THE FEEDING RESPONSES OF SOME PHYTOPHAGUS INSECTS TO CHEMICAL LEAF CONSTITUENTS

A Thesis

Presented to

the Department of Entomology

Faculty of Agriculture and Home Economics

The University of Manitoba

In Partial Fulfilment

of the Requirements for the Degree

Master of Science

by

Percy John Procter

May 1961

ABSTRACT

by

Percy John Procter

THE FEEDING RESPONSES OF SOME PHYTOPHAGUS INSECTS TO CHEMICAL LEAF CONSTITUENTS

The purpose of this study was to isolate plant chemical feeding stimulants of some oligophagous insects and to determine the role of some common plant chemicals as to their effect on feeding stimulus, both by themselves and in conjunction with known chemical attractants. All tests were conducted under laboratory conditions.

Experiments with the Colorado potato beetle (Leptinotarsa decemlineata Say) larvae showed that extracted chemicals from potato leaf foliage could induce feeding, but no non-nutrient feeding stimulant was obtained. Isolates of flavone glucosides were found unattractive to larvae and inhibitory to the response produced by other nutrient chemicals. Some plant nutrients, notably saccharides and amino acids, could initiate a feeding response, but when presented in certain mixtures were inhibitory to this response. Tests of individual saccharides and amino acids show variation in the response to common plant chemicals by the larvae.

Feeding studies with the sweet clover weevil (Sitona cylindricollis Fahr.) indicate chemical leaf

constituents were enhanced by the presence of their sugars.

Effects of nutrient chemicals fed to the diamond back moth larvae (Plutella maculipennis Curt) indicate that a feeding summation occurs when presented with a known phagostimulant. Some nutrients, notably l-alanine, produced no cumulative effects in feeding trials.

ACKNOWLEDGMENTS

I am very grateful to my advisor, Professor A. J. Thorsteinson, who suggested the subject of this study, and whose guidance was an invaluable asset during the course of this investigation. Also, I would like to extend my thanks to other members of the Department of Entomology, for their co-operation and helpfulness.

In addition, I would like to express my appreciation to Dr. T. A. Angus, Department of Agriculture, Science Service, Insect Pathology Laboratory, Sault Ste. Marie, Ontario. The late Professor T. P. M. Payne, Department of Microbiology, University of Manitoba, gave generous assistance and advice on potato beetle disease. For work done in X-ray diffraction of some plant extracts, I would like to thank Professor Ferguson, Department of Geology, University of Manitoba.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES PAGE

CHAPTER I

INTRODUCTION

The instinctive motivations of insects as guided by their five senses, hearing, sight, touch, taste and smell are, under normal conditions, sufficient to direct them to their niche in the environment, and to satisfy nutritional and reproductive requirements. All phases of these motivations are of equal importance to the insect insofar as its continuation of numbers and species is concerned. However, one aspect of this life process which is of particular concern to humans, especially agronomists, is the ability of some of the phytophagus insects to choose, from many species of plants, a few which can satisfy their biological needs. With some phytophaga the host requirements are met by the plant species of many botanical orders. In others, the relationship has become so stereotyped that only plants from one family, or even a few from one genus, are acceptable as host, even though others which would appear to have similar nutritional qualities are present in abundance.

The discrimination of plants on the part of the phytophaga is now generally conceded to be the result of stimuli to the olfactory and gustatory receptors, effected by the odors and tastes arising from plant chemicals and

producing the resultant ingestion of the leaf material. Olfaction and gustation, the discriminating senses involved, are allied activities difficult to separate physiologically, but gustation is presumably the final critical sense determining acceptability. It is the investigation of this fraction of the entire feeding impulse which was experimented with here to determine what effect the common and specific plant substances have on the biting response and ingestion of food material by some phytophagus insects as related to their host plants.

Problem

These investigations had the following objectives: (1) to isolate plant chemical feeding stimulants of the Colorado potato beetle, Leptinotarsa decemlineata (Say), and to determine the role of some of the common plant nutrients in its feeding response; (2) to study responses of the sweet clover weevil, Sitona cylindricollis (Fahr), to refined chemicals extracted from the foliage of sweet clover; and (3) to determine the effect of a few nutrient chemicals used in conjunction with a known stimulant on the feeding of the diamond back moth, Plutella maculipennis (Curt), and to attempt to isolate additional chemical feeding stimulants.

Organization of the Thesis

The thesis is divided into five chapters. The introduction is contained in Chapter I. Chapters II, III and IV describe the work done with each of the three insects, and Chapter V contains a summary of the investigations of all three insects.

Importance of Study

The plant preferences exhibited by many of the phytophagus insects, and the regularity and specificity involved in this relationship have been observed for a great number of years. Unfortunately, this relationship also exists among many of the plants which man regards favourably, and has resulted in serious inroads into the productivity of many crops cultivated by him for his subsistence and well-being. The extent to which this damage has been minimized by human efforts has, in the past, been guided only by ingenuity and need; these efforts, however, have been unsuccessful in achieving any permanent means of control. The development of the synthetic organic insecticides was at first thought to end further need for other controls. However, because these insecticides are losing their effectiveness through the acquired resistance built up in some insects, other means of obtaining sure and more lasting control are desirable.

Although it is not the purpose of this project to deal with the control of insect populations affecting the agricultural crops, the results of these feeding experiments may have some future use in the development of plants which have resistance "built" into their genetic constitution, making them immune, or at least more resistant, to the attacks of certain insects.

CHAPTER II

THE COLORADO POTATO BEETLE

Introduction

The Colorado potato beetle is one of the more important insects of the world, from an economic viewpoint. The number of institutes which have been formed and which function specifically for its study attest to its importance in the potato industry. The result of this notoriety has been to make the potato beetle one of the most intensely studied insects, and, because of the large amounts of literature published on it, one of the best known, from a biological aspect. Its oligophagous feeding habit is well known. In its original environment in North America the beetle was confined to a wild species of Solanum (S. rostratum), but with the introduction of the potato, the insect adopted a new and very favourable host. This allowed it to follow this crop to other potato-growing areas of the globe. With its spread to new areas, access to many other plant species was given; however, it still remains confined to a few species of Solanum. Other Solanaceous plants will serve as alternate hosts, but are not as readily accepted as the Solanum species, in particular, S. tuberosum, the potato.

As a group, the Solanaceous plants contain a number of poisonour alkaloid compounds, whose presence in certain members add a limiting factor to the beetle's ability to develop on them.(3). Initial attraction in this insect was claimed to be the odours of some of the acceptable species (9), but Trouvelot et al (15) and Jermy (7) consider that there is no olfactory attraction, at least for the adult. The chemical constituents of the foliage condition the feeding response. (10) (1) (2) Therefore, final determination of host, in the absence of possible repellent substances is primarily a function of the gustatory sense.

This form of discrimination functions in both adult and immature forms of this insect. In adults, which probably have a more highly developed olfactory sense, plant odours may possibly provide the stimuli required in oviposition, but these have not been investigated, Larvae, although they have their food pre-selected by the ovipositing female, will refuse to feed on hosts containing inhibitory chemicals. The discrimination is subject to the variations in larval age, in rearing temperatures and inanition, so that under some conditions unacceptable plants will be consumed. Feeding under these conditions usually results in poor development and growth, so that normal maturation is not attained. (3)

In these experiments the chemical leaf constituents

were tested with the larval forms only. This phase of the beetle's life history is primarily one of feeding and growth, but still discriminatory as to host. Therefore, feeding will not be affected by the more complex functions occurring in the adult stages, and, at the same time, this larval consumption gives an indication of the response that might be expected in the mature forms.

Potato Beetle Cultures

As these experiments were primarily concerned with the feeding responses of the immature stages of the Colorado potato beetle, cultures were maintained to supply a constant year-long source of larvae. Eggs were obtained from adult beetles, which had been prevented from entering winter dispause by a technique of de Wilde's. (18) His methods of continuous rearing utilized the adult beetle's sensitivity to its photoperiod for the avoidance of this condition. Long-day photoperiods (eighteen hours) supplied by artificial lights, plus a constant supply of fresh young potato foliage as food were sufficient to keep adult beetles in a state favourable for oviposition.

Adults were confined in 24 inch long cylindrical cellulose acetate cages. The top ends of the tubes were covered with screening. Open ends had a diameter of 8 inches to fit the pots in which the plants were grown.

Screened windows and a door were made in the sides of each cage to prevent moisture condensation and to facilitate egg removals. Illumination was supplied by four 40 watt tube type fluorescent lamps at room temperature, with a photoperiod of 20 hours per day. Approximately 25 adult insects could be confined with each potted potato plant. When defoliated, plants were exchanged for new ones from supplies grown in the greenhouse for this purpose.

Eggs were collected from the adult cages daily and the ensuing larvae reared by methods similar to those used by de Wilde. When larvae had grown to "test size", the required number were removed from the rearing cages and placed in other dishes without food, to evacuate the alimentary tract prior to use in experiments. Some larvae were allowed to complete their development to maintain the numbers of ovipositing adult beetles.

Selection of Experimental Insects

The fourth instar larvae not older than 20 hours after the third moult were used in the experiments. This limitation insured that test insects used were of a uniform physiological age, and thus prevented variations in feeding reactions caused by age differences. First and second instar larvae showed little response to substances proven attractive to the older larvae. This seemed to be

caused either by the inability of the smaller instars to bite the material of the artificial leaves, or by the lack of the necessary feeding stimulus required to promote biting by young larvae. Third instar larvae would show limited response to active substances, but were not used because of their smaller feeding capacity in comparison with fourth instar larvae, which displayed a more vigorous feeding response to substances tested.

Disease

Disease became a problem in the potato beetle cultures during January 1959. The problem first became apparent in the excessive mortality rates, and the state of moribund larvae suggested a bacterial infection. The sick larvae became unco-ordinated in their movements and then inactive. This was followed by death. A color change was also noted in the affected insects. Microscopic examination of body fluids revealed the presence of active motile rods, which, in stained slides, appeared gram-negative. A gram-positive coccus was also present in these preparations. The instars showed differing degrees of susceptibility to the disease, with the majority of the deaths occurring in the second.

Attempts were made to control the disease by sterilizing possible sources of contamination - rearing

dishes, forceps, etc. However, mortality rates still remained at levels of 95 to 100 per cent. The continuation of the infection in the culture was probably due to bacteria air-borne on dust particles, the contamination of greenhouse foliage used in feeding, or the emerging larvae's habit of eating the remnant of the egg case, contaminated by adult faeces.

As sterile conditions could not be maintained in larval rearing, antibiotics were fed to prevent the occurrence of the disease in the larvae. Preliminary tests using sprayed applications of polyatin and streptomycin to to the leaves fed to the young stages of the potato beetle showed that the latter chemical, at concentrations of 0.25 gm/100 mls. of water, was able to prevent the disease. Polyatin was detrimental to growth at concentrations of 2 tsp./100 mls., and lower concentrations were not effective in lowering the death rate of larvae fed the treated foliage.

