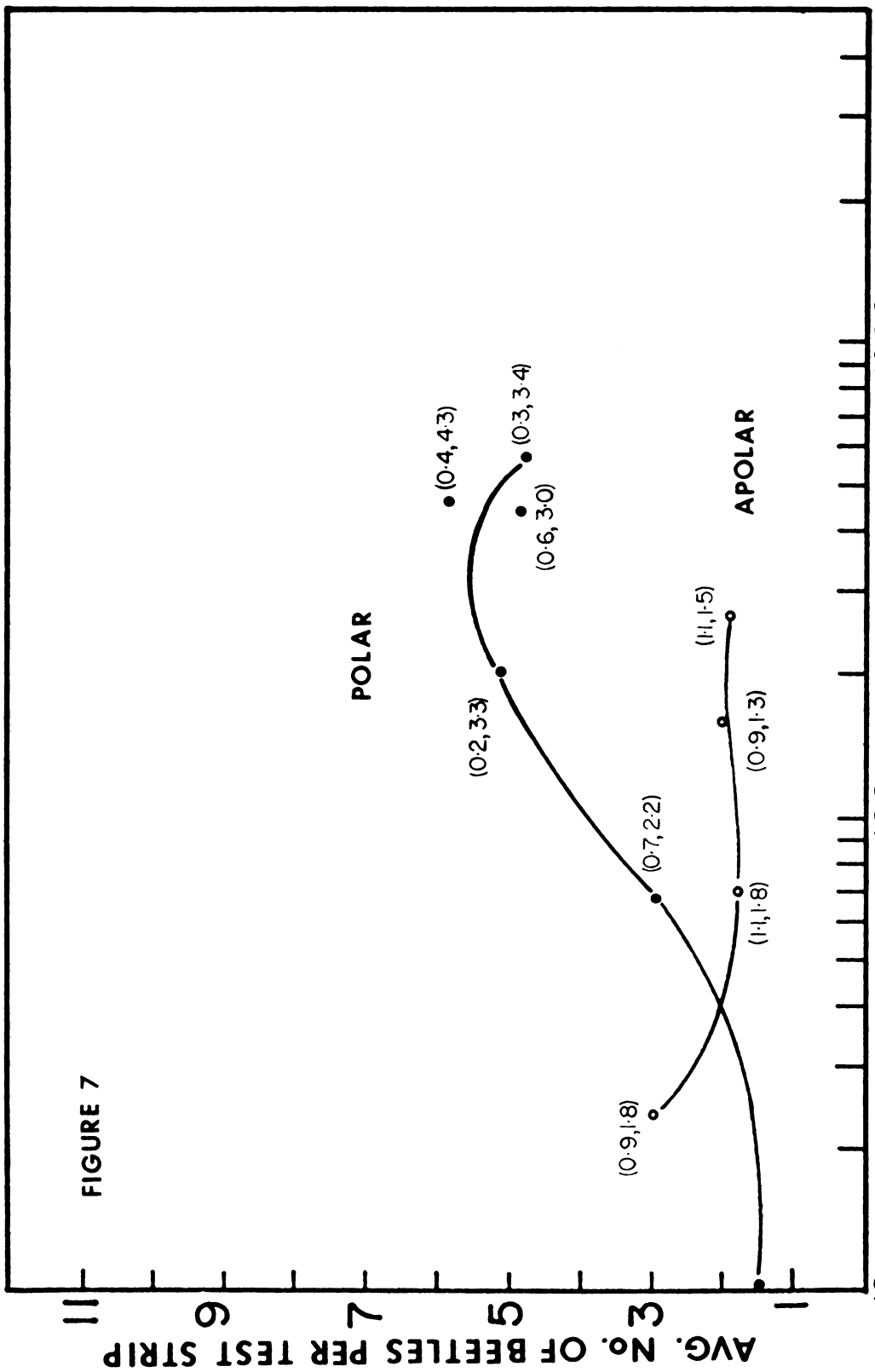
AVG. No. OF BEETLES PER TEST STRIP
11
9
7
5
3
1
10
100
1000
LOG PPM EXTRACT
POLAR
APOLAR
(0·4, 4·3)
(0·3, 3·4)
(0·6, 3·0)
(0·2, 3·3)
(0·7, 2·2)
(0·9, 1·8)
(1·1, 1·5)
(0·9, 1·3)
(1·1, 1·8)

FIGURE 7

Table 18. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO DEWAXED PEA SEEDLING APOLAR AND POLAR HYDROPHOBIC COMPOUNDS WITH AND WITHOUT BARLEY CRUDE EXTRACT IN THREE PER CENT AGAR*.

Fraction	PPM	Pea Extract Avg. Count[a] Cont.	Pea Extract Avg. Count[a] Test	Pea Extract Avg. Activity[b] Cont.	Pea Extract Avg. Activity[b] Test	Pea extract plus barley[R] Count[a] Cont.	Pea extract plus barley[R] Count[a] Test	Pea extract plus barley[R] Activity[b] Cont.	Pea extract plus barley[R] Activity[b] Test
Controls	0.0	1.3	0.8	0.8	0.4	0.9	7.6[14]	0.8	4.3[13]
Ap	43.6	1.5	1.9	0.4	0.3	0.6	4.5	0.4	2.2
Ap	73.7	0.3	0.7	0.0	0.0	0.9	3.1	0.1	1.0
Ap	97.0	1.6	0.3	0.4	0.2	1.7	3.7	0.4	1.1[3]
P	19.4	1.0	1.4	0.9	1.2[3]	---	---	---	---
P	43.6	1.9	1.2	0.5	0.2[3]	---	---	---	---
P	131.0	1.3	2.8	0.6	1.3[10]	1.7	7.8	1.2	3.5[7]
P	229.0	0.6	1.4	0.3	0.8	---	---	---	---
P	291.0	2.7	1.1	0.5	0.4[3]	---	---	---	---
P	411.0	1.3	1.7	0.2	0.6	0.7	5.6	0.4	3.3
P	547.0	1.6	0.2	0.1	0.2	0.8	4.1	0.7	2.6[3]

*25 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per strip per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

Ap (dewaxed apolars), P (dewaxed polars), R (482 ppm barley crude extract).

[3-14]Superscript applies to all values to its left until superseded, indicating the number of days in the computed average if not four days.

Table 19. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY EXTRACT FRACTIONS INCORPORATED WITH PEA SEEDLING CRUDE EXTRACT INTO THREE PER CENT AGAR*.

Treatment Mixture			Average Count[a]		Average Activity[b]	
Barley Fraction	PPM	PPM Pea Extract	Cont.	Test	Cont.	Test.
None	0.0	None	1.9	1.6 (K)	0.6	0.7
Philic	1260.0	None	1.1	4.7 (L)	0.4	2.9
Philic	1260.0	291.0	0.9	3.6 (KL)	0.1	0.5
Philic	1260.0	582.0	1.9	2.1 (K)	0.0	0.3
Phobic	582.0	None	2.5	7.8 (M)	1.2	3.3
Phobic	582.0	291.0	1.8	3.4 (KL)	1.1	0.8
Phobic	582.0	582.0	2.4	2.7 (KL)	0.3	1.1
None	0.0	None	1.6	0.8 (P)	1.0	0.5
Philic	1260.0	None	1.8	3.0 (QR)	0.7	2.6
Philic	1260.0	582.0	1.0	1.5 (PQ)	0.0	0.8
Philic+W	1260.0+70.0	582.0	1.1	4.6 (R)	0.2	2.6
Philic+P	1260.0+586.0	582.0	1.4	4.2 (R)	0.0	1.8
Philic+A	1260.0+196.0	582.0	1.8	1.5 (PQ)	0.0	1.0
Philic+W+P+A	1260.0+852.0	582.0	2.0	3.1 (QR)	0.3	3.0

*25 laboratory reared beetles, one control and one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per strip per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[1]Means sorted by Duncan's MRT. Values opposite the same letter were not significantly different at the 5% level. Transformation: $(Y+1)^{\frac{1}{2}}$

Philic (hydrophilic compounds); Phobic (total hydrophobic compounds); A (dewaxed apolar compounds); P (dewaxed polar compounds); W (surface wax).

a whole sample of hydrophobics. Barley (H-W) apolar compounds were significantly less effective than the others at overcoming the deterrence of pea extract. Barley epicuticular wax was the most effective fraction. Barley (H-W) polars were significantly better than the (H-W) apolars, but significantly less effective than the wax. All three fractions added together resulted in good recovery of activity.

E. (H-W) Polar Compounds

The (H-W) polar materials were separated into glycolipids and phospholipids on silicic acid columns. The phospholipid fraction contained a non-phosphorous compound, gramine, for which a means of separation was not immediately available. It was found that both (H-W) subfractions were stimulants (Table 20). Different combinations of glycolipids and phospholipids were made and bioassayed at various times in the study (Table 21). There was no consistent pattern of either count or activity data due to changes in the glycolipid/phospholipid ratio.

The CLB response to glycolipids from barley and pea seedlings is compared in Figure 8. The barley data clearly showed a dose-dependent response, while the pea data indicated only a low response at all levels tested. The middle curve of Figure 8 represents pea glycolipids minus monogalactoslydiglyceride. A slightly better response was found in this instance than for other pea total-glycolipid bioassays, but it remained a low response. The monogalactosyldiglyceride was bioassayed separately in that case and is reported below.

1. Individual Glycolipids

The mono- and di-galactosyldiglycerides and the sulfolipid of both barley (Table 22) and pea (Table 23) were isolated and bioassayed. For

Table 20. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY GLYCOLIPIDS AND PHOSPHOLIPIDS IN THREE PER CENT AGAR*.

Treatment		Average Count[a]		Average Activity[b]	
Fraction	PPM	Control	Test	Control	Test
PL	97.0	0.6	3.6	0.4	3.2
PL[t]	97.0	0.8	2.2	0.5	3.2
PL[t]	155.0	1.1	3.2	0.5	3.6[3]
GL[t]	194.0	1.2	2.8	0.6	3.1
GL	242.0	1.9	3.0	0.5	3.5

*25 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[t]20 beetles per test unit.

[3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

PL (phospholipid); GL (glycolipid).

Table 21. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO VARIOUS RECOMBINATION RATIOS OF BARLEY GLYCOLIPID TO PHOSPHOLIPID IN THREE PER CENT AGAR*.