After several generations of larvae had received treatment with streptomycin, the antibiotic was no longer effective in combatting the organisms causing the disease. Higher concentrations were then used, but, again, were effective only for a short time. The pathogenic bacteria seemed to have built up resistance to this antibiotic. It therefore appeared that further identification of the causal organism was needed for future control in the culture.

Samples of moribund larvae were sent to the Insect Pathology Laboratory at Sault Ste. Marie. There, the insect pathogen was tentatively identified as Bacillus leptinotarsae, causal agent of potato beetle septicemia. The fact that the symptoms of the infected larvae were comparable with those in White's (17) description of the disease tended to support this identification. As a further test, another sample of sick larvae was given to the Microbiology Department of the University of Manitoba, where three other possible pathogens were isolated - Streptococcus, Proteus and Pseudomonas. The Microbiology Department also tested the antibiotic sensitivity of these organisms, and recommended that aureomycin and terramycin be used to reinforce the streptomycin employed in previous treatments.

Methods of Testing

Plant extracts and other chemicals tested were presented to the larvae in artificial leaves of Japanese elder pith, a method devised by Raucourt and Trouvelot (10), and later improved upon by Chauvin (1) and Thorsteinson (13). Slices of pith, 240 microns thick and die-cut to a diameter of one-half inch, were saturated by immersion in a solution of the test substance. Excess solution was removed by draining. The cellular structure of the pith made it absorbent and tender enough for the larvae to bite.

Expansion and contraction with changing moisture content did not distort disc area for later measurements. After treatment the discs were dried and then clipped in the ends of split swab sticks.

Tests with the larvae were carried out in plastic dishes, 7 cm. in diameter by 1.5 cm. deep. A circle of filter paper was held to the bottom of each dish by impregnating a section across the centre of the paper with hot paraffin wax, and then allowing it to cool and adhere to the bottom of the test dish. The areas of untreated paper on either side of this area served as an absorbent for water added later to maintain humidity. Each dish contained two paired discs, the untreated one serving as the negative control. The free ends of the holding swab sticks were sealed with wax to the central area.

Before the larvae were placed in the dishes, each disc was sprayed with distilled water from an atomizer until saturated. The unwaxed area was also sprayed. Five test-size larvae, starved for 3 hours, were then placed in each dish. The dishes with the larvae were put into a water-saturated air-tight container for 20 hours at a temperature of 27°C. At the end of this time, the larvae were removed and discarded. The discs were detached, stained with methylene blue, and then permanently mounted on glass slides in order to allow estimations of the eaten areas.

Evaluation of Disc Consumption

The gustatory stimulus of the substances offered to the larvae in artificial leaves can be measured only by computation of the areas eaten from these discs. Several methods were used in this evaluation, each suited to a particular phase of the experimentation.

Visual Evaluation

The evaluation of component parts of a plant extract as to their acceptability with the larvae was done visually. This was essentially a screening process to eliminate non-active fractions. After an extract fraction had been tested with the larvae, the treated and negative control discs from each testing container were mounted on sheets of note paper or cardboard with Scotch tape for a permanent record. Staining with methylene blue allowed a better differentiation between disc and background. The criteria of activity in extracts were the number of treated discs eaten in the replicates and the amount consumed. If three treated discs from as many replicates were partially consumed, or if two out of three were almost totally consumed, the fraction was considered attractive.

A refinement of this evaluation involved a visual comparison of the eaten discs from a test series, with discs whose areas had been previously computed by a

mechanical method described below. The standard measured discs with which the comparison was made were divided into groups of equal percentages, so that most feeding patterns were represented. This method was used only when small amounts of feeding had taken place, as the error in judgment increased with the percentage of the discs consumed.

Mechanical Evaluation

This method of disc measurement is the most accurate. However, it is time-consuming and does not lend itself to the evaluation of a large series of tests involving several replicates and the many dilutions which were used with the pure chemicals.

The discs were stained and mounted as described above, and projected with an epidiascope on a flat surface serving as a screen. The enlarged images were traced on sheets of paper fastened to the screen, and the magnified disc areas were computed with the use of a compensating planimeter. Readings of eaten discs were compared with those from uneaten ones, for conversion to a percentage basis. The limitations of the planimeter necessitated the enlargements, as the boundaries of normal-sized discs were difficult to follow accurately with this instrument.

Weight Evaluation

Comparisons of disc weights taken before and after feeding trials were too variable because of contamination with larval faeces. As areas were unaffected by this source of error, weights of enlarged tracings were used for estimations. After discs had been enlarged and traced as described above in Mechanical Evaluation, the tracings were cut from the paper and their weights determined. However, because of difficulty in obtaining paper of uniform weight distribution, this method was abandoned.

Light Intensity Evaluation

A faster and more efficient method of evaluation was later developed for disc measurement. Light transmission, through photograph negatives of discs, was measured to determine the degree of larval consumption.

Contact photographs of opaquely stained discs were made on contrast film. Exposure and development time were standardized for all groups of film. Light from a constant source was shone through these cleared disc images onto the photo-electric cell of a Weston light-meter (Model No. 756), and the intensity was measured for a determination of disc area with a quartz filter. After standardization of the light-meter with discs of a known size (as determined by a planimeter), readings were converted into percentages of the

uneaten area of untreated control discs.

Construction of Histograms from Light-Meter Readings

The light-meter readings obtained with this method were proportional to the areas of discs remaining after feeding. In order to account for feeding which had occurred on untreated discs, and to convert to areas consumed, the following formula was used:

$$100 - \left[\frac{\text{Sum of photometer units of similarly treated replicate discs}}{\text{Sum of photometer units from the paired untreated discs in the replicates}} \times 100 \right]$$

= Percent of discs eaten attributed to treatment given

Areas of the discs covered by the disc holders of split swab sticks were previously subtracted from the sum of the photometer units of both treated and untreated discs before the formula was applied.

The values obtained with this formula were used to construct all histograms presented in this thesis.

Materials and Methods

Preparation and Partition of Plant Extract

Preliminary tests have shown that larvae of the Colorado potato beetle will feed on artificial leaves treated with an aqueous or alcoholic potato leaf extract.

The isolation of the chemicals in such an extract should indicate those responsible for this gustatory response.

The initial extract was made from fresh or fresh frozen Pontiac potato foliage. A 1:4 ratio, w/v of leaves and 95% ethanol was macerated in a Waring blender and then boiled for 15 minutes. After boiling, the coarser leaf particles were removed by straining, and the liquor cooled to room temperature. The material dissolved in the alcohol was filtered through No. 1 Whatman filter paper and the filtrate concentrated to a gummy, tarry residue.

A scum of chlorophyl formed on the surface as the material was concentrated, and was removed with several washings of benzene. This benzene soluble fraction had no activity when tested with the larvae. This concentrated tarry residue was then taken up with water, and filtered to eliminate other inactive water-soluble substances, one of which was more chlorophyl.

The water-soluble extract was concentrated to a thin, brown, syrupy liquid, and then applied in bands to rectangular pieces of 27 mm. Whatman chromatographic paper (of a size conveniently fitting a tall 400 ml. beaker). The bands were formed by drawing a loaded pipette along a line, 1 inch from the bottom of the paper. The application of the extract was repeated until the material had spread for one-quarter inch on either side of the drawn line.

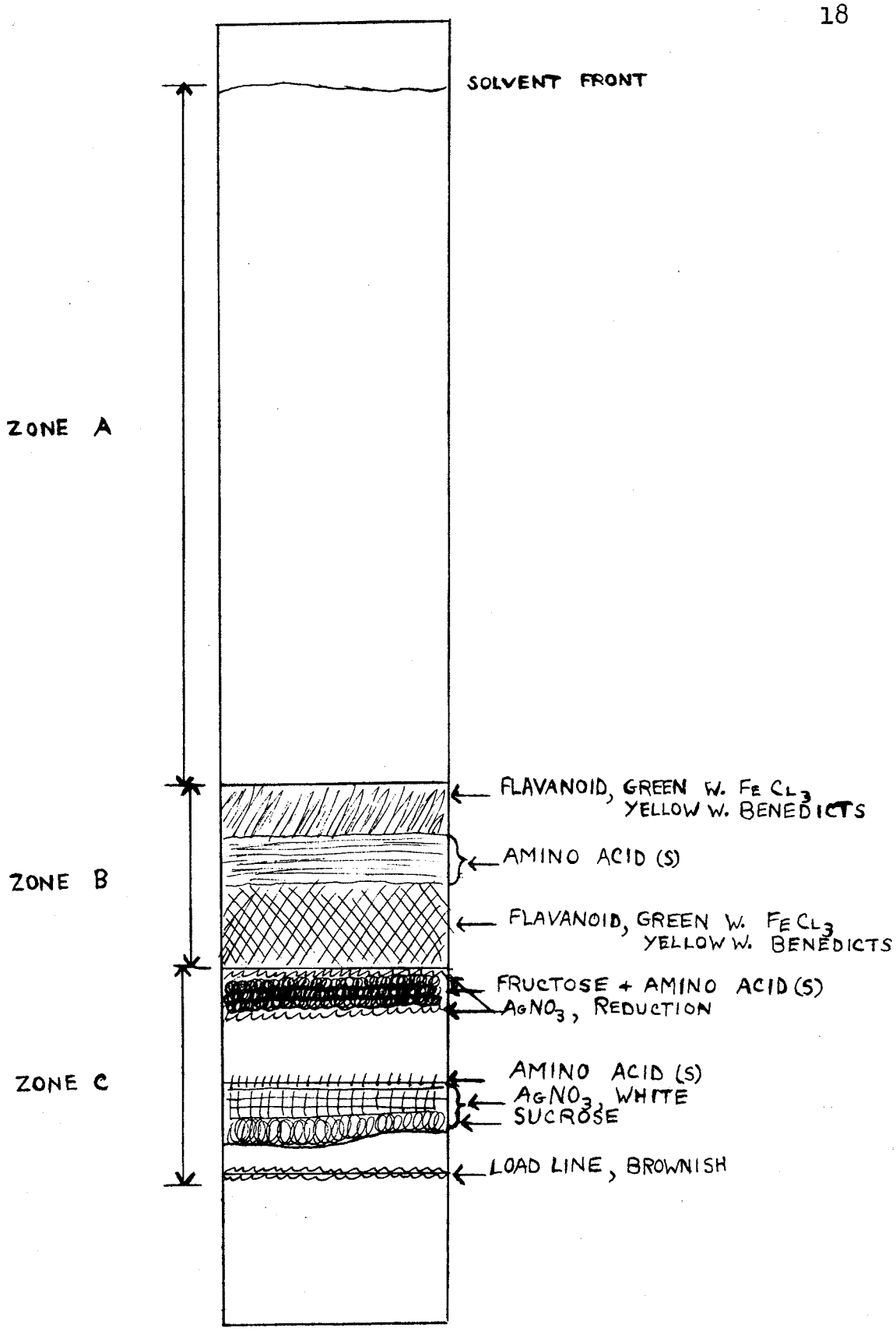


FIGURE 1

CHROMATOGRAPH OF ETHANOL EXTRACT OF POTATO LEAVES

Butanol: acetic acid: water (4:1:5)

When the paper with its absorbed extract material had been thoroughly dried in a stream of warm air, it was placed in a beaker containing one-half inch of solvent. The area of paper below the absorbed band served as a wick to enable the solvent to pass up through the band by capillary ascent; it also prevented the material from coming in direct contact with the solvent reservoir in the beaker. As this liquid passed up the paper, a fraction of the extract was dissolved in it, and was deposited at the top of the paper as the solvent evaporated. The volatile solvents used required shielding, so that evaporation would take place only along the upper edge. The top portion of the paper, which held the deposited material, was cut off and the contents eluted with a suitable solvent.

The first solvent run through the band of extract in this manner was benzene. It removed remaining chlorophyl and other soluble substances which were again inactive when eluted and tested with the larvae. In preliminary methods, acetone was used in place of benzene. This solvent not only removed the chlorophyl, but also deposited a substance which, on testing with the larvae, appeared to have a slight inhibitory effect on feeding. This material produced a dark red color on exposure to ammonia fumes and on treatment with Benedict's solution.

After drying, the remainder of the paper sheets were placed again in the beakers now charged with 95% ethanol. This solvent carried a yellowish-brown material to the top of the paper. This was cut from the paper and the beaker re-charged with ethanol. This process was repeated until no visible material was deposited by a new charge of solvent.

The strips cut from the paper containing the ethanol-deposited material were eluted with hot methanol and then hot water. The eluates were bulked and concentrated to an oily consistency. On standing, the concentrate formed a mass of crystals easily removed by filtration and washing with hot methanol. The crystalline material gave no response to the potato beetle larvae, and no further attempts were made at identification after it was shown to be inorganic in nature.

The concentrated fraction from which the crystals were removed still caused feeding activity by the larvae. Further separation of its chemical contents was obtained by partition chromatography.

The brown coloured material which remained at the position of the original load line of the paper, and which was not moved by either solvent, was eluted with water, but yielded only a slight response with the larvae.

The chromatographic separations of the active ethanol fraction were performed in a multiple sheet ascending-type chamber. The extract was banded on 20 x 20 cm. sheets of 3 MM Whatman paper with a capillary pipette, and developed with the alcoholic phase of a mixture of N-butanol, acetic acid and water, in the proportions 4:1:5 respectively. The solvent front was allowed to advance to 4 cm. from the top edge of the paper before the sheets were removed for drying in a warm stream of air.

Test strips were cut from one of each group of the developed chromatograms for spraying with one of the following chromogenic reagents: 0.25% ninhydrin in acetone, 1% ferric chloride, and alpha-napthol - H_3PO_4 (1:10w/v). These reagents were to detect amino acids, flavones and sugars, respectively. These treated strips permitted localization of substances on the unsprayed chromatographs, and provided for comparisons between the different batches of developed sheets.

The division of the chromatographs was performed by marking boundaries of chemical zones as shown by their coloration under ultra-violet light, and by comparison with the chromogenically treated strips. The divisions were cut from the sheets and eluted for larval feeding tests and further separation.

A general diagram of the chromatograph resulting from the development of the active ethanol fraction can be seen in Fig. 1.

Zone A of this chromatograph did not react with any of the three standard spray-reagents; however, treatment with ammoniacal silver nitrate revealed one reducing band. The contents of the entire zone were eluted with hot water and tested with the larvae. No gustatory response was obtained from this zone.

Zone B contained two bands of flavonoid material and one of amino acid. The lower band of the flavones was seen as a clear yellow zone without treatment, but treatment with ferric chloride was necessary to show the upper band in ordinary light. Both of these bands produced a greenish-yellow fluorescence in ultra-violet light, characteristic of flavone glycosides (8). As the amino acid was located between the two flavone bands, separate elutions of these pigments each contained a portion of it, due to over-lapping. Larval tests gave no response to individual flavones or to the eluate of the entire zone.

Zone C eluate gave the only active larval feeding response of this fractionation. A characteristic of this area was the complex over-lapping of the component bands, preventing their individual elution for larval tests. The

chromogenic spray for amino acids showed their presence and their considerable over-lapping with bands of other substances. Alpha-napthol revealed two distinct bands of sugars. A rescorcinol spray of similar strips confirmed the presence of the two sugars but did not indicate others. Ammoniacal silver nitrate indicated several bands near the top band of sugar, and an area near the lower sugar band which remained paper-white, even after the treated strip was heated to 90 C. Several pigments remained at the original load line of the chromatograph and were included in the elutions of this zone. The entire zone was eluted several times with hot water, and the eluates bulked to form one sample.

Sugar Identification

Attempts at identification of the saccharide bands in Zone C were made using ascending chromatography of spot samples. Spots of the Zone C eluate were developed with N-butanol, acetic acid and water (4:1:5) with control spots of pure sugars. The position of the sugars in the extract on the chromatograph agreed with those of sucrose and fructose. These sugars were later tested in pure form and the results are described in a later section.

Amino Acid Identification

As amino acids were conspicuous in the active

chromatographic fractions, an attempt was made to determine the more prominent of these substances, so that they might be fed to the larvae in a pure form.

The identification was performed with two-dimensional chromatographs of spot applications of the material obtained from a hot 95% ethanol extract of potato leaves. This extract was not purified in any way other than by filtration of the concentrated material. Ten microliters of this standard extract were applied as spots, with control spots of pure amino acids. The sheets were developed with 1) N-butanol: acetic acid: water (4:1:5), and 2) phenol: water (100:39 w/v). After drying, the sheets were treated with 0.25% ninhydrin.

The most prominent of these substances in the extract were aspartic and glutamic acids. Serine, glycine and gamma-aminobutyric acid were also present, but apparently in much smaller amounts. Several other amino acids were also detected, but in such minute amounts that their position was difficult to determine.

Pure Chemicals Used in Feeding Trials

As the analysis of the potato leaf extract had been confined to sugars and amino acids, only these groups were tested with the larvae in pure form. The sugars used in these trials were: sucrose, fructose and dextrose; the amino

acids were: aspartic, glutamic, alanine, glycine, serine, gamma-aminobutyric and glutamine.

These compounds were presented to the insects in pith discs, impregnated with the solution. Concentrations of each substance tested were in a graded molar series. Certain mixtures of these nutrients were also presented, with two or more substances from each group in each treatment.

Results and Discussion

The results of the feeding responses to the potato leaf extracts provided a guide for the progressive purification of active fractions. The elimination of these non-active substances entailed the risk of removing some active material, but as long as one of the fractions from the separation remained active, this risk was disregarded.

The final active isolation obtained is represented in Zone C of Fig. 1. The spray reagents used on this chromatogram indicated the presence of a large number of components in this band, all of which, either singly or collectively, could have produced the stimulus necessary for the feeding response. However, the limited attempts made at identification of these components, confined to amino acids and sugars, revealed that sucrose and fructose, along with several amino acids, were present in the active

zone. Sucrose and some amino acids have the ability to induce a feeding response by themselves, as will be shown later. Therefore, the attractiveness of this zone was accounted for by the presence of this group of substances, and further separation of the fractions was abandoned.

One inactive fraction, the B zone of Fig. 1, was of particular interest. This area contained the flavonoid substances which Chauvin (2), in his isolates of potato leaves, suggested as the gustatory stimulus inducing the feeding response. The zone contained two bands characteristic of this material; neither showed any activity when tested with the larvae separately. Further tests were tried, using the eluate from the entire zone, in combination with sucrose and gamma-aminobutyric acid, to determine whether or not there would be an additive response, such as is exhibited by Plutella maculipennis (Curt), to nutrients when presented in combination with the gustatory stimulant, sinigrin, as illustrated in a later section.

In Figs. 2 and 3, the response to a constant concentration of gamma-aminobutyric acid with a dilution series of the flavone solution is shown. The two highest concentrations appear to lower the amount of feeding which would otherwise be expected with the amino acid by itself. At the third highest concentration, or 1/16 stock solution,

the same comparison shows that the amount of feeding exceeds that of the positive control. However, a comparison of this control with that shown in Fig. 3 indicates that the amount of feeding of the positive control in Fig. 2 may be too small. A statistical analysis of variance between the treatments in both these graphs indicates that there is no significant difference between them.

Fig. 4 shows the response of a dilution series of the flavonoid solution with a constant 0.004 M. solution of sucrose. The effect of this plant substance on feeding under these conditions is variable. Analysis of variance shows no significant difference between treatments.

From these experiments there is no indication that the flavones, partially isolated in these separations, have any cumulative effect on the feeding response of potato beetle larvae, under these conditions; the experiments indicate that there may be a slight inhibiting effect at higher concentrations.

Results of Larval Feeding with Pure Chemicals

These feeding trials with pure substances were done because of the marked feeding responses produced by them with a polyphagous grasshopper, Camnula pellucida (14) (11). Also, some have been identified in plant extracts, and their effect on the feeding behaviour made it desirable to

determine their stimulatory role in plant extracts.

SUGARS

Sucrose, fructose and dextrose were presented to the larvae in pure form. The first two of these substances were identified in the final active isolate, present in the active Zone C of the chromatographic separation shown in Fig. 1.

The response to sucrose was very pronounced, and, as shown in Fig. 5, it varied with concentration. Although the most active of the concentrations shown in this graph are unrealistic as plant analysis has shown, the response was so consistent and pronounced that 0.1 M. sucrose was used as a positive control for other experiments, to provide an additional check on the appetite of the insects. Statistical analysis of responses comparing treated discs with untreated ones, using Student's t-test, gave a significant difference among concentrations, and a P value smaller than .01. Fructose and dextrose yielded no pattern of response after six replications of concentrations similar to those that were used with sucrose. The former, present in plant extracts, was tested from two different samples of analytical grade to insure that contamination of supplies had not taken place.

Fructose was used again in two identical molar series, with the addition of sucrose. The levels of sucrose

added to each series were .1 M. and .02 M. No disc measurements were recorded for these two series of tests, but visual estimation showed that fructose does not enhance the feeding response to sucrose.

AMINO ACIDS

The presence of amino acids in the plant extracts has been mentioned. Aspartic and glutamic acids were the most conspicuous of the amino acids found in the extract. These were presented to the larvae in pure form, and the results of the feeding responses and the concentrations used can be seen in Figs. 6 and 7.

The amount of feeding on aspartic acid was consistently negative, i.e. more feeding occurred on the negative controls presented with each treated disc. The exception was the 0.0008 M. concentration. A statistical analysis using the Student's t-test gave a P value of .02 for the probability of the negative feeding response. Differences between concentrations had no significance when an alysis of variance was used as a statistical test.

Glutamic acid (see Fig. 7), when presented in concentrations of the pure form, yielded erratic feeding responses. The percentage values obtained were highly variable, fluctuating from 23% to -16%, but they seemed to show an indirect response to concentration. The t-tests

gave a P .05 value for effects of the treatment, but it is difficult to interpret this.