GL/PL Ratio	GL	PL		Average Count[a]		Average Activity[b]	
	PPM		Sum-PPM	Control	Test	Control	Test
0.8[t]	164	205	369	1.0	6.2	1.0	4.5
1.9[t]	243	126	369	0.7	7.1	0.6	3.9
2.0[t]	194	97	291	1.3	4.3	0.8	3.8[1]
2.5	242	97	339	0.9	7.3	0.3	4.6
4.2	242	58	300	1.6	6.7	0.5	4.5[3]
4.6[t]	299	70	369	0.7	4.2	0.7	3.8

*25 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[t]20 beetles per test unit.

PL (phospholipid); GL (glycolipid).

[1,3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

Figure 8. Response of newly emerged, unfed, adult cereal leaf beetles to the concentration of barley and pea seedling glycolipids incorporated into three per cent agar.

Data summarized from randomized complete and incomplete block experiments: 25 laboratory reared beetles per test unit; one test unit per concentration per day.

Counts were averaged from three hourly observations of beetles per day over four days.

Activity (selected values in parentheses, control left, test right) was averaged from four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

Superscript indicates the number of days in the computed average if not four days.

The curve of best fit for barley glycolipids was described by a 1st degree polynomial equation:

Coefficients	Index of determination: 0.25
-0.024	Standard error of estimate for Y: 1.62
2.242	

The curve of best fit for pea total glycolipids was described by a 2nd degree polynomial equation:

Coefficients	Index of determination: 0.03
5.5	Standard error of estimate for Y: 1.10
-4.3	
1.0	

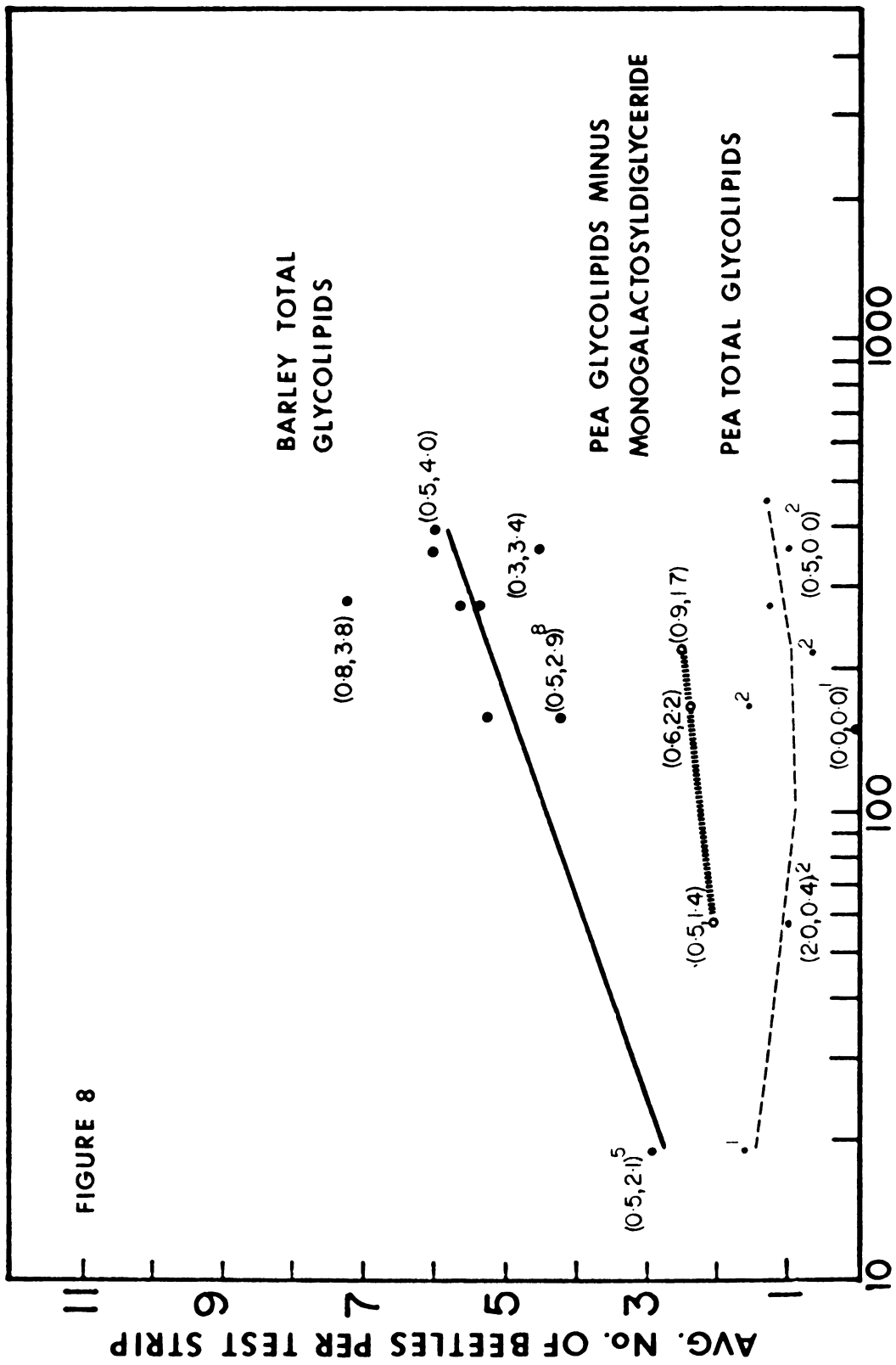
FIGURE 8
AVG. No. OF BEETLES PER TEST STRIP
LOG PPM EXTRACT
BARLEY TOTAL GLYCOLIPIDS
PEA GLYCOLIPIDS MINUS MONOGALACTOSYLDIGLYCERIDE
PEA TOTAL GLYCOLIPIDS
(0·8,3·8)
(0·5,4·0)
(0·3,3·4)
(0·5,2·9)
(0·5,2·1)
(0·6,2·2)
(0·9,1·7)
(0·5,1·4)
(2·0,0·4)
(0·9,0·0)
(0·5,0·0)
11
9
7
5
3
1
10
100
1000

Table 22. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO GLYCOLIPIDS INDIVIDUALLY ISOLATED FROM BARLEY AND INCORPORATED INTO THREE PER CENT AGAR*.

Glycolipid	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
MDG	1.9	0.9	1.2	1.4	1.6
	19.4	0.3	2.4	0.7	2.3
	19.4	0.6	2.1	0.4	2.5
	97.0	1.8	2.7	1.1	4.4_1
	136.0	0.0	3.7	1.5	3.7^1
	177.0	0.5	2.4	0.4	1.8
	214.0	0.4	2.8	1.0	3.4
DGD	1.9	2.7	1.1	1.2	0.5^3
	19.4	0.4	2.2	0.2	2.4
	19.4	0.8	2.9	0.3	3.4
	97.0	0.4	2.4	0.3	4.4
	97.0	0.0	3.6	0.3	3.0
	194.0	1.3	3.2	0.2	3.1
SUL	0.2	1.2	2.7	2.2	2.0
	0.2	1.2	1.8	0.8	0.7
	1.9	1.7	2.8	1.1	1.9
	1.9	2.6	2.0	1.9	2.2
	19.4	0.7	3.1	0.5	4.4_3
	19.4	1.2	4.0	1.5	4.3^3
	19.4	1.3	2.0	1.1	2.0

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

MDG (monogalactosyldiglyceride); DGD (digalactosyldiglyceride); SUL (sulfolipid).

[1,3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

Table 23. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO PEA SEEDLING GLYCOLIPIDS INDIVIDUALLY ISOLATED AND INCORPORATED INTO THREE PER CENT AGAR*.

Glycolipid	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
Complete	142.0	0.9	2.2	0.6	0.8[3]
MDG	29.0[P]	0.6	4.1	0.5	4.2[3]
	97.0[$]	0.5	2.8	0.5	1.7
DGD	97.0[$]	0.8	2.7	0.3	1.4[3]
SUL	X[$]	0.9	1.3	0.3	1.4[3]
	22.0[#]	3.2	1.3	1.0	1.1[2]

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[P]First isolated from silicic acid column with chloroform:acetone, 1:1 (v/v) followed by preparative TLC on silica gel H, and then eluted with chloroform:methanol, 2:1 (v/v).

[$]Complete glycolipids applied to second silicic acid column (5 ml Unisil). Elution: 50 ml chloroform:acetone (90:10, v/v) (MDG); 50 ml chloroform: acetone (25:75, v/v) (DGD); 75 ml acetone (SUL).

[#]Eluted from a silicic acid column in last 200 ml of 300 ml used.

MGD (monogalactosyldiglyceride); DGD (digalactosyldiglyceride); SUL (sulfolipid).

[2,3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

each class of glycolipid, the dose-response relationship was relatively flat. Based on activity scores, there seemed to be a stronger dose-response for sulfolipid than for mono- or di-galactosyldiglyceride. The CLB also seemed more sensitive to the former than to the latter two classes. On one occasion, the monogalactosyldiglycerides of pea seedlings did stimulate a high level of feeding activity by the CLB. A possible explanation for this unique event is presented in the DISCUSSION.

2. Phospholipids

The phospholipids of pea and barley seedlings were bioassayed (Table 24). The data confirmed earlier observations that pea-derived, (H-W) polar compounds did not stimulate CLB feeding behavior to a significant extent. The data for the barley phospholipids in Table 24 is the same as that in Table 20.

a. Acidic Phospholipids

Barley acidic phospholipids, without further treatment after elution from the DEAE column, showed a low stimulatory effect (Table 25). Three experiments were performed to determine whether the change of associated cations such as occurs with the acid phospholipids during silicic acid column chromatography (123), could alter CLB response in this study (Table 26). When washed with deionized water, a remarkable positive effect on response to the acidic phospholipids was seen compared to any other wash. A slight positive dose-response was indicated when the acid phospholipids were in either the Ca^{+2} or Na^{+1} form compared to whatever their state was directly from the DEAE column, but many more tests would be required to confirm this indication.

Acid phospholipids of pea seedlings were bioassayed without alteration of their cation content (Table 27). Like nearly all previous

Table 24. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY AND PEA SEEDLING PHOSPHOLIPIDS INCORPORATED INTO THREE PER CENT AGAR*.

Phospholipid Source	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
Pea Seedlings	56.0	1.9	1.8	0.5	0.3[3]
	72.0	1.2	0.8	1.6	1.4
	151.0	1.6	2.1	0.6	1.2
Barley Seedlings[R]	97.0	0.6	3.6	0.4	3.2
	97.0	0.8	2.2	0.5	3.2
	155.0	1.1	3.2	0.5	3.6[3]

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[R]Data taken from Table 20.

[3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

Table 25. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY SEEDLING NEUTRAL AND ACID PHOSPHOLIPIDS INCORPORATED INTO THREE PER CENT AGAR*.

Treatment	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
APL	0.0	0.4	0.4	0.5	0.9
	19.4	0.3	1.8	0.4	1.8
	136.0	1.9	1.4	0.4	1.3
NPL[c]	19.4	0.7	2.5	0.6	3.6
	34.2	1.1	2.6	0.4	0.9
	136.0	0.5	4.6	0.2	5.0
	180.0	1.6	6.8	0.2	3.0
	291.0	0.9	3.1	0.3	2.3
APL+NPL	15.5+71.8	1.6	4.1	0.3	4.7

*20 beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

APL (acid phospholipids); NPL (neutral phospholipids).

[c]Contained gramine.

Table 26. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY ACID PHOSPHOLIPIDS TREATED TO ALTER ASSOCIATED CATIONS AND INCORPORATED INTO THREE PER CENT AGAR*.

Washing Solution	APL (PPM)	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
Na_2-EDTA	19.4	0.2	1.6	0.3	1.2
	58.0	0.1	1.2	0.1	2.6
Deionized Water	19.4	0.8	4.3	0.8	5.3
	58.0	1.0	3.0	0.5	5.3
Unwashed	9.7	1.7	1.2	2.4	1.2
	19.4	1.0	1.8	0.6	2.0
	55.0	1.0	1.5	0.8	1.4
$CaCl_2$	9.7	1.1	1.3	2.4	1.5
	19.4	0.6	1.2	0.6	1.4
	55.0	0.2	2.2	0.7	2.7
$CaCl_2$, then Na_2-EDTA	9.7	0.9	2.2	0.6	1.4
	19.4	0.7	1.0	1.2	1.1
	40.7	0.7	0.9	0.9	1.2

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

APL (acid phospholipids).

Table 27. RESPONSE OF NEWLY EMERGED, UNFED ADULT CEREAL LEAF BEETLES TO PEA SEEDLING ACID AND NEUTRAL PHOSPHOLIPIDS INCORPORATED INTO THREE PER CENT AGAR*.

Phospholipid Fraction	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
APL	0.0	1.5	1.1	0.6	0.8
	21.0	1.2	2.0	0.4	1.1
	132.0	1.6	2.7	0.6	1.1
NPL	34.0	0.7	1.1	0.7	0.9
	180.0	1.5	2.4	0.8	0.6
APL, NPL	21.0+34.0	2.1	2.1	0.7	0.8

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

APL (acid phospholipids); NPL (neutral phospholipids).

bioassays of pea hydrophobic compounds, there was only a marginal response which was consistent with earlier observations that no strong deterrence was associated with (H-W) polar compounds of pea seedlings.

It was hoped that elution of the DEAE column with conc. acetic acid would separate the suspected phosphatidyl serine from the remaining acid phospholipids. However, an absolutely pure sample of phosphatidyl serine was not obtained. The remaining acid phospholipids and the phosphatidyl serine were bioassayed separately at several concentrations and together at one concentration (Table 28). Based on the activity scores, it can only be repeated that the acid phospholipids of barley stimulated a light to fair feeding response by the CLB.

b. Neutral Phospholipids

When bioassayed simultaneously, the neutral phospholipids of barley were far more effective than the acid phospholipids (Table 25). Gramine was present in the neutral phospholipids during these bioassays. Bioassay of the neutral phospholipids of pea plants showed them to be ineffective (Table 27).

The individual neutral phospholipids of barley were obtained from the DEAE column in various degrees of purity and bioassayed without further separation attempted. In general, barley neutral phospholipids stimulated an inconsistent, low level of feeding behavior when bioassayed separately (Table 29). No combination of two barley neutral phospholipids was found to act as a strong stimulant (Table 30). All combinations of two were, on the average, significantly more effective than their respective blank controls when gravimetric amounts were available for bioassay. The combination of phosphatidyl ethanolamine with acid phospholipids (Table 30) was very effective, having produced a degree of response

Table 28. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY PHOSPHATIDYL SERINE AND THE REMAINING ACID PHOSPHOLIPIDS INCORPORATED INTO THREE PER CENT AGAR*.

Phospholipid Fraction	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
PS(?)	4.8[1] 5.2[3] 10.5[2] 12.0[1] 12.0[4] 13.0[1] 17.0[1] 21.0[2] 25.0[4]	0.8 1.4 1.0 0.5 1.1 0.8 0.8 1.1 1.6	2.2 3.1 1.2 2.6 1.3 1.5 2.0 2.6 0.9	0.4 0.3 0.5 0.4 1.1 0.4 0.0 0.8 1.4	2.3 2.8 0.8 2.4 0.7 1.2 2.5 0.9 0.7
R	20.0 22.0 27.0 60.0 68.0	1.8 0.6 1.4 1.2 2.0	2.8 2.2 2.1 2.1 1.1	1.0 0.5 0.6 0.3 1.1	1.4 2.2 1.7 2.2 1.0
PS, R	5.2,22.0	0.5	2.1	0.3	2.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

PS(?) Believed to be phosphatidyl serine. R (remaining acid phospholipids).

[1]By TLC, contained one unidentified phospholipid.

[2]By TLC, contained lysophosphatidyl ethanolamine plus unidentified acid phospholipid.

[3]By TLC, contained lysophosphatidyl ethanolamine.

[4]By TLC, contained several unidentified acid phospholipids.

Table 29. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY NEUTRAL PHOSPHOLIPIDS IN THREE PER CENT AGAR*.

Phospholipid	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
PE	X	0.6	1.5	0.6	1.9
	X	1.1	2.8	0.8	1.7
	5.8	0.9	1.8	0.5	1.4
	9.7	0.8	0.5	0.5	0.8_2
	13.6	1.3	0.8	0.4	0.5^2
PC	X	1.2	1.4	0.3	1.4
	13.0	0.4	1.1	0.3	1.4_2
	23.3	0.0	0.8	0.4	0.9^2
LPE	X	0.2	0.3	0.2	0.3^3
LPC	X	1.4	0.6	1.0	0.8
	1.7	1.9	2.0	1.1	1.5
	3.5	1.7	2.0	0.8	1.7
	6.7	0.6	3.1	0.5	3.2

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

X (weightless sample).

[2]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

PE (phosphatidyl ethanolamine); PC (phosphatidyl choline); LPE (lysophatidyl ethanolamine); LPC (lysophosphatidyl choline).

Table 30. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO COMBINATIONS OF TWO BARLEY NEUTRAL PHOSPHOLIPIDS IN THREE PER CENT AGAR*.

Phospholipids	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
PE + PC	12.0 + 32.0	0.8	0.8	0.7	1.3
	1.9 + 13.0	0.9	1.6	0.6	1.3[2]
	13.6 + 23.3	0.6	1.6	0.6	1.2[2]
PE + LPE	12.1 + X	1.4	2.8	0.6	2.5
PE + LPC	X	0.5	1.4	0.4	0.8
	6.0	1.0	1.4	0.3	1.2
	8.4	1.2	2.0	0.9	1.3
	12.0 + X	1.6	2.5	0.9	2.2
	14.2	0.8	1.3	1.3	0.8
	16.5	0.8	1.8	0.7	1.6
	23.0	0.8	2.0	0.2	1.8
PE + APL	23.0 + 44.0	1.4	3.4	0.6	3.2
PC + LPE	X	2.5	1.4	1.1	1.1
	32.0 + X	1.3	1.7	0.5	1.9
PC + LPC	36.9 + X	0.8	1.3	0.4	1.7[2]
	32.0 + X	0.5	2.0	0.3	1.3
PC + U	9.7	1.2	1.2	0.1	0.6[2]
	27.0	1.6	2.5	1.5	2.7
LPE + LPC	X	1.0	1.5	0.4	2.1

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[2]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

PE (phosphatidyl ethanolamine); PC (phosphatidyl choline); LPE (lysophosphatidylethanolamine); LPC (lysophosphatidyl choline); APL (acid phospholipids); U (non-phosphorous compound later identified as gramine).

that indicated an interaction had occurred. Similarly, phosphatidyl choline, which had been a poor stimulant alone or in dual combination, produced a response of 2.7 when bioassayed with the gramine at a combined concentration of 27 ppm.

When the bioassay mixture approached the composition of the native neutral phospholipid fraction, the response became consistently significant (Table 31). A positive dose-response was indicated, but not strongly. The combination of neutral phospholipid mixture and phosphatidyl serine or acid phospholipid minus phosphatidyl serine gave a consistently good response. While interaction was indicated, it appeared to be of an additive nature.

To investigate the possibility that the fatty acid composition might affect the response of the CLB to phospholipid, three species of lecithin were bioassayed (Table 32). One species, L-α-1-stearoly-2-oleoyl lecithin, was significantly more effective than the other two lecithins.

F. Gramine

Since the non-phosphorous contaminant of neutral phospholipids was involved in every bioassay reported in Table 31, it became necessary to isolate, identify and bioassay this substance. A combination of infrared and mass spectrometry, as well as available literature, helped to identify the isolated compound as gramine. The selected series of masses from the mass spectrum for the isolated and commercial gramine were identical and are presented in Table 33. The proposed fragmentation patterns of major interest are shown in Figure 9. A definite positive response was obtained with isolated and commercial gramine (Table 34),

Table 31. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO THREE OR MORE NEUTRAL PHOSPHOLIPIDS OF BARLEY IN THREE PER CENT AGAR WITH A NON-PHOSPHOROUS COMPOUND*.

Phospholipids	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
PC + PE + LPE + U	19.4	1.8	1.7	0.9	1.7
	38.8	1.0	3.0	0.8	2.1
	52.4	0.9	3.0	0.5	3.1
PC + PE + LPE + LPC + U	53.0	0.9	4.6	0.5	2.4
	58.0	1.1	3.7	0.3	3.4
	60.0	0.8	2.6	0.5	2.1
	83.0	1.7	2.8	0.9	2.4
	96.0	1.2	4.0	0.9	2.8
	102.0	0.5	5.5	0.5	4.8
PC + PE + LPE + U + (G)	60.0 + (20.0)	1.2	3.8	0.7	3.4
	58.0 + (22.0)	1.4	3.5	0.3	3.4
PC + PE + LPE + U + (PS?)	58.0 + (5.0)	0.6	4.2	0.3	4.2

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

PE (phosphatidyl ethanolamine); PC (phosphatidyl choline); LPE (lysophatidy ethanolamine); LPC (lysophosphatidyl choline); PS (phosphatidyl serine) G (acid phospholipids minus phosphatidyl serine); U (non-phosphorous compound later identified as gramine).