The feeding trials with l-alanine, glycine, l-serine, gamma-aminobutyric acid and l-glutamine are shown in the graphs of Figs. 8 to 12. Their presence in the potato leaf extracts was in very minute amounts, according to ninhydrin treatment of chromatograms, and hence positive positional identification of them was uncertain. The response of the first three mentioned substances is very apparent, with the plateau of good response dropping off at approximately 0.004 to 0.02 molarity. From the histogram of gamma-aminobutyric acid, the response appears to be less pronounced at the higher concentrations, but activity exists over a wider range. L-glutamine's effect on the consumption of the discs is indeterminable. The t-tests performed on trials of this material gave P values of .5, and any feeding which did occur was extremely variable.

COMBINATIONS OF AMINO ACIDS

Mixtures of amino acids that have been shown to produce a feeding response by themselves appear to be antagonistic to the response when presented in combination with each other. L-serine, gamma-aminobutyric acid and l-alanine, each with equal molarity and at the concentrations indicated in Fig. 13, produced the feeding pattern indicated in this graph. With this mixture series,

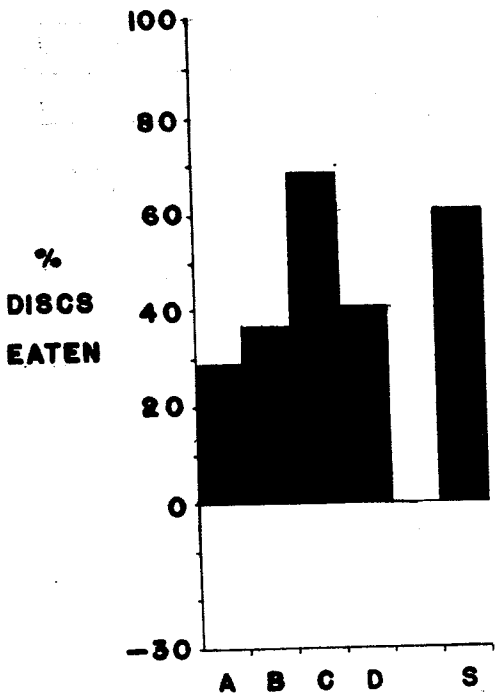
consumption falls off rapidly below the 0.004 M. concentration, and falls again more slowly at concentrations above .004 M. The small amounts of feeding which did occur on the amino acid mixture, as shown in Fig. 13, can be weighted by the percentages of consumption in the .1 M. sucrose control. Using this as a comparison, responses at .02 and .004 M. concentrations of amino acids surpass that of sugar. However, the expected amount of feeding from a mixture of such individually active substances was not attained.

COMBINATIONS OF AN AMINO ACID AND SUCROSE

The results of combinations of 0.004 M. gamma-aminobutyric acid with .02 M. sucrose are shown in Fig. 14A, and a replication of these substances together with a lowered sugar content in Fig. 14B. It appears from these graphs that the combined substances are less attractive to the larvae when presented together. However, a comparison of responses to 0.004 M. gamma-aminobutyric acid and controls in Figs. 13, 14A and 14B reveals considerable variation in feeding response to the same treatment in different experiments.

Thus, although it is difficult to interpret these results as showing that the mixtures of sucrose and gamma-aminobutyric acid are antagonistic to the feeding response,

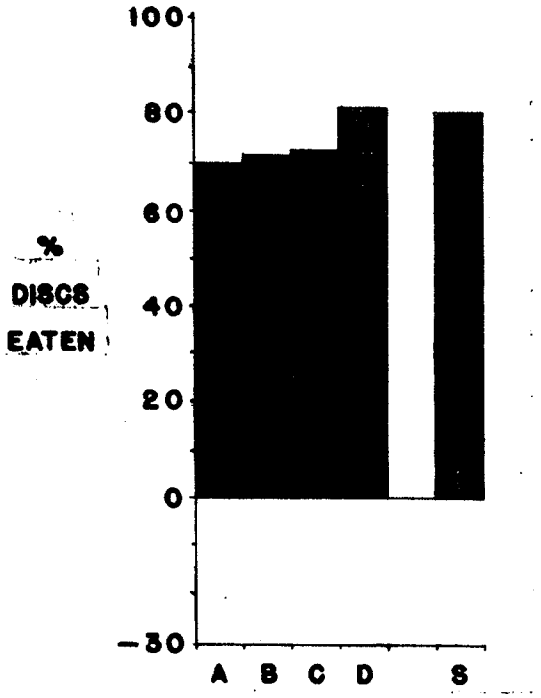
it is obvious that no summation is gained by combining these two active substances, which can induce feeding by themselves.



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO

A = .004M ɣ-AMINOBUTYRIC ACID
+ STOCK FLAVONE SOLUTION
B = .004M ɣ-AMINO. + 1/4 STOCK FLAV.
C = .004M ɣ-AMINO. + 1/16 STOCK FLAV.
D = .004M ɣ-AMINO.
S = .1M SUCROSE
5 REPLICATES

FIGURE 2

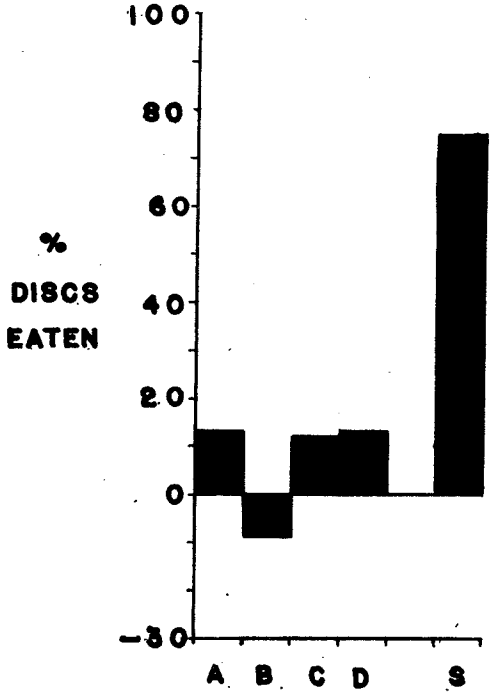


FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO:

A=.004M δ-AMINOBUTYRIC ACID
+ 1/64 STOCK FLAVONE SOLUTION
B=.004M δ-AMINO + 1/256 STOCK FLAV.
C=.004M δ-AMINO. + 1/1024 STOCK FLAV.
D=.004M δ-AMINO.
S=.1M SUCROSE

4 REPLICATES

FIGURE 3

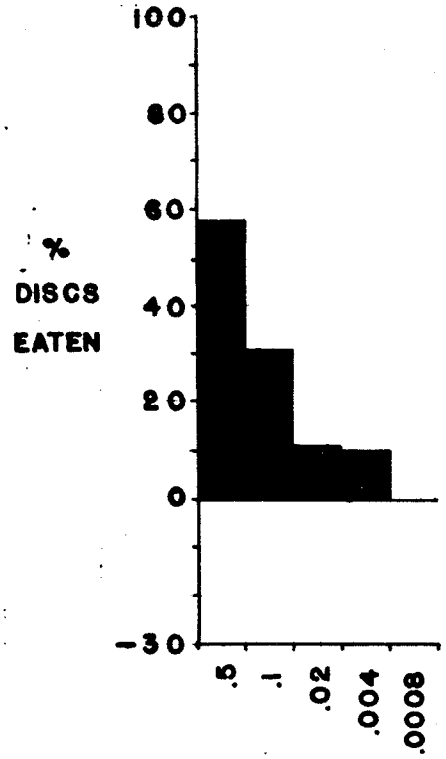


FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO:

A=.004M SUCROSE + STOCK FLAVONE SOLUTION
B=.004M. SUC. + 1/4 ST. FLAV.
C=.004M. SUC. + 1/16 ST. FLAV.
D=.004M SUCROSE
S=.1M SUCROSE