Table 32. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO THREE SPECIES OF COMMERCIALLY OBTAINED PHOSPHATIDYL CHOLINE WITH AND WITHOUT GRAMINE IN THREE PER CENT AGAR*.

Treatment Mixture			Average Count[a]		Average Activity[b]	
Phospholipid	(PPM)	Gramine PPM	Control	Test	Control	Test
1-s-2-o	155.2	0.0	2.0	2.3	1.4	2.1
Dilin.	97.0	0.0	2.1	1.3	1.2	1.4
	155.2	0.0	1.2	1.0	1.2	1.2
	97.0	58.2	1.5	4.2	0.5	3.4
	0.0	58.2	1.1	4.4	0.5	3.0
Distear.	155.2	0.0	0.9	2.5	0.4	1.1
	0.0	38.8	1.7	4.6	0.8	1.9
	155.2	38.8	2.3	2.8	1.2	2.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

1-s-2-o (L-α-1-stearoyl-2-oleoyl lecithin).

Dilin. (L-α-dilinoeloyl lecithin).

Distear. (L-α-distearoyl lecithin).

Table 33. A SELECTED SERIES OF MASS INTENSITIES FROM THE MASS SPECTRUM OF GRAMINE 3-(DIMETHYLAMINOMETHYL)-INDOLE.

Mass/e	Relative Intensity	Mass/e	Relative Intensity
41	5.0	102	13.6
43	21.1	103*	9.2*
44	8.9	104	1.2
50	5.5	127	0.8
77*	10.0*	128	6.5
78	2.8	129	35.6
79	1.3	130*	100.0*
81	1.5	131	32.4
83	3.6	132	3.5
85	4.5	142	1.3
86	2.1	156	2.0
87	7.8	173	8.2
89	1.1	174 (M+)	60.6*
100	0.6	175	8.9
101	2.2		

*Peaks corresponding to fragments shown in Figure 9.

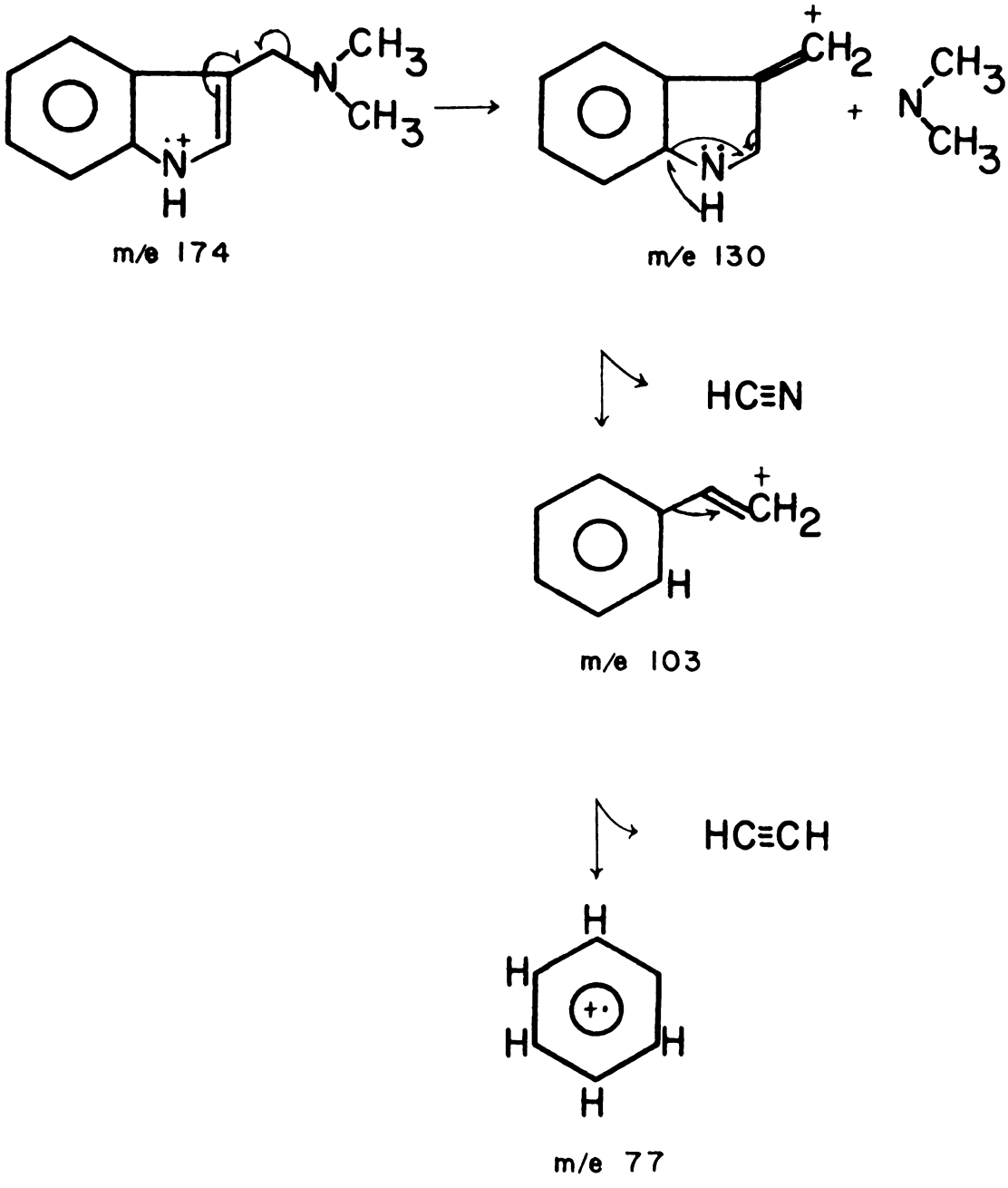

Figure 9. Major ions represented in the mass spectrum of gramine, 3-(dimethylaminomethyl)-indole.

Table 34. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO GRAMINE IN THREE PER CENT AGAR*.

Gramine Source	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
Extracted from barley	3.9	2.0	2.0	1.9	1.9
	9.7	1.2	3.0	0.9	2.4
Commercial	9.7	2.2	2.6	0.7	1.3
	19.4	1.4	2.1	0.8	1.9
	19.4	1.5	1.5	0.3	1.6[2]
	38.8	2.0	2.8	1.3	1.7[2]
	97.0	0.9	1.5	0.7	1.9
	116.0	2.1	1.6	1.1	1.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[2]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

but like that of the epicuticular wax, it was a fairly flat response with little concentration effect.

Because gramine can be classified as a secondary plant compound, it was bioassayed in combination with other compounds present in barley leaves to look for interactions (Table 35). Gramine produced a significant increase in response to the barley glycolipids, but only a slight interaction was seen with 1-hexacosanol. Indole-3-acetic acid (IAA) was bioassayed alone and combined with gramine due to its similar structure. Judged by TLC, gramine was more abundant than other indoles in barley. IAA was, therefore, tested at lower concentrations than was gramine. It showed marginal activity by itself and, in every combination with gramine, reduced the response relative to the gramine control. This reduction was judged to be insignificant overall.

To assess the effect of gramine on CLB response to the neutral phospholipids, gramine was separated from that fraction during elution from the DEAE column. Two bioassays of neutral phospholipids (143 ppm), gramine (38.8 ppm), and the two combined were performed (Table 36). Without gramine, the neutral phospholipids evoked a significantly reduced response from the CLB from that usually observed. With gramine readded, the response became more typical of that found for the neutral phospholipids plus the former unknown contaminant; cf. Table 25 and Table 31. Since the response to the combination was greater than to either alone, an interaction was clearly established.

The effect of gramine with glycolipids and phospholipids of pea seedlings was investigated. Neither fraction had been an effective CLB stimulant (Figure 8, Tables 23 and 24). Gramine transformed the two pea

Table 35. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO COMBINATIONS OF GRAMINE WITH OTHER PLANT BIOCHEMICALS IN THREE PER CENT AGAR*.

Treatment Mixture			Average Count[a,1]		Average Activity[b]	
Gramine ppm	Other	ppm	Control	Test	Control	Test
0.0	GL	112.0	1.7	4.5	0.2	3.8
31.0	GL	112.0	0.8	6.2	0.1	5.2
0.0	C-26	19.4	0.8	3.6	1.1	4.1
19.4	C-26	19.4	1.1	4.7	1.0	4.7
38.8	---	0.0	1.3	3.7	0.2	2.3
38.8	IAA	13.6	1.5	2.1	0.4	1.8
38.8	---	0.0	0.8	4.2 (M)	0.6	3.3
38.8	IAA	1.9	1.4	2.4 (L)	1.0	2.8
38.8	IAA	3.8	2.4	2.4 (L)	1.0	3.1
38.8	IAA	7.6	1.2	3.1 (LM)	0.7	3.0_3
0.0	IAA	1.9	1.9	1.4	0.8	1.3^3
0.0	IAA	3.8	1.3	1.0 (K)	1.3	1.7
0.0	IAA	7.6	1.4	2.6 (L)	0.7	1.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

GL (barley total glycolipids); C-26 (1-hexacosanol); IAA (indole-3-acetic acid)

[3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

[1]Means associated with letters have been sorted by Duncan's MRT. Means opposite the same letter were not significantly different at the 5% level. Transformation: $(Y+1)^{\frac{1}{2}}$

Table 36. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY NEUTRAL PHOSPHOLIPIDS WITH AND WITHOUT GRAMINE IN THREE PER CENT AGAR*.

Treatment Mixture		Average Count[a,1]		Average Activity[b]	
NPL - ppm	Gramine - ppm	Control	Test	Control	Test
143.6	0.0	0.8	1.3 (K)	0.5	1.4
0.0	38.8	1.7	4.6 (L)	0.8	1.9
143.6	38.8	0.9	3.6 (L)	0.8	3.9
143.6	0.0	0.5	1.6 (S)	0.3	2.1
0.0	38.8	0.0	0.5 (S)	0.3	0.9
143.6	38.8	0.5	1.4 (S)	0.3	3.8

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

NPL (neutral phospholipids).

[1]Means sorted by Duncan's MRT. Means opposite the same letter were not significantly different at the 5% level. Transformation: $(Y+1)^{\frac{1}{2}}$

(H-W) polar fractions from unpreferred substrates to a degree of palatability previously found for barley extracts only (Table 37).

Gramine was bioassayed in combination with two of the three species of commercially obtained phosphatidyl choline which differed in their fatty acid composition (Table 32). There was no significant increase of response compared to gramine alone.

Further investigation of the influence of gramine was made by introducing the highly deterrent pea (H-W) apolar fraction to barley hydrophilic extract and three concentrations of gramine (Table 38). The pea compounds deterred the usual CLB feeding response to the barley hydrophilic compounds. Addition of gramine enabled the test agar to again stimulate the response to a level comparable to that of the barley hydrophilic compounds alone. A slight inconsistency of this effect was seen at the 58.2 ppm concentration of gramine, but the effect was irrefutable.

V. Hydrophilic Compounds

A. Commercially Obtained Chemicals

Several bioassays of individual amino acids and amino acid combined with sucrose or gramine were performed (Table 39). Tryptophan, from which gramine is derived, was not active at the levels tested either by itself or combined with gramine, relative to gramine alone. β-Alanine was not active as a stimulant by itself, while it significantly increased the response with sucrose compared to the sucrose control. Additional study would be required to confirm this apparent interaction. Proline gave only a slight response when bioassayed alone and did not significantly increase the response to sucrose compared to a sucrose control.

Table 37. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO PEA SEEDLING GLYCOLIPIDS AND PHOSPHOLIPIDS WITH AND WITHOUT GRAMINE IN THREE PER CENT AGAR*.

Treatment Mixture		Average Count[a,1]		Average Activity[b]	
Lipid Class-ppm	Gramine-ppm	Control	Test	Control	Test
Glycolipid 194.0	0.0	0.6	1.1	0.5	0.8[2]
0.0	57.0	2.0	2.9	0.8	2.8
194.0	57.0	2.4	7.0	0.9	3.4
PL - PC 151.3	0.0	1.4	2.1 (S)	0.6	1.2
0.0	48.0	1.4	2.3 (S)	1.0	2.6
151.3	48.0	2.2	4.1 (S)	0.9	3.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[1]Means sorted by Duncan's MRT. Means opposite the same letter were not significantly different at the 5% level. Transformation: $(Y+1)^{\frac{1}{2}}$

[2]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

Table 38. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO A DETERRENT FRACTION OF PEA SEEDLING EXTRACT IN BARLEY HYDROPHILIC EXTRACT WITH AND WITHOUT GRAMINE IN THREE PER CENT AGAR*.

Treatment Mixture			Average Count[a,1]		Average Activity[b]	
B.H. ppm	P.A. ppm	Gramine ppm	Control	Test	Control	Test
660.0	0.0	0.0	0.7	2.2 (S)	0.5	1.9
660.0	38.8	0.0	0.7	0.8 (S)	0.4	0.8
660.0	38.8	9.7	0.5	2.6 (S)	0.5	1.5
660.0	38.8	29.0	1.6	2.3 (S)	0.7	1.8
660.0	38.8	58.2	1.3	2.0 (S)	0.3	1.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

B.H. (barley hydrophilic fraction); P.A. (dewaxed pea apolar compounds).

[1]Means sorted by Duncan's MRT. Means opposite the same letter were not significantly different at the 5% level. Transformation: $(Y+1)^{\frac{1}{2}}$

Table 39. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO SELECTED AMINO ACIDS IN THREE PER CENT AGAR WITH OR WITHOUT SUCROSE OR GRAMINE*.

Amino Acid	(PPM)	Sucrose (PPM)	Gramine (PPM)	Average Count[a] Control	Average Count[a] Test	Average Activity[b] Control	Average Activity[b] Test
L-Tryptophan	97.0	0.0	0.0	1.6	0.8	0.5	0.2^{2}
	199.0	0.0	0.0	1.3	0.4	1.1	0.3^{2}
	0.0	0.0	38.8	1.1	2.1	0.6	2.8
	97.0	0.0	38.8	1.0	1.9	0.7	2.1
	199.0	0.0	38.8	1.6	1.9	0.9	2.0
β-Alanine	691.0	0.0	0.0	2.6	0.5	1.8	1.2
	432.0	0.0	0.0	1.3	0.6	1.3	0.6
	346.0	776.0	0.0	0.3	6.0	0.5	3.6
	0.0	776.0	0.0	2.0	3.1	0.8	2.8_{3}
	259.0	0.0	0.0	2.1	1.9	1.4	1.0^{3}
L-Proline	55.8	0.0	0.0	2.1	1.3	0.9	1.5
	111.6	0.0	0.0	0.8	2.1	0.5	0.5
	892.0	0.0	0.0	1.1	2.0	1.2	1.9
	0.0	776.0	0.0	1.0	3.4	0.8	2.1_{2}
	97.0	0.0	0.0	1.0	1.1	1.3	1.5^{2}
	97.0	776.0	0.0	3.1	4.6	0.5	2.5

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[2,3]Superscript applies to all values to its left, indicating the number of days in the computed average if not four days.

Amino acid mixtures corresponding to the free amino acids of a CLB host-plant, orchard grass, and a non-host, alfalfa (38) were bioassayed. Accommodating to a reduced number of laboratory reared beetles, the number of beetles used per test unit was reduced to 20. Field-collected beetles were also used. Results under both conditions are presented in Table 40. There was no evidence of a positive or a negative response to either plant-simulated amino acid mixture presented to the laboratory beetles. Nor was there any indication that the amino acids interacted with sucrose when field beetles were used. There seemed to be a greater response to gramine when the orchard grass amino acid mixture was combined with it than to gramine alone, but further tests with laboratory reared beetles would be appropriate.

B. Extracted Chemicals

Most portions of the bioassays of the fractions derived from the Sephadex G-10 column (MATERIALS AND METHODS, section IV.C.2.) had to be deleted after one, two, or three days since the laboratory beetles were in the last days of their seasonal production and the number available became erratic. Results of the first experiment are presented in Table 41. Fraction No. 1 contained all compounds not held back by adsorption effects on the column; it was active at both concentrations tested. Fraction No. 2 was considered inactive and fraction No. 3 was active. Addition of sucrose (485 ppm) to fraction No. 1 (1145 ppm) did little to increase CLB response to this fraction compared to 563 ppm of No. 1 alone. Gramine (38.8 ppm) added to 1145 ppm of fraction No. 1 significantly increased the response compared to 2,250 ppm of this fraction alone.

Table 40. RESPONSE OF NEWLY EMERGED AND FIELD COLLECTED ADULT CEREAL LEAF BEETLES TO PLANT-SIMULATED AMINO ACID MIXTURES IN THREE PER CENT AGAR*.

Plant Mixture	PPM	Additive	PPM	Average Count[a]		Average Activity[b]	
				Control	Test	Control	Test
NEW BEETLES							
Dac	369.0	----	----	2.3	0.8	1.7	1.2[2]
Med	369.0	----	----	1.2	0.6	0.3	0.9[3]
FIELD COLLECTED BEETLES[c]							
----	----	Gramine	38.8	1.3	2.0	0.8	1.0
Dac	369.0	Gramine	19.4	0.6	2.7	0.9	1.8
----	----	Sucrose	776.0	0.8	1.2	0.4	0.9
Dac	369.0	Sucrose	776.0	0.4	0.4	0.0	0.3[2]
Dac	369.0	----	----	0.1	0.8	1.0	0.2[2]
----	----	Sucrose	776.0	1.2	3.1	0.7	2.3[3]
Dac	369.0	Sucrose	776.0	0.8	3.5	1.0	2.5

*20 beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[c]Beetles fasted, but watered, two days prior to test; daily exposure to test materials was 23.5 hours after which visual analysis was made.

[2,3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

Dac (Dactylis glomerata L.); Med (Medicage sativa L.). Reference: (38).

Table 41. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO FRACTIONS OF BARLEY HYDROPHILIC COMPOUNDS SEPARATED ON A SEPHADEX G-10 COLUMN AND INCORPORATED INTO THREE PER CENT AGAR*.

Fraction No.	PPM	Average Count[a]		Average Activity[b]	
		Control	Test	Control	Test
1st: 25 ml[c]	563.0	1.0	2.0	0.1	2.1[2]
1st: 25 ml[c]	2250.0	0.8	2.6	0.6	2.6
2nd: 37 ml[d]	21.3	1.1	1.0	0.5	0.3[2]
2nd: 37 ml[d]	171.0	2.0	3.8	0.2	0.7[2]
3rd: 75 ml[e]	13.2	0.3	2.4	0.0	2.2[2]
1st plus S	1145.0 + 485.0	2.2	2.2	0.5	2.7[2]
1st plus Gr	1145.0 + 38.8	1.1	4.6	0.7	3.4

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[c]Contained sugars, amino acids, other.

[d]Contained gramine, other.

[e]Contained majority of saponarin, other.

S (sucrose); Gr (gramine).

[2]Superscript applies to all values to its left, indicating the number of days in the computed average if not four days.

From another Sephadex column, only two fractions were collected. The first 25 ml were separated into a cation fraction and a neutral plus anion fraction by a cation exchange column. Due to lack of beetles, the bioassay results of these fractions (Table 42) were inconclusive for the neutral and anionic compounds. The cations were surely a source of stimulation as was the last fraction (200 ml) from the Sephadex column.

The activity of the saponarin-containing fraction of barley seedlings was examined (Table 43). There was little indication among field beetles that the saponarin may have had much influence either alone or combined with sucrose. In one instance where laboratory beetles were used, the saponarin had been obtained as a precipitate from 10% methanol in water after the remainder of the hydrophilic compounds had been separated by the Sephadex column. The test generated a very significant response on both the test and control agar strips even though successively fewer beetles were used each day.

Table 42. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY HYDROPHILIC COMPOUNDS SEPARATED BY COMBINED GEL FILTRATION AND ION EXCHANGE COLUMN CHROMATOGRAPHY AND INCORPORATED INTO THREE PER CENT AGAR*.

Fraction	Column	PPM[c]	Average Count[a]		Average Activity[b]	
			Control	Test	Control	Test
Last 200 ml[d]	G-10	----	1.1	2.4	0.8	2.4[3]
Cations (9%)	Dowex-50	----	3.2	1.3	0.4	1.6[2]
Anions, Neutrals (91%)	Dowex-50	----	0.3	0.0	0.7	0.0[1]

*20 laboratory reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.