6 REPLICATES

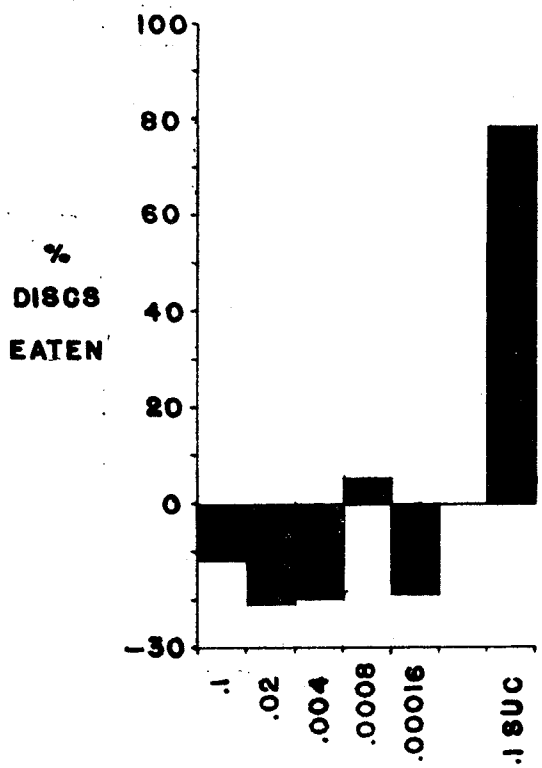
FIGURE 4



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF SUCROSE

3 REPLICATES

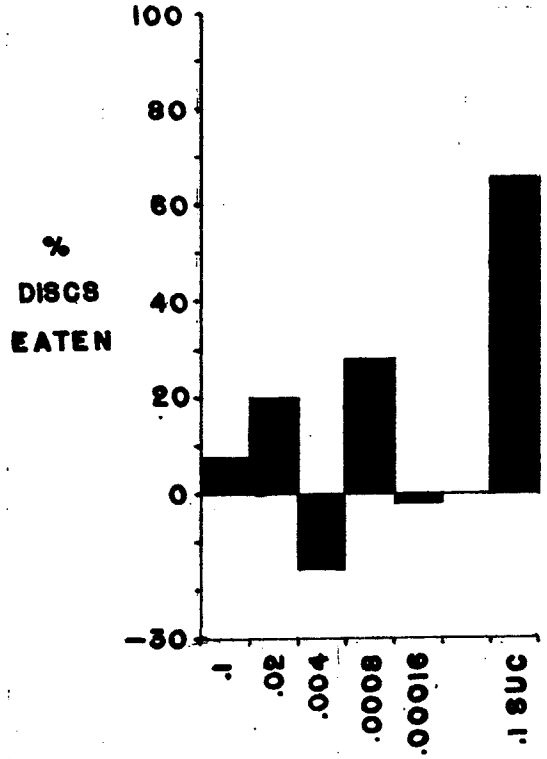
FIGURE 5



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF ASPARTIC ACID

4 REPLICATES

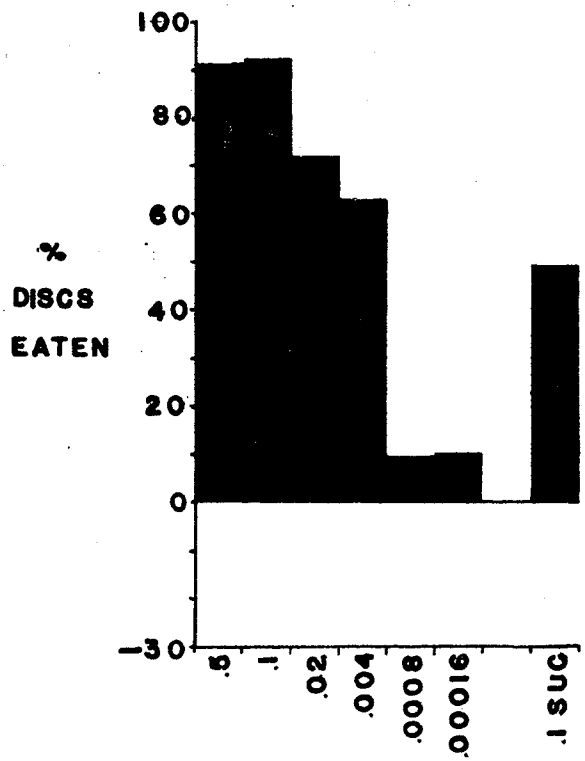
FIGURE 6



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF GLUTAMIC ACID

5 REPLICATES

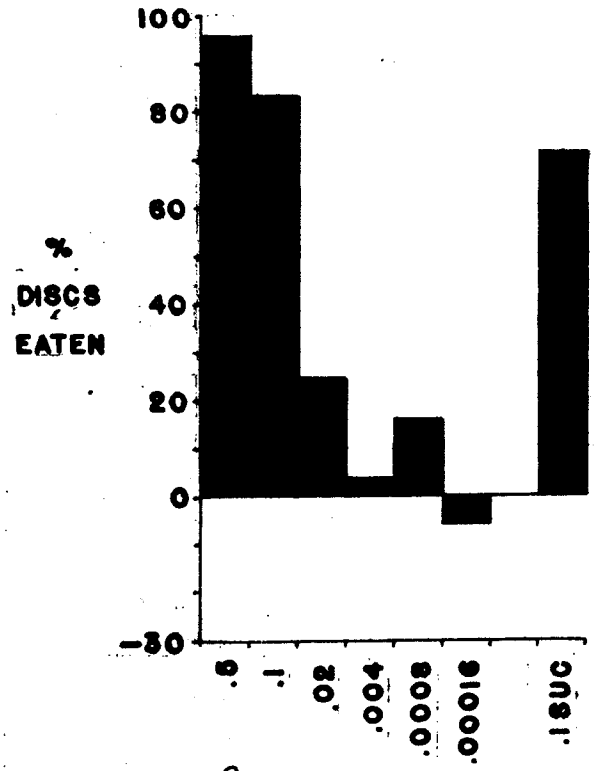
FIGURE 7



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF L-ALANINE

5 REPLICATES

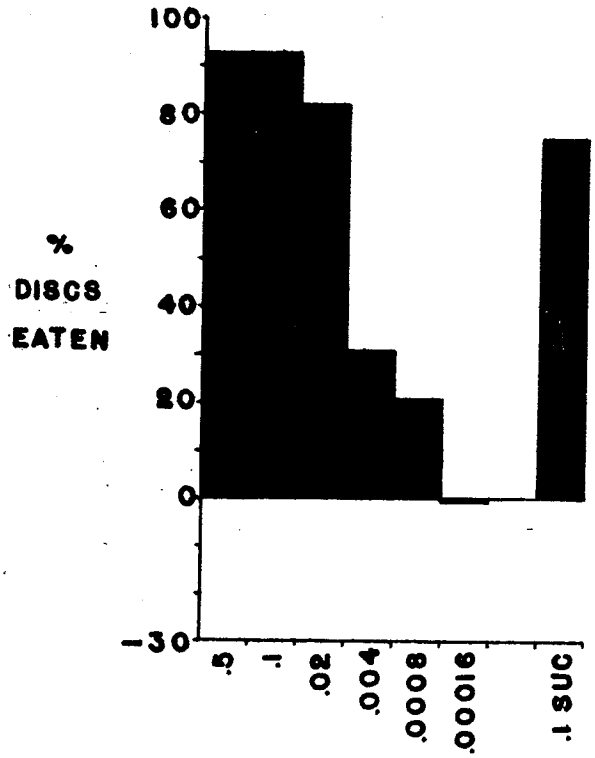
FIGURE 8



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF GLYCINE

7 REPLICATES

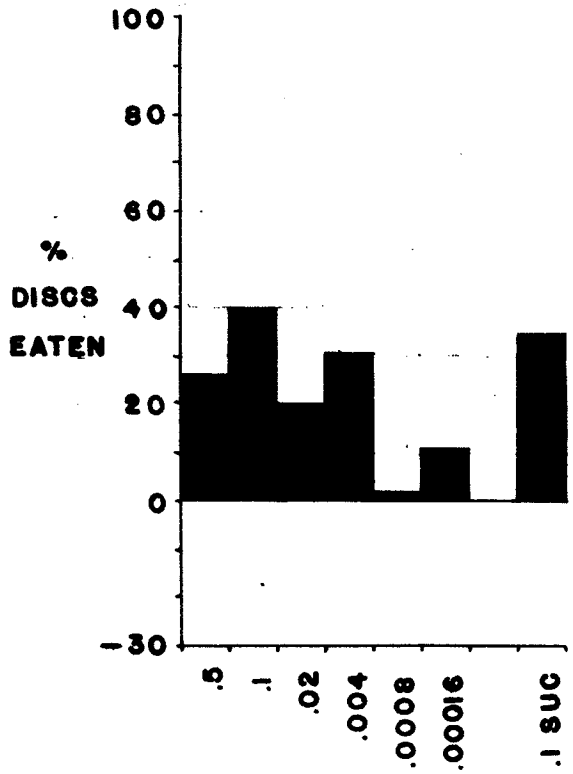
FIGURE 9



FEEDING RESPONSES OF LARVAL L. DECEMLINEATA TO GRADED MOLAR CONCENTRATIONS OF L-SERINE

4 REPLICATES

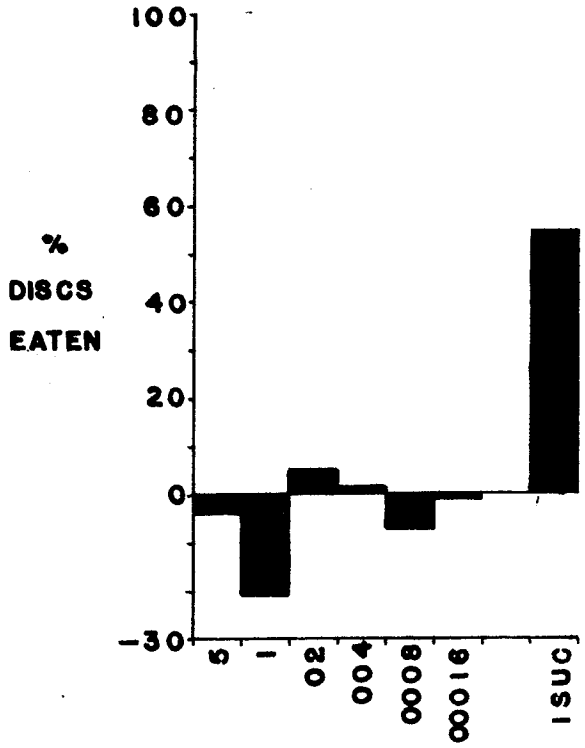
FIGURE 10



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF δ-AMINOBUTYRIC ACID

3 REPLICATES

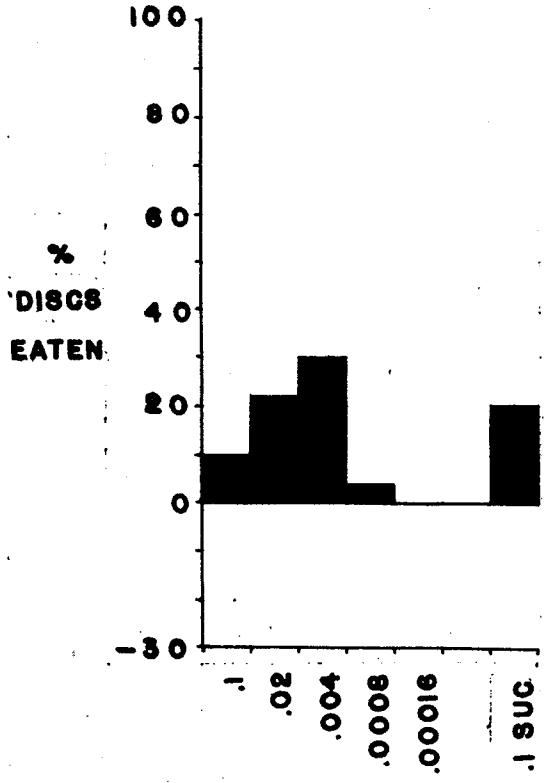
FIGURE 11



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO GRADED MOLAR
CONCENTRATIONS OF L-GLUTAMINE

7 REPLICATES

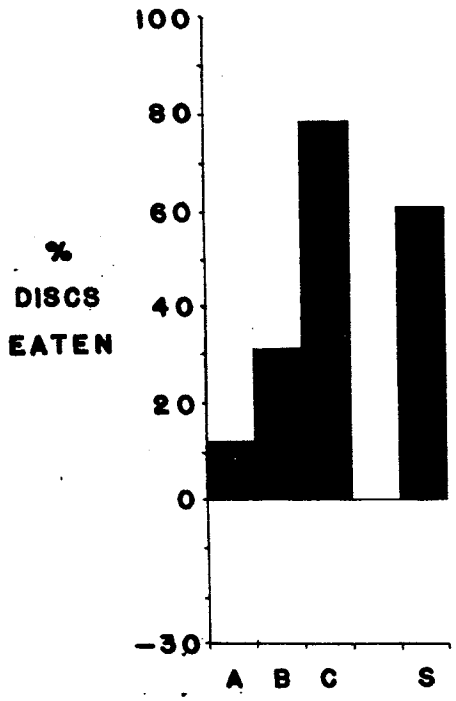
FIGURE 12



FEEDING RESPONSES OF L. DECEMLINEATA TO A GRADED COMBINATION OF THE MOLAR SOLUTIONS OF L-SERINE, δ-AMINOBUTYRIC ACID AND L-ALANINE

3 REPLICATES

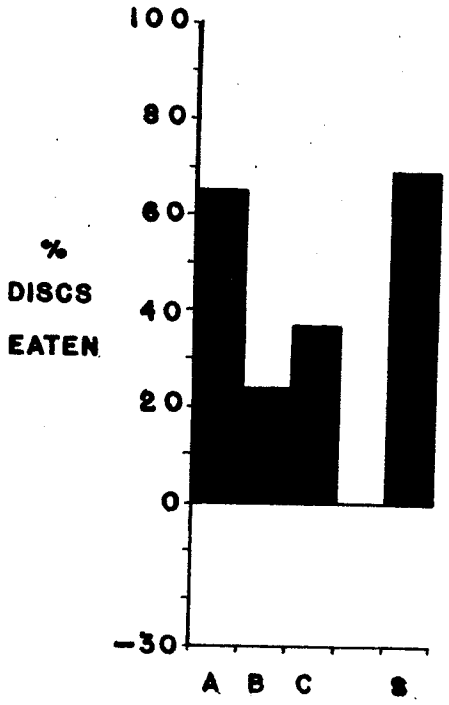
FIGURE 13



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO:

A=.004M γ-AMINOBUTYRIC ACID
B=A + .02M SUCROSE
C=.02M SUCROSE
S=.1M SUCROSE
3 REPLICATES

FIGURE 14A



FEEDING RESPONSES OF LARVAL
L. DECEMLINEATA TO:

A=.004M δ-AMINOBUTYRIC ACID
B=A+.004M SUCROSE
C=.004M SUCROSE
S=.1M SUCROSE

3 REPLICATES

FIGURE 14B

CHAPTER III

THE SWEET CLOVER WEEVIL

Introduction

The production of sweet clover in the southern areas of Manitoba is seriously affected by the damages inflicted by the sweet clover weevil, Sitona cylindricollis (Fahr.). Previous to the presence of this insect, clover played an important role in crop rotations as green-manure and as a forage crop in connection with the beef and dairy industry. Now that weevil damage is so severe, substitutes are taking its place in farming programs, and the versatility of this easily established and quickly maturing crop is being lost to production of plants of a more resistant but perennial nature such as alfalfa. Although some success has been achieved in controlling the weevil on sweet clover with insecticides, these measures of control are still unsatisfactory, and a problem is presented by the residues left after their use on the crops destined for livestock fodder.