[a]Average of three hourly counts of beetles per day over four days.

[b]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[c]No weight recorded. Equivalent volumes of each were used.

[d]Contained saponarin.

[1-3]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

Table 43. RESPONSE OF NEWLY EMERGED AND FIELD-COLLECTED, ADULT CEREAL LEAF BEETLES TO SAPONARIN-CONTAINING EXTRACT FROM BARLEY SEEDLINGS INCORPORATED INTO THREE PER CENT AGAR*.

Fraction Mixture		Average Count[b]		Average Activity[c]	
Sap. ppm	Suc. ppm	Control	Test	Control	Test
NEW BEETLES[a]					
38.8	----	2.0	4.0	1.5	2.9[2]
38.8	776.0	0.7	4.7	1.7	3.0[1]
87.3[d]	----	0.8	0.6	3.2	2.9
FIELD BEETLES[e]					
155.0	----	0.3	0.4	0.6	0.9
0.0	776.0	0.8	0.9	0.5	1.6
155.0	776.0	0.7	1.6	0.3	1.6
155.0	----	0.1	0.6	0.5	0.5
0.0	776.0	0.1	0.8	0.5	1.4
155.0	776.0	0.1	1.2	0.3	2.0

*One control, one test agar strip per test unit; one test unit per concentration per day.

[a]20 laboratory reared beetles per test unit.

[b]Average of three hourly counts of beetles per day over four days.

[c]Average of four daily agar damage scores: 1 (light), 2 (fair), 3 (good), 4 (quite good), 5 (very good), 6 (excellent).

[d]This saponarin obtained from a washed precipitate. Remaining saponarin fractions contain unidentified substances.

[e]15 beetles per test unit; 23.5 hour exposure per day to test materials.

[1,2]Superscript applies to all values to its left, indicating the number of days in computed average if not four days.

DISCUSSION

I. Validity of the Bioassay

The agar medium employed in this study stimulated a feeding response by the CLB only when extract of a host-plant was incorporated with it, but not when extract of non-hosts were used (Table 4). This result suggests that conclusions drawn from the laboratory studies reflected the molecular ecology of the insect/host relationship in the field, and, therefore, have increased the understanding of the chemical basis of CLB host selection.

II. The Parallel Study of Pea Seedling Extracts

Leaves of non-host plants have commonly been used in studies of the feeding response of phytophagous insects. There are few examples, however, in which extracts of non-hosts have been used. Jermy (79) used leaves and the juice from non-hosts applied to host leaves of eight phytophagous insects. Gilbert et al. (47) used extract fractions of host and non-hosts of the smaller European elm bark beetle, S. multistriatus.

The bioassay of pea fractions was an asset to this study. Some extract fractions of this plant deterred the CLB, i.e., Figure 4 and Tables 7 and 9, while other fractions stimulated feeding (Table 6). The deterrence was partly overcome by certain barley fractions, i.e., Figure

4 and Tables 17 and 19. These observations made two facts apparent. First, pea is not a host of the CLB, in part, because it contains deterrent chemicals. This conclusion lends support to the contention of Jermy (79) that the host range of a phytophagous insect is strongly limited by the distribution of deterrent chemicals. Second, there must have been a host-specific, recognizable quality about the barley extract which caused the CLB to feed, at reduced activity, in the presence of the deterrent(s). This conclusion supports those who believe that host selection depends on both the presence of specific stimulants and the absence of strong deterrents (11, 73, 162).

III. Estimation of Beetle Response

For many of the early experiments in this study, CLB response was assayed only by count data for the time periods indicated in the Tables and Figures. Later, it was found that response measurement could be improved by visually examining and scoring the damage to the agar strips on a daily basis. The value of a visual analysis is readily apparent. Two means of the count response were not significantly different at the 5% level in Table 7 (means indicated by "M"), while their respective activity scores were significantly different (1.9 and 2.8). Count means found significantly different at the 5% level (L, M) are paired with activity scores not significantly different (1.7, 1.9). A difference of 0.7 in activity was considered significant. In another instance (Table 9), crude extract from barley was bioassayed by itself and combined with three concentrations of pea seedling epicuticular wax. No significance was found among those count means analyzed statistically (bottom four). Yet, activity was significantly reduced in this group when pea surface

lipids were present. In Table 12, insignificant differences in count data were associated with activity differences considered significant to the observer. Based on the considerations presented above, it was concluded that activity estimates by an experienced observer were a valid basis of judging CLB response.

IV. Functions of Barley Stimuli, and Corresponding Pea Fractions

A. Hydrophobic Compounds

1. Epicuticular Wax

Little has been done to relate insect feeding behavior to the nonvolatile plant cuticular chemicals. Yet, secondary substances are found free on leaf surfaces in amount easily detected by insects (143).

Barley: The leaf alcohols of barley, 1-hexacosanol in particular, stimulated feeding by the laboratory reared beetles and field-collected beetles (Tables 15 and 16). Other wax fractions bioassayed alone were ineffective, and they did not interact in a detectable manner with the alcohols (Tables 11, 12, 13, 14). Due to the low numerical response obtained in the alcohol bioassays shown in those Tables and from samples of whole barley wax (Table 8), it was concluded that the function of these alcohols was neither to attract CLB through olfaction nor to arrest their movement per se after contact with the test agar had been made.

The function of the leaf alcohols of barley was to incite a biting response. Evidence for this conclusion came from visual analysis of the test agars after the test period. Several categories of agar damage were recognizable: bites, nibbles, channels, and rashes of close nibbling over a variable amount of surface area. When whole surface lipids or purified

alcohols were bioassayed, a great amount of random biting, nibbling and rashing predominated. There were some channels of short to medium length. If a blank agar strip were in the same test unit, the blank often showed greater damage than usually seen in other test situations. This effect probably resulted because the beetles were incited to bite by the alcohols, but, not finding other chemicals to stimulate continued feeding, they continued to seek these chemicals. Their movements brought them to the control strip where biting also occurred as a carry-over response.

An indication of the strength of the influence of the leaf alcohols is found in Table 19. Barley wax in combination with barley hydrophilic compounds overcame some of the deterrence of pea seedling crude extract. The waxes also produced a pronounced effect by increasing the response to hydrophobic compounds of barley from which the wax had been previously removed (Figure 4).

(Note: Inclusion of the silicic acid coating from a preparative TLC plate, silica gel H, with barley wax significantly reduced the response compared to untreated wax (Table 12). Such materials should be excluded from the bioassay medium.)

Although other reports of non-volatile, primary alcohols as feeding stimulants have not been made, volatile alcohols have been shown to serve as attractants for such insects as Epilanchna fulvosignata (120), for B. mori (179), and for A. grandis (108). Primary alcohols of C-28 and C-30 chain length did not arrive from their commercial source in time to be bioassayed.

Pea: Extensive qualitative and quantitative differences exist between the epicuticular waxes of barley and pea (Table 44, this section).

Table 44. THE SURFACE LIPIDS OF BARLEY AND PEA LEAVES.

Lipid Class	Barley[a] Chain length and % of Class	Barley[a] Class %	Pea[b] Chain length and % of Class	Pea[b] Class %
Hydrocarbons	C-26 (15.8), C-27, C-29, C-31 (5.0) C-33 (66.9)		C-31 (99)	42.0
Esters				
Acids	C-16 - C-22 C-22 - C-28		C-16 - C-22 C-26, C-28	
Aldehyde	None		C-26, C-28	5.0
Secondary Alcohols	None		C-31	7.3
Primary Alcohols	C-20 (0.5), C-22 (5.3), C-24 (5.2), C-26 (87.0), C-28 (2.0)	72.0	C-24 (2.0), C-26 (57.7), C-28 (38.8)	20.0
Acids	Present		Not mentioned	

[a]From von Wettstein-Knowles (185). Per cent composition by TLC.

[b]From Kolattukudy (91). Per cent composition by column and thin-layer chromatography.

The primary alcohols, 1-hexacosanol and 1-octacosanol, comprise about 20% of pea surface lipids, yet pea wax was repellent or, at least, deterred CLB feeding (Tables 9 and 10).

Using field-collected beetles, it was found that 1-hexacosanol in combination with 1-docosanol was far more effective than the 1-hexacosanol alone, either with or without sucrose in the medium (Table 16). The C-26 and C-28 primary alcohols are found in most plant waxes (92), but the ratio of C-26:C-X primary alcohol, as well as total concentrations, may be very important to the CLB sensory system. If future research does not demonstrate a qualitative basis for the negative CLB response to pea epicuticular lipids, then the ratio of these alcohols should be considered.

2. Internal Compounds

a. Total Hydrophobic Fraction Minus Wax

Barley: Apolar hydrophobic compounds (minus wax) did not stimulate significant feeding by CLB (Figure 7). In contrast, locusts responded to such apolar compounds as triglycerides, fatty acids and sterols (102). β-Sitosterol was a biting factor for B. mori (54). The apolar compounds contained in an ether extract of potato, Solanum tuberosum L., leaves stimulated feeding by larval Colorado potato beetles (71).

Polar compounds were the glycolipids and the phospholipids. Each fraction was an effective stimulant of the CLB feeding response (Figure 8 and Table 20). As discussed later, interaction of the phospholipids with gramine was responsible for the activity demonstrated by the phospholipids.

Visual examination of the agar strips of both glycolipid and phospholipid treatments revealed similar responses. Both differed from

alcohol bioassays by having a greater proportion of rashing and channeling. The significance of the rashing is not understood, but may represent a degeneration of behavior to the larval level. When feeding, CLB larvae do not eat entirely through the leaf, but remove irregular areas of epidermis and the middle parenchymous layer of the leaf - a type of rashing results.

A stronger parallel can be found between agar channeling and the normal adult pattern of feeding damage on barley leaves. Adult CLB eat longitudinal holes completely through the leaves between the vascular bundles. The greater proportion of channeling efforts evoked by the glycolipids and the phospholipids relative to the alcohols was interpreted to mean that the beetles had been brought closer to continued feeding, a step in the feeding sequence.

One experiment was performed to determine if the ratio of total glycolipids/total phospholipids might affect CLB response in the bioassay (Table 21). No definite effect was found, which implied that the ratio of these two lipids within a plant's leaves would not contribute to host-plant resistance.