With the failure of usual control methods, attempts at some biological control might in some way prove useful. In this regard, one of the weak points in the biology of the weevil is its host preference for the sweet clovers; other

legumes grown here are much less attractive. The weevil's oligophagous nature strongly suggests that its feeding habits may be governed by chemical feeding stimuli, peculiar to its hosts, which regulate the sweet clover's acceptability to the insect. This chemical basis of host plant selection has been demonstrated with other oligophaga, for example the diamond back moth, and any knowledge of such a chemical relationship with the sweet clover weevil might be used in a clover breeding program aimed at developing resistance in new varieties.

Methods and Materials

Preliminary testing with adult sweet clover weevils has shown that they will feed on extracts made from sweet clover foliage. Therefore, chemical separations were performed on these extracts in an attempt to isolate fractions causing this form of response.

Methods of testing used with this insect were similar to those described under the Methods and Materials section of the previous chapter on the potato beetle. Test dishes and artificial leaves were the same, except that the thickness of the discs was reduced to 160 microns, to correspond to the thinner leaves of the sweet clover plant. Discs were treated with extracts in a manner similar to that already described. Prior to testing, the weevils were

starved for at least three hours.

All weevils used in these experiments were field-collected from volunteer and cultivated sweet clover plants growing around the University. Female weevils were preferred for testing, as these showed a more ravenous appetite, and thus were more responsive to the treatments offered to them on the discs. The collection of weevils was subject to seasonal limitations, as adults are present on the plants only in the spring and fall. Spring adults were preferred, as they were sexually mature, and their developing ovaries seemed to give them more voracious appetites. Because feeding response to treated pith discs is limited under the best conditions, the increased appetites were an aid in obtaining results. An additional check on appetites was especially necessary with the late fall adults, which were losing their appetites prior to entering diapause. The weevils were presented with sweet clover leaf discs, stamped with a die of the same diameter as the pith discs, to determine non-feeders present in the replicates.

No accurate measurements of consumption were made in these experiments, for, even with the most active of the treatments, the amount of feeding occurring was quite small in comparison with the remaining uneaten portion of the disc.

Therefore, the activity of a treatment was judged visually from at least three replicates of the same treatment. Only conspicuous differences, easily detected by visual examination, were taken into account.

Plant Extraction and Partition

The initial extract was made from fresh sweet clover leaves. One hundred grams of leaves were macerated and boiled with 250 ml. of distilled water. After heating, the pulp was separated from the liquid fraction and again extracted with a similar quantity of water. The two liquid fractions were combined and then placed on a water bath and heated. This caused a flocculation of a peculiar gel-like substance which included the chlorophyl. This material was removed by filtration. The filtrate was then evaporated to dryness.

The dried extract was then washed several times with methanol until no colour appeared in the washings. On concentration and testing, this methanol extract caused a slight feeding reaction with the weevils. This process was repeated four to five times. The insoluble residue left after these washings was combined into one fraction, and on testing with the weevils proved inactive as a feeding stimulus. The methanol-soluble fraction still retained its activity with the weevils.

The methanol concentrate was then washed with acetone. This was repeated several times, and the soluble material tested with the weevils; it was found unacceptable to them. The residue after the acetone washing was extracted in the same way with cellosolve. This was repeated until no colour appeared in the washings. Both the insoluble and the soluble materials were active on testing, but as the soluble material gave a more pronounced feeding response, further separations were performed with this fraction.

The cellosolve-soluble fraction was treated with lead sub-acetate to effect separations by precipitation. After this treatment the supernatant fraction appeared to be more attractive to the weevils in feeding trials than it had been, previous to this precipitation. Further separations were performed on this fraction by chromatography. The substances precipitated with the lead acetate were regenerated with hydrogen sulphide, filtered and dried. The filtrate was taken up with water and tested with the weevils. It produced no response.

Preliminary procedures, used in extracting the plant material, involved the ascent of solvents through a band of extract applied to filter paper, as described under Methods of the potato beetle section. Benzene and then acetone were

allowed to run through the extract. Material moved to the top of the paper by both these solvents was not active with the weevils. Iso-propyl alcohol, run after these solvents, dissolved an active substance. Cellosolve, run after the iso-propanol, also gave an attractive fraction. However, the evaporation rate of both the latter chemicals was comparatively slow, taking several days to exhaust the supply in the reservoir. As the sweet clover weevil was present in the fields for a limited time only, the procedure was abandoned in favour of the quicker methods of separation already described.

Chromatographic Separation of Plant Extracts

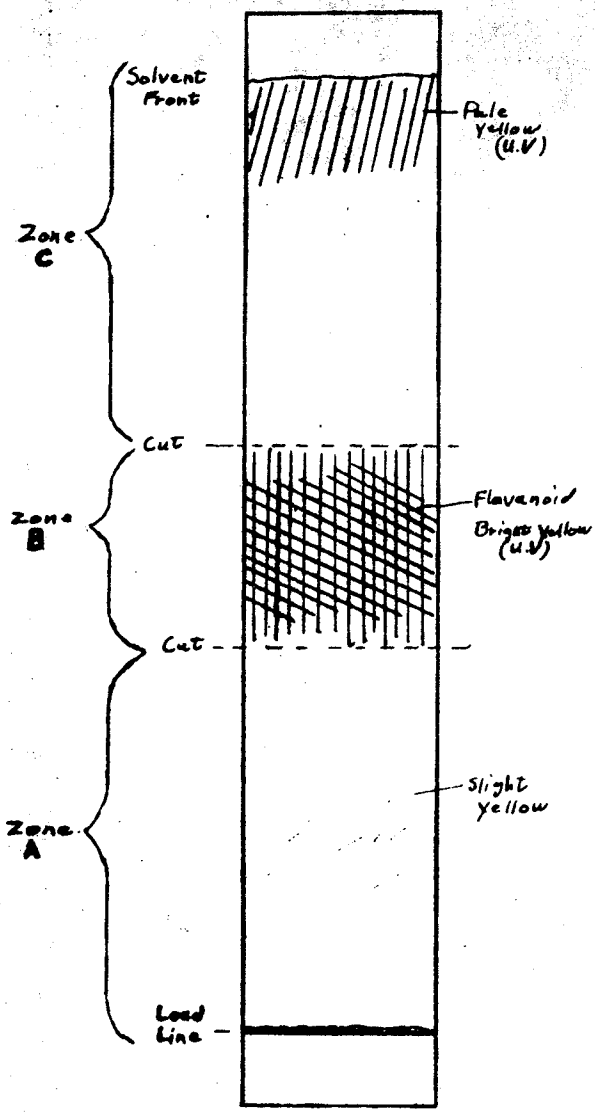
Ascending partition chromatography was used for further separation on the active material obtained after the lead acetate treatment. The fraction was placed in a band on sheets of 27 mm Whatman filter paper, and developed with the solvent, N-butanol, acetic acid and water (4:1:5). The thickness of this paper allowed a large quantity of extract material to be separated for testing with the weevils. However, the separations obtained with it were not as distinct as those produced with a thinner No. 1 Whatman paper. The final grade of paper used was of an intermediate thickness, No. 3 Whatman. This allowed a fairly large amount of the fraction to be separated, and, at the same

time, gave good separation of the components.

The division of the resulting chromatograms, for elution and testing with the weevils, was at first performed visually. Divisions were made along bands of pigments, mainly flavonoid substances, as seen under natural lighting (see Fig. 15). Each of the zones were eluted first with hot methanol and then water. The two elutions from each zone were bulked, concentrated and tested with the weevils. Zones C and B produced no feeding response. The eluate of Zone A was active in feeding trials, and gave a response which, although no measurements were taken, seemed equal to that obtained before this fractionation was performed.

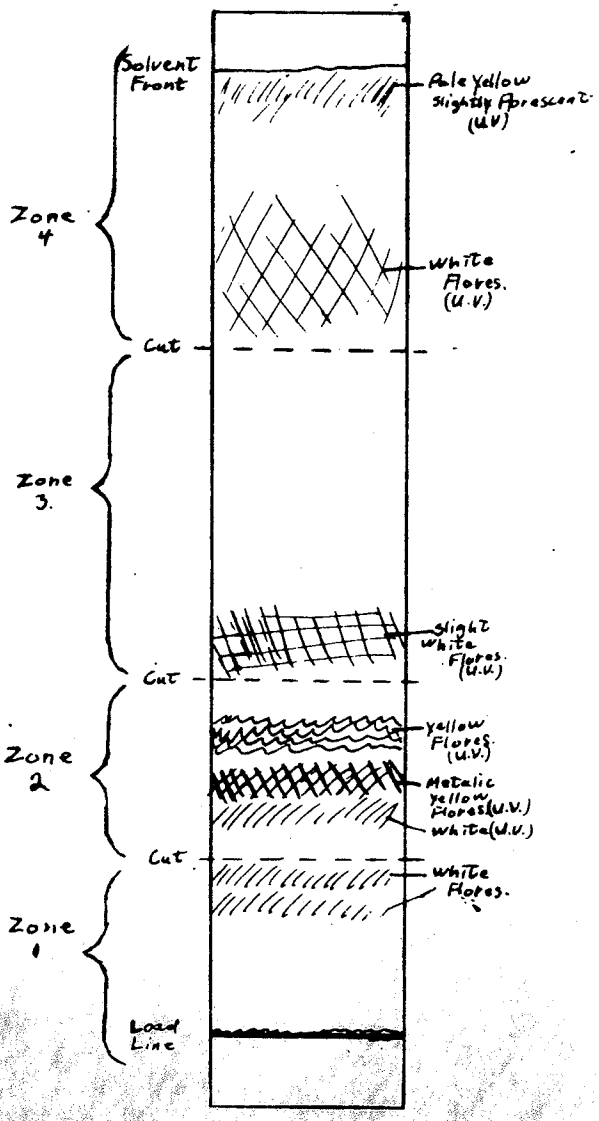
With the localization of the active area of chromatograms developed in this way, an attempt was made to narrow the active band and thus decrease the quantity of inert substances present. Using similarly developed chromatographs was the same extract, ultra-violet light was used to determine the position of additional bands. The further division is represented in Fig. 16.