Pea: The apolar hydrophobic compounds (minus wax) were a source of strong deterrence. As little as 44 ppm of these compounds significantly reduced CLB response to 482 ppm of barley crude extract (Table 18), and to 660 ppm of barley hydrophilic compounds (Table 38). The ultimate basis of deterrence in this fraction was not determined, but its identity is required before the usefulness of its effect can be properly judged.

The polar hydrophobic compounds were not stimulants (Table 18). Nor were they strongly deterrent. Only at the highest concentration tested (547 ppm), did they exhibit a strong deterrent effect.

b. Glycolipids

(1) Mono- and Di-galactosyldiglycerides of Barley

These fractions were effective stimulants when bioassayed individually, but they were less effective than the total glycolipids (cf. Table 22 and Figure 8). There was no apparent difference in CLB response to these two glycolipids (Table 22). Whether or not they were perceived as the same entity by the CLB is unknown. If so, then considering the flat numerical response to concentration for each fraction (Table 22), some other factor was required to produce the greater dose-response found for total glycolipids (Figure 8).

(2) Sulfolipids of Barley

This fraction was a strong stimulant to the CLB (Table 22). The one instance where activity was down (2.0) relative to other tests at 19.4 ppm may be explained by the isolation procedure used. The sulfolipid zone was scraped from the preparative TLC plate and placed in a "c" sintered glass funnel through which solvent was then passed. It is suspected that materials from the gel were incorporated with the sample and acted as a deterrent. The other samples were eluted by centrifugation which effectively removed the contaminants. Based on its low concentration (8% of the glycolipids), the sulfolipid was the most efficient stimulant of the three glycolipids. The sulfolipid probably interacted with the mono- and di-galactosyldiglycerides to produce the increased numerical response with concentration (Figure 8).

(3) Total Glycolipids of Pea

Except in one case, pea glycolipids were non-stimulative (Figure 8, Table 23). Three possible explanations can be offered to account for this inactivity. First, contamination of glycolipids with deterrent

apolar compounds on elution from silicic acid columns. Second, a polar deterrent(s) which chromatographed with these polar lipids. Third, some inherent quality of the glycolipids which distinguished them from those of barley.

Evidence for all three situations can be found in Table 23 and Figure 8. One mono-galactosyldiglyceride sample indicated in Table 23 gave an activity rating of 4.2 out of 6.0. This sample was separated from total glycolipids on a silicic acid column by elution with chloroform:acetone, 1:1 (v/v), which would have removed any tailing apolar deterrents not completely eluted with chloroform. Further purity of this sample was obtained by preparative TLC which would have eliminated any apolar deterrents. A low, but definite, activity was stimulated by the remainder of that glycolipid sample (Figure 8). This fact supports the alternative of having removed a deterrent with the mono-galactosyldiglyceride during elution from the silicic acid column. However, this activity was low relative to that evoked by glycolipids of barley. This fact implied either a second deterrent not observed during analytical TLC or an inherent difference in the remaining glycolipids.

(4) Mono- and Di-galactoslydiglycerides of Pea

With the exception discussed above, the mono- and di-galactosyldiglycerides from pea seedlings did not significantly stimulate CLB feeding. Reasons for this result have been discussed in the section above.

(5) Pea Sulfolipids

Insignificant activity was obtained from this fraction (Table 23) in contrast to the sulfolipid from barley (Table 22).

The overall inactivity of the pea glycolipids remains an interesting topic. Did each glycolipid co-chromatograph with its own undetected

deterrent? Was there, instead, some quality about the glycolipids, themselves, that was discernable to the CLB, providing the basis of rejection? The latter situation would have major implications to the subject of host-plant selection.

c. Phospholipids

Barley phospholipids were highly effective stimulants without further treatment after they were isolated by silicic acid column chromatography (Table 20). At this time, gramine (DISCUSSION, section IV.A. 1. c.) was present during the bioassay. Phospholipids from pea seedlings were inactive under the same conditions (Table 24) .

(1) Acid Phospholipids of Barley

When the acidic phospholipids were examined, they were found to have low effectiveness as stimulants (Table 25). The phospholipids with altered cation content did not significantly alter the CLB response to this fraction, while a plain deionized-water wash improved the activity a great deal (Table 26). It is possible that the deionized water removed a water-soluble contaminant picked up from the silicic acid or DEAE columns, including ammonium acetate used to elute the acid phospholipids from DEAE, though precautions were taken to prevent this occurrence. Washing with Na_2-EDTA or $CaCl_2$ may have recontaminated the acid phospholipids, thus depressing the activity of the CLB.

Several efforts were made to isolate and bioassay phosphatidyl serine. However, pure samples were not obtained. Therefore, definitive conclusions about the effect of this compound cannot be drawn (Table 28). The data merely confirmed that acid phospholipids were not strong stimuli to CLB feeding. They did not interact with barley neutral phospholipids

in a synergistic way, though an additive interaction was indicated (Tables 25, 30, 31).

(2) Neutral Phospholipids of Barley

The neutral phospholipids bioassayed without gramine were not highly active (Table 36). Under that condition, the activity of neutral phospholipids was no better than that of the acid phospholipids, which produced only a mild feeding response.

When gramine was present, the neutral phospholipids were good feeding stimulants (Tables 25 and 36). On an indidivual basis, they were much less effective (Table 29) and were far less effective than the individual glycolipids (Table 22). Hsiao (69) graded as moderate, the response of larval Colorado potato beetles to commercially obtained lecithin at 0.01M and 0.1M. Assuming a molecular weight of 780 for lecithin, 0.01M and 0.1M amount to ca. 600 ppm and 5790 ppm, respectively, relative to the present bioassay. The maximum concentration of lecithin tested alone in the present study was 23.3 ppm for native lecithin (Table 29) and 155 ppm for synthetic lecithin (Table 32). If tested at the higher concentrations used by Hsiao (69), then lecithin might be expected to be a moderate CLB stimulant.

A significant fact materialized from the trend noted in Tables 29-31. As more of the neutral phospholipids were combined, the response increased. This observation agrees with the effect seen when glycolipids were separated to their individual components (Figure 8, Table 22), and to that when the surface wax of barley was removed from the hydrophobic compounds (Figure 6). It is clear that the feeding response of the CLB upon barley is based upon a multicomponent system of stimulants, each contributing to the whole response. Similar conclusions were reached for the Colorado potato beetle (70) and for the boll weevil (160).

A report by Mehrotra and Rao (102) indicated that locusts discriminated among the phospholipid fractions of different vegetable oils as the CLB has done between pea and barley phospholipids in the present study. The presence of deterrents, the presence of unobserved feeding stimulants of a different chemical class, or a difference in the composition of the phospholipid fraction are the only alternative explanations. The possibility that the fatty acid composition might influence CLB response was examined (Table 32). Based on the experiments completed, a differential response was indicated in favor of L-α-1-stearoyl-2-oleoyl lecithin. The matter of influence of fatty acid composition constitutes an area for further research.

(3) Acid Phospholipids of Pea

Acid phospholipids of pea did not stimulate feeding in the CLB (Table 27). This fact is interesting in the same manner as the ineffectiveness of the pea glycolipids (cf. section IV.A.2.a.(3).

(4) Neutral Phospholipids of Pea

The neutral phospholipids from this plant were not feeding stimulants when bioassayed without further treatment after isolation from a silicic acid column. They were not separated into their individual components for bioassay (Table 27).

c. Gramine

In every bioassay, gramine was found to stimulate the feeding response of newly emerged, adult CLB (Tables 34-40). When larval CLB (mixed instars) were left overnight in one test unit of blank vs. gramine, or one blank test unit vs. one gramine test unit, overwhelming preference was shown for the gramine agar. The most significant data obtained from studies of gramine involved its interaction with extracts of pea plants.

Gramine altered both the glycolipid and phospholipid fraction of pea from non-stimulatory to highly stimulatory (Table 37). Gramine also overcame the strong deterrence of pea seedling hydrophobic compounds (minus wax) (Table 38). It is concluded that gramine acted as a host-recognition factor through stimulation of taste receptors.

To be a factor in host selection by the CLB in the field, gramine must be present in the plant at a detectable level when the beetles attack. The evidence presented below is indirect, but it supports the contention that these conditions can be satisfied.

Gramine has its own phenology during the growth of the plant. According to Bowden and Marion (16), Brandt et al. (18) found that gramine disappeared from barley after about one month of growth. Schneider and Wightman (139) indicated that it would disappear by day 50. The assumption will be made that in spring-planted barley, gramine will be present for 40 days. Castro et al. (22) found adult CLB first in roadside grasses (early to late March over a two year period), then in winter-planted crops and finally in spring-planted crops as they became available. It is not unreasonable that spring-planted crops would be sown after the month of March (104, 181). Since the CLB post-diapause adults are in the field before spring crops are planted, gramine must be present in those cultivars which produce it when the beetles invade the emergent growth.

Gramine was not quantified in the present study. Bowden and Marion (16) extracted 0.2456 g of gramine from 80 g of dried barley 11 days of age. Converting this value to a fresh weight basis, assuming 90% water content, that level was 307 ug/g (0.31 ppm). Schneider et al. (140) determined gramine levels in two cultivars of barley at 14 days of age. The values were 535 ug/g (0.54 ppm) and 623 ug/g (0.62 ppm). The least

concentration of gramine bioassayed by itself was 3.9 ppm (Table 34). A low, but definite, response was obtained. However, the response to gramine alone was fairly flat or independent of concentration above the threshold level (Table 34). It was in combination with other feeding stimulants that gramine was most effective, i.e., Tables 35 and 36. The flat dose-response would be useful where concentration in the plant changes over time. Since Bowden and Marion (17) demonstrated that all of the gramine was located toward the tip of the leaf, it is obvious that its concentration there will be perhaps several times greater than the figures presented above, making its detection and effects very likely. The positive phototropic behavior of the CLB would help to bring them into the vicinity of the tips of the younger leaves, which is the most preferred tissue (22).

In contrast to the leaf alcohols of barley, gramine is apparently not widely distributed in nature. Among the grasses, it has been reported only from the Genera Hordeum, Phalaris, and Arundo (132). This restricted distribution lends support to the contention that gramine acted as a host-recognition factor in this study.

Phytophagous insects sensitive to alkaloids have generally shown a negative response (142). An exception was the aphid, Acyrthosiphon spartii (Koch), which was reported by Smith (154) to respond positively to sparteine. Mohyuddin (117) found, but did not identify, a nitrogen containing feeding stimulant in the water extract of Calystegia sepium (L.) for a moth, Oidaematophorus monodactylus (L.), and a beetle, Deloyala guttata (Oliv.). While gramine had not previously been reported to be a feeding stimulant/sign stimulant for any insect species, it had been

reported to increase feeding by sheep on hay sprayed with "low levels" of gramine (5).

B. Hydrophilic Compounds

The numerical response of CLB to barley hydrophilic compounds indicated a decreased sensitivity relative to hydrophobic substances (Figure 5). After the recognition of gramine in the hydrophobic fraction, gramine was found to be present in those hydrophilic fractions preserved from earlier bioassays. The relative distribution of gramine between the aqueous and organic phases at the time of extraction was unknown, but some gramine must have been present in the hydrophilic fractions as they were bioassayed.

One curious aspect of the hydrophilic compounds was the type of agar damage that they evoked. It was this fraction that produced the highest proportions of channels, which was taken to indicate highly directed efforts at feeding. It is suspected that substances in this fraction form the final link in the chain of chemical stimuli which bring about continued feeding. Even the hydrophilic fraction of pea seedlings released the feeding response to a lesser degree (Table 6), which indicated that botanically restricted substances were not required at this level of the feeding response.

1. Sucrose

One must assume that sucrose contributed to the feeding response on the hydrophilic substances. No values for the sucrose content of barley leaves were found in the literature, but Smith (155) summarized the seasonal averages for several grasses and these ranged from 3-5.4% wet weight. From a 10 g leaf sample, which was typically extracted in

this study, 30-50 mg of sucrose might be present. If the entire sample were used as a single treatment, some other factor(s) must also have acted as a stimulant since the standard 40 mg of sucrose often used in this study, i.e., Tables 10 and 11, never attained the high numerical response reached by the barley hydrophilic fraction (Figure 5).

2. Amino Acids

Three amino acids were studied individually (Table 39). Tryptophan was selected because of its structural relationship to gramine and IAA. β-Alanine was selected because it was a deterrent to the European corn borer larvae (14). Proline was selected because it was a stimulant for the Colorado potato beetle (70).

Only β-alanine gave an indication of being a stimulant, but the concentrations used appear to be higher than should be required. Data in Table 42 reveal that the total cationic compounds comprise about 9% of the hydrophilic fraction. For a 100 mg sample (ca. 1,900 ppm), only 9 mg (ca. 175 ppm) could be amino acid and any individual amino acid would be well below that concentration.

The cationic fraction and the neutral-anionic fraction of the hydrophilic compounds were bioassayed, but insufficient data was obtained to justify definite conclusions (Table 42). However, the possibility that the cationic fraction may have been a stimulant was indicated. Gramine was believed to have been removed by the Sephadex G-10 column before the sample was applied to the cation exchange resin.

The simulated amino acid mixtures of a host plant and a non-host (Table 40), would have been informative had an adequate supply of laboratory beetles been available. Given the present information on the per

cent of cationic compounds in the hydrophilic fraction, meaningful bioassays could be made in the future.

3. Saponarin

The "last 200 ml" fraction off the Sephadex column demonstrated significant activity (Table 41) part of which must have been due to gramine. This fraction also contained the glycoflavone, saponarin. Zielske et al. (191) found that a glycoflavone of alligatorweed, Alternanthera phylloxeroides was a feeding stimulant for the chrysomelid, Agasicles sp. No clear indication was obtained in the present study that saponarin stimulated feeding by the CLB, but the results did suggest that further work in this area would be warranted (Table 43). One must assume that if saponarin were a feeding stimulant, it still would be of less significance than gramine. The basis for this conclusion is found in Table 19 where data show that crude extract of pea plants deterred feeding upon barley hydrophilic compounds (gramine deficient, saponarin concentrated), and in Table 38 where data show that gramine added to the hydrophilic fraction overcame the deterrent influence of pea apolar hydrophobic compounds (minus wax).

V. A Model of Cereal Leaf Beetle Host Selection and Feeding Response

A conceptualization of the barley-CLB relationship based on feeding stimulants has been derived from the results of this study. The adult CLB locates the spring-planted barley in some manner (aggregating pheromones released at a food source have been found for pine beetles, Dendroctonus brevicomis Lec. (189) and for the boll weevil (57). Stimulation by the alcohols of the surface wax causes biting through the leaf. Deterrents

are absent on the leaf surface or present at ineffectual levels. Detection of gramine triggers recognition of a suitable host directly, or indirectly by lowering feeding stimulant thresholds, or by raising thresholds to internal deterrents. Glycolipids (of the type found in cereal plant leaves?) are effective stimulants by themselves, while phospholipids require some potentiating factor, in this case, gramine. Together, these chemicals reinforce the biting response and possibly lower the threshold to stimulation by hydrophilic chemicals. As yet unidentified hydrophilic substances act with sucrose to bring about the behavior whereby the beetles produce feeding holes parallel to the leaf axis between the vascular bundles.

VI. Suggested Areas of Future Research

A. Questions Immediately Posed by the Present Study.

1. Since the epicuticular wax of pea seedlings have the long chain, primary alcohols which incite CLB biting response, why did samples of this wax deter feeding under the bioassay conditions employed? Were there actual deterrents involved, or was the particular combination of alcohols not suitable?

2. Why were the individually isolated glycolipids of pea plants so ineffective as feeding stimulants? Were there discrete deterrents which co-chromatographed with the pea glycolipids, or were there other species differences discernable to the CLB sensory systems, possibly related to the fatty acid composition?

3. What was it about the apolar hydrophobic compounds (minus wax) which caused that fraction from pea plants to be so highly deterrent to the CLB?

4. Within the hydrophilic fraction of barley, what was the identity of the substance(s) which caused the agar damage in those bioassays to be dominated by channeling rather than random biting and rashing?

5. Can the results of this study be applied to develop an artificial diet for the CLB which would allow successive generations to be reared, ultimately with the diet as the sole food source?

6. Can gramine be demonstrated in spring-planted barley crops when the CLB enter these fields?

7. Would there be any effect of barley resistance to the CLB if barley cultivars either very high or very deficient if gramine were tested in the field? According to Schneider et al. (140), Brandt et al. (18) found that gramine-free varieties of barley correlated with greater nematode susceptibility.

B. Questions Arising Through Interaction of this Study With the Literature

1. Since wheat, oats and rye have not been shown to produce gramine, are there other substances which serve as sign stimulants to CLB, perhaps alkaloids? The CLB host, rye grass, Lolium perenne L. contains the alkaloid, perloline, for instance (77).

2. Could the tryptamine alkaloid content of barley be manipulated to become a mechanism of resistance to CLB infestation? The only "overtly toxic" alkaloids known in grasses are the tryptamine alkaloids (26). Like gramine, these alkaloids are derived from trytophan (139). Castro et al. (22) did not mention Phalaris tuberosa L. [or its proper name, P. aquatica L., (2)] among the recorded host plants for the CLB. P. tuberosa was reported to attain concentrations of tryptamine alkaloids toxic to sheep (45, 46). Schneider et al. (140) reported tryptamine alkaloids in barley

seedlings. Guerra (50) found several indole compounds to be deletereous to growth and development of larval Heliothis spp. when incorporated into the diet.

3. The concept of electrophysiological investigations of CLB feed-response mechanism is one which could bear fruit if properly undertaken. It has confirmed results of behavioral studies of insect feeding stimulants (109). It has helped to formulate hypotheses regarding insect interpretation of chemical stimuli, and therefore, host selection by insects (34).

VII. Closing Statements

The collective mind of the human race patiently striving for a solution, may be the most powerful force that the cereal leaf beetle and its brethern have ever faced. We must assure that this is so. Work must progress to reshape the present selection-feeding model to a more precise representation of the cereal leaf beetle/host relationship, thereby exposing its vulnerabilities, which it surely must possess.

SUMMARY

1. The cereal leaf beetle required chemical stimuli extracted from barley seedlings to stimulate feeding on agar.

2. For a good feeding response to occur, an absence of deterrence was also required.

3. Some chemical stimulants from barley and their function included:

A. Hydrophobic Substances

The beetles were more sensitive to these substances than to the hydrophilic substances. Maximum feeding from this unfractionated group of compounds occurred at about 300 ppm and declined beyond 2,000 ppm.

1. Primary alcohols of the leaf surface (C-22, C-24, C-26) incited a biting response and it was concluded that this was their function in the field as well. Effective down to 1.0 ppm, the alcohols were the only active fraction of the epicuticular wax. A relatively flat response to concentration was found.

2. Glycolipids, especially sulfolipid, reinforced the biting and helped stimulate continued feeding. Mono- and di-galactosyldiglyceride were very similar stimulants based on CLB response. They were effective at about 20 ppm and above and produced a flat dose-response curve. Sulfolipid was active at the 1.0-2.0 ppm level and above. It interacted with the mono- and di-galactosyldiglycerides to produce a positive dose-response curve.

3. Neutral phospholipids as a group were stimulants, but they required the presence of gramine to synergize the CLB response to them. They were effective above 20 ppm. It was suggested, and some evidence was offered, that the fatty acid composition of phospholipids may affect the CLB response to them.

4. Acid phospholipids did not show a positive dose-response, but there were stimulants of low effectiveness.

5. The predominant agar damage observed in bioassays of glycolipids and phospholipids was comprised of biting and rashing. It was concluded that these compounds function to reinforce the biting response and/or to lower the stimulation threshold for hydrophilic compounds.

6. Gramine acted as a sign stimulant having caused CLB to feed in the presence of deterrent compounds and to feed upon formerly unaccepted non-host extracts.

B. Hydrophilic Substances

Maximum sensitivity to these compounds occurred at about 2,000 ppm. The adequate threshold level was about 300 ppm. Pure sucrose elicited a positive, but low, response at 0.002M (776 ppm). Except for sucrose, other stimulants from this fraction remain unidentified.

4. The only extract fraction of pea seedlings which stimulated feeding, but at a low level of response relative to the corresponding barley preparation, was the hydrophilic fraction. It was concluded that compounds in this fraction function beyond the sensory level of host recognition and serve to produce highly directed efforts toward continued feeding.

5. Both epicuticular wax and apolar hydrophobic compounds (minus wax) of pea seedlings were deterrent fractions to the CLB. The source of this deterrence was not identified.

6. The glycolipids and phospholipids of pea were not strongly deterrent, but they did not stimulate feeding. The reason for this is not known.

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