After this division (Fig. 16) of the chromatograph, the zones were cut from it as shown, and each eluted with methanol and water. The two eluates were combined for testing. Zones 3 and 4 produced no feeding, as was



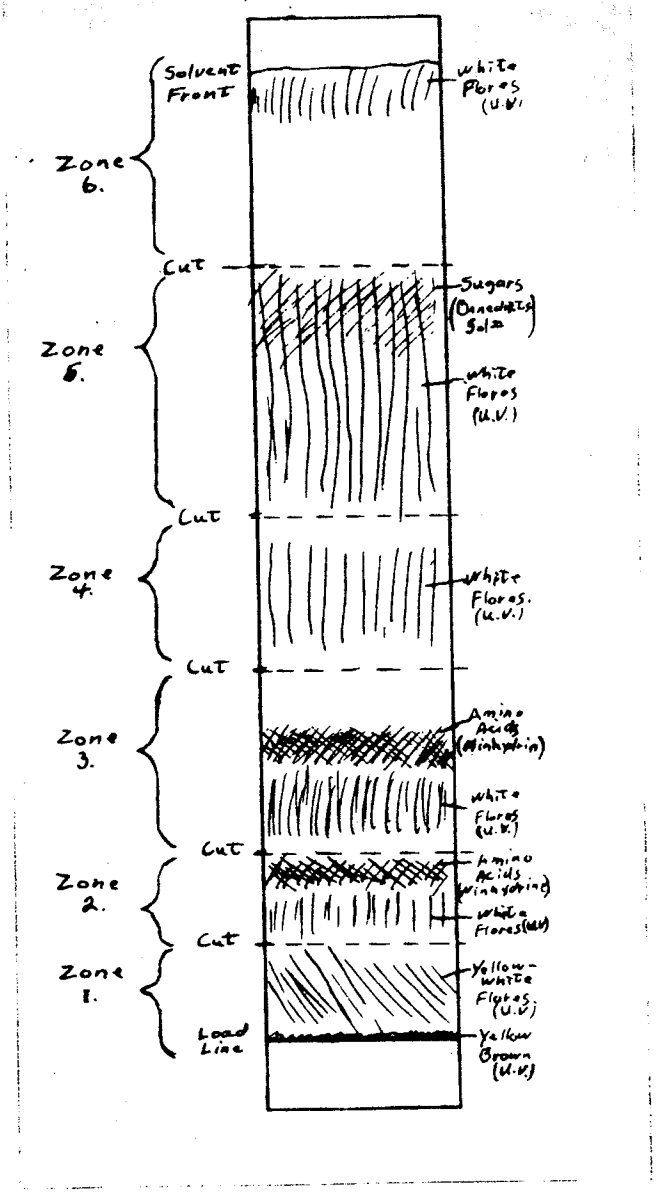
GENERAL DIAGRAM OF THE CHROMATOGRAPH RESULTING FROM THE DEVELOPMENT OF THE ACTIVE EXTRACT FROM SWEET CLOVER FOLIAGE AS SEEN UNDER NATURAL LIGHT

FIGURE 15



GENERAL DIAGRAM OF THE CHROMATOGRAPH RESULTING FROM THE DEVELOPMENT OF THE ACTIVE EXTRACT FROM SWEET CLOVER FOLIAGE AS SEEN UNDER ULTRA-VIOLET LIGHT

FIGURE 16



DEVELOPMENT OF ZONE A OF FIGURE 15

FIGURE 17

expected from the previous separation, and the feeding activity was now present in Zones 1 and 2. This indicated either that two areas of attractive material were present, one in each of the active zones, or that the zone of activity had been separated in the division of the chromatogram. The lower area, Zone 1, coincided with an area of sugar, as revealed by Benedict's reagent, present in a zone centering below the cut of the division between Zones 1 and 2.

One of the active feeding zones, Zone 2 of Fig. 16, was re-chromatographed in an attempt to further isolate and purify the chemical feeding stimulant. The eluate from this zone was applied as a band to chromatograph paper, and developed with the solvent mixture, phenol and water (100:39 w/v). The developed paper was divided under ultra-violet light, and the different sections so formed were eluted. This division resulted in five component bands, each of which, on elution and testing, gave no feeding response with the weevils. The disappearance of the feeding stimulus in this separation could have been caused by various factors: lack of sufficient activating material, inhibition of feeding by residues of the developing solvent (phenol), chemical reactions either interfering with the response or masking its effector, or the instability of the material under these conditions.

To try to overcome some of these factors, the eluate of the entire active zone of the chromatographic separation, represented as Zone A in Fig. 15, was developed in the solvent mixture; pyridine: isobutanol: ammonia (4:1:2). In this chromatographic separation, the higher volatility of the developing solvent ensured that no residues remained in the eluates which might interfere with the feeding response. The developed chromatographs were divided under ultra-violet light, as shown in Fig. 17. Each zone was eluted with methanol and water, and then combined for testing with the weevils. The only responsive area of the chromatograph was Zone 4. Benedict's reagent revealed the presence of sugars in the top portion of Zone 5, but this zone was not active.

The results of this separation concluded extract testing with the weevils, as checks of their appetites with sweet clover leaf discs showed considerable variation, and indicated that they were entering fall diapause.

Results and Discussion

The continued fractionation of these extracts was entirely dependent on the activity of the separations obtained previously. Therefore, most feeding results, as they were an integral part of the procedure used, are mainly included in the section on Methods and Materials.

The observations on these feeding results indicate that the partially refined plant extracts obtained from these procedures will induce feeding in adult weevils. The substrate on which they were offered showed no attraction. Thus, the feeding response observed can be directly attributed to the chemicals which the discs received from treatment with plant extracts.

The identity of the chemicals inducing this response was not determined. However, many of the active extracts produced later probably contained a high carbohydrate content, because some preliminary steps, such as the precipitation with lead acetate, tended to increase the relative carbohydrate content of these extracts. In addition, the active fractions obtained from the chromatographic separations, as represented by Zone A of Fig. 15 and Zones 1 and 2 of Fig. 16, were from areas also containing sugars. The presence of the sugar may have had a magnifying effect on the other feeding stimulants (cf. Diamond back moth, Chapter IV).

The chromatographic separation, represented in Fig. 17, shows the sugars present in Zone 5. As Zone 4 was the responsive area, this is some evidence of phagostimulant activity produced by an extract non-reactive to saccharides.

CHAPTER IV

THE DIAMOND BACK MOTH

Introduction and Literature Review

The host plant relationship of the diamond back moth, Plutella maculipennis (Curt.), is a classical piece of evidence in support of the chemical basis to the discrimination of host in phytophagus insects. Its implications are important, not only for its practical applications, but also for the academic interest it has aroused in this field of study. It has provided a clearer understanding of such terms as monophagy and oligophagy. These definitions, which formerly described the degree of host specificity in an insect, as compiled from observational evidence, are now more suitably used in connection with the number of specific chemical groups contained in the hosts and causing their attraction. It has also aroused academic speculation about the evolutionary basis of this relationship (4) (6).

The first work leading to the discovery and proof of a host-specific phagostimulant was performed by Verschaffelt (16). His observations that the hosts of Peiris rapae and P. brassicae, the cabbage butterflies, correspond to distribution of mustard oil glucosides in

plants led him to surmise that the odorous fission products of these plant chemicals are responsible for edibility of the host plants. Dethier's (5) review of chaemoreception in insects contains an adequate summary of this work. Much later, Thorsteinson (12) confirmed and extended this study. Working with P. rapae, P. brassicae and the diamond back moth, he made isolations of these glucosides from plant material, showing that they induced feeding per se, and that fission products contributed little if anything to the feeding stimulus. In addition, he showed that sufficient nutrients must also be present in the diet along with the olfactory and gustatory stimulants for their full effects to be realized in a feeding response. It is the importance of the nutrient dietary content in phagostimulation of Plutella which was the main investigation of the present study.

Materials and Methods

Rearing and Testing Methods

All larvae used in the following experiments were obtained from cultures maintained in the laboratory. The cultures were originally started from field-collected adult moths, placed in a cage with potted wild mustard plants for oviposition. The foliage of these plants, on

which the eggs were laid, was stripped from the plants and placed in half-pint paper cartons. In winter months, Brussels sprouts were also used as oviposition plants. After the eggs had hatched, the larvae were reared in the same boxes, by placing fresh mustard or Brussels sprouts foliage on top of that on which the eggs had originally been laid. Renewal of larval food was continued in this manner until larvae had pupated or reached the third instar, when they were removed for testing purposes. Pupae were returned to the oviposition cages to maintain the numbers of egg-laying adults. Mould and other putrefying growths which formed on old foliage and frass were controlled by allowing old food supplies to dry before the new was added to the boxes. It was not necessary to regulate the room temperatures or lighting conditions under which cultures were kept.

The measurement of the feeding responses obtained from the chemicals was accomplished by an evaluation of consumption occurring on the artificial leaves used in presentation. The substrate used for this purpose was sliced Japanese elder pith, 160 microns thick. A description of its preparation has been given in the previous chapter on the potato beetle. The discs were arranged in pairs in the test dishes, an untreated or negative control with a treated disc, except when pure

chemicals were being tested. In the latter case, a control was not presented with each test dish, but included with each series of the dilutions as a separate blank treatment. Before larvae were allowed access to the tests, the moisture content of discs was adjusted by saturation with distilled water applied from an atomizer sprayer. After the completion of the larval test period, the discs were stained, removed from their mountings and permanently mounted on sheets of paper with cellulose tape.

Treatment of discs with substances to be tested was performed by soaking them in a solution of the desired concentration. After thorough saturation, they were removed, drained and dried, and then stored in preparation for use with the larvae.

Pure Materials Tested

These investigations of the feeding effects produced by pure nutrients were limited by the short supply of the available phagostimulant. Sinigrin, the glucoside used, was not available commercially at the time these experiments were performed, and its use was essential for the evaluation of the response of the chemicals used in conjunction with it. A sample prepared from black mustard seed in 1946 (by Thorsteinson, 1953) was used. (12).

Sucrose was the first pure nutrient which was tested in these experiments. Discs impregnated with this substance were presented to the larvae in two paired series of concentrations:

1) .5, .1, .02, .004 and .0008 M. sucrose, each paired with an untreated control disc in the same dishes;

2) two discs of the same series of sucrose molarities but with .1% sinigrin added to one of the pairs. A control of .1% sinigrin paired with an untreated disc was also included in this series.

Pure l-alanine was presented in a similar number of paired concentrations. Discs impregnated with this substance were presented in two paired series of concentrations:

1) .5, .1, .02, .004 and .0008 M. l-alanine, paired with negative controls in the dishes;

2) two discs of the same series of alanine molarities, but with .1% sinigrin added to one of the pairs. A control of .1% sinigrin, with .1 M. sucrose added, was paired with an untreated disc and added to this series.

Glucose was also tested at similar concentrations, but with the failing supply of sinigrin it was not possible to determine its response with the phagostimulant added.

Methods of Glucoside Isolation

Isolations of mustard oil glucosides were attempted,

from botanical sources in order to replenish the supply of sinigrin.

The method used was a variation of the extraction process of Thorsteinson (12). Alcoholic extractions of the source material were similar, but, in place of the alumina column of his procedure, partition paper chromatography was used in the final attempts to purify the glucosides. The presence and position of the desired chemicals on the chromatographic sheets was determined by larval feeding tests. Positive responses obtained with any separation were interpreted as an indication of the presence of the glucoside chemicals. Eluates of chromatograms and other active isolates were allowed to stand so that crystallization of the glucosides might occur.

A commercial preparation of mustard flour was the first source of glucosides used in these extractions. This flour was a mixture of white and black mustard seeds, and therefore contained both sinigrin and sinalbin. Alcoholic extractions of this material gave excellent feeding responses with the Plutella larvae. Partition chromatography fractions also gave active responses, and the most active of these elutions were set aside for crystallization.

The second source of glucoside used was a commercial preparation of dried horseradish root. An isolation

procedure similar to that of mustard flour was used on this material. Active alcoholic fractions were subjected to chromatographic separations, and attempts made to obtain an active crystalline material.

Results and Discussion

Pure Chemicals Tested

The discs resulting from these tests were not measured accurately to determine their consumption. Evaluation of the eaten areas was performed by visual estimation.

The results of these trials indicated that sucrose, at some concentrations, produces a slight feeding response by itself. At the .1 M. level of sucrose the feeding reaction was apparent, although the amounts consumed were small. The identical test series, which had the addition of .1% sinigrin to all concentrations of sugar (see Fig. 18, Feeding of *Plutella* larvae on discs treated with various sucrose molarities plus .1% Sinigrin), showed feeding through all levels with a peak in consumption at .1 M. From this point, going down the concentrations, the amount eaten from each disc decreased, and reached its lowest quantity at the 0 level of sucrose. This level contained only the sinigrin treatment. A

comparison of the two test series shows that although neither sucrose nor sinigrin are particularly active by themselves, when used together they produce a total response exceeding expectation. The summation of the combined materials confirms Thorsteinson's results, which showed the necessity of glucoside and nutrient in feeding response, and indicates that sucrose may be a factor in these required nutrients.

The results of these trials suggested another series of tests with an amino acid, l-alanine. This substance produced no response when fed by itself at the concentrations of .5 to .0008 M., and also no additive response when used with one of the most attractive concentrations of the previous experiment, .1 M. sucrose and .1% sinigrin. The results obtained with glucose showed that this sugar, unlike sucrose, provoked no response by itself.

Thus, the stimulatory effect of nutrient and glucosides is not induced by all nutrient substances, nor are all sugars capable of producing a feeding stimulus in themselves.

Glucoside Isolations

Many of the extracts and eluates obtained in these separations showed a feeding response attributed to glucoside content, but no active crystalline material was produced (Fig. 20).

Some crystalline material resulted from an alcoholic extract of horseradish root, and, on testing with the larvae, gave an excellent feeding response. However, X-ray diffraction identification revealed that this substance was sucrose, the feeding response of which has been described. The large amounts of feeding obtained with this material were an increase over that of the optimum level of pure sucrose. The difference in response could have been caused by an increased sugar concentration used with this isolate, but as the highest level of pure sucrose tested, .5 M., decreased feeding, it was assumed that this sample also contained some mustard oil glucosides. No purified forms of this glucoside were isolated.

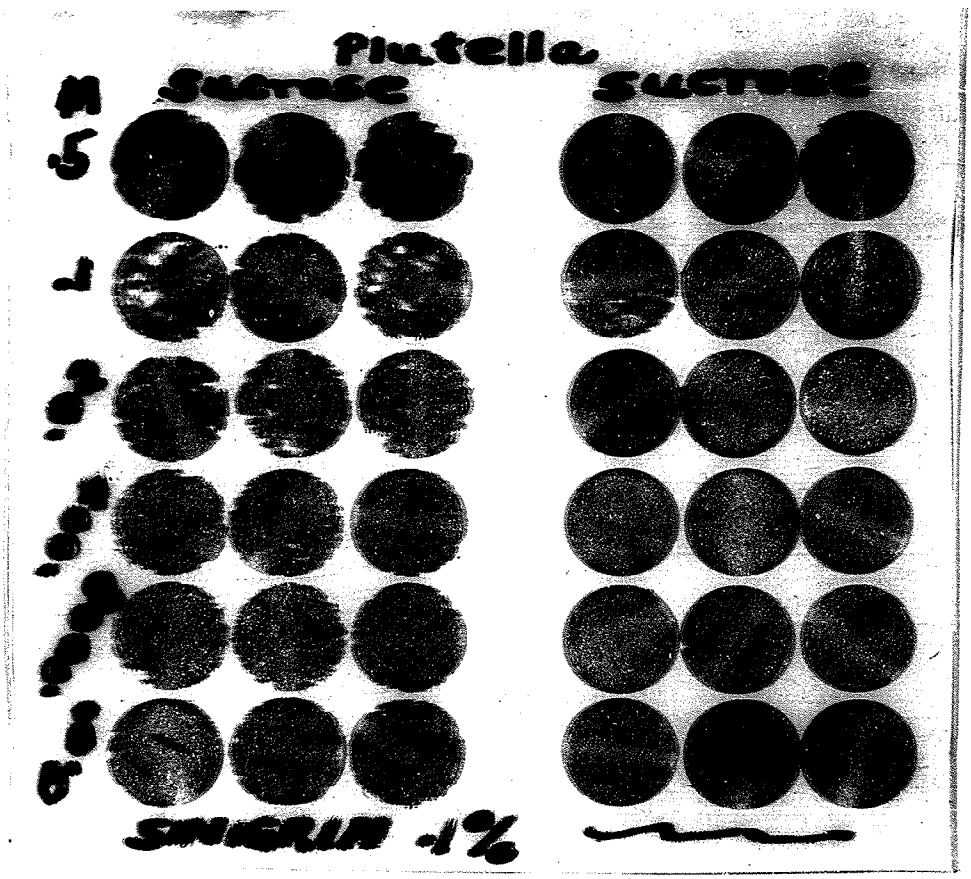


FIGURE 18

FEEDING RESPONSES OF PLUTELLA LARVAE TO DISCS TREATED WITH VARIOUS CONCENTRATIONS OF SUCROSES WITH 0.17, SINIGRIN

CHAPTER V

SUMMARY AND CONCLUSIONS

The investigations of the chemosensory regulation of the potato beetle larva's feeding responses had two objectives: to isolate a phagostimulant peculiar to potato foliage, and to determine the effects some common plant nutrients have on the feeding responses of these insects.

The results obtained from experimentation on the first of these objectives showed that active substances could be extracted from potato foliage. It was shown that some of the nutrient constituents stimulate feeding, but no non-nutrient feeding stimulant was demonstrated. Isolates of flavone glucosides were found unattractive to the larvae and inhibitory to the responses which could be produced by other nutrient chemicals.

Investigations performed with some common plant nutrients in pure form showed that some of these stimulate feeding, but antagonism to the response of each could develop when presented in certain mixtures. In addition, larvae were found to be able to discriminate between certain saccharides.

The feeding results obtained with the sweet clover weevil from partially refined chemical isolates of sweet

clover indicate that the host does contain certain substances capable of inducing feeding, and that the attractiveness of these isolates is probably enhanced by their carbohydrate content.

It is known that the presence of a phagostimulant and suitable dietary nutrients are necessary to induce feeding in larvae of M. plutella. The purpose of these experiments was to determine a few of these dietary substances contributing to this response. In the limited trials performed, it was found that sucrose, under these conditions, would evoke feeding summation, as well as being slightly active in itself. The addition of an amino acid to this attractive mixture of sugar and stimulant produced no cumulative feeding effects, nor was this nutrient active when presented by itself. Another sugar, glucose, tested alone, induced no response, but indicated that a saccharide discrimination occurs in the larvae.

BIBLIOGRAPHY

1. Chauvin, R. Premiers essais de purification de la substance qui attire le doryphore vers les feuilles de pomme de terre. Comps. Rend. Acad. Sc. 221: 713 - 714. 1945.

2. Chauvin, R. Nouvelles recherches sur les substances qui attirent le doryphore (Leptinotarsa decemlineata Say) vers la pomme de terre. Annales de L. I.N.R.A. 3: 303 - 308. 1952.

3. Chin, Chun-Teh. Studies on the physiological relations between the larvae of L. decemlineata Say and some solanaceous plants. H. Veenman and Zonen, Wageningen, Netherlands. 1950.

4. Dethier, V.G. Chemical factors determining choice of food plants by Papilio larvae. Amer. Naturalist 75: 61 - 73. 1941.

5. Dethier, V.G. Chemical Insect Attractants and Repellents The Blakestone Co., Phil. Pa. 289 pp. 1947.

6. Fraenkel, G.S. The Raison d'etre of secondary plant substances. Science, Vol. 129: 1466 - 1470. 1959.

7. Jermy, T. Entomol. exptl. et appl. 1: 197 - 208. 1958.

8. Lederer E. and M. Lederer. Chromatography. Alsevier Publishing Co., N.Y. 1954.

9. McIndoo, N.E. The relative attractiveness of certain solanaceous plants to the Colorado potato beetle. Proc. Ent. Soc., Wash. 37: 36 - 42. 1935.

10. Raucourt, M. and B. Trouvelot. Les principes constituants de la pomme de terre et le doryphore. Ann. Epiphyt. 2: 51 - 98. 1936.

11. Tauber, M. The feeding behaviour of the clear-winged grasshopper Camnula pellucida (Scudder) (Orthoptera: Acrididae) with special reference to the chemotactic influence of some organic constituents found within food plants. 1959.

12. Thorsteinson, A.J. The chemotactic responses that determine host specificity in an oligophagous insect (Plutella maculipennis (Curt.) lepidoptera). Can. Jour. of Zoo. 31: 52 - 72. 1953.

13. Thorsteinson, A.J. The experimental study of the chemotactic basis of host specificity in phytophagous insects. Can. Ent. 87, No. 2. 1955.

14. Thorsteinson, A.J. Host selection in phytophagous insects. Annual Review of Entomology, Vol. 5. 1960.

15. Trouvelot, B., J. Raucourt and J. Castets. Remarques sur le mode d'action physiologique des principes actifs de Solanum tuberosum envers les larves de L. decemlineata. Compt. Rend. 199: 684 - 686. 1934.

16. Verschaffelt, E. Proc. Acad. Sci. Amsterdam. 13: 536 - 542. 1910.

17. White, G.F. Agricultural Research. 51: 233 - 234. 1935.

18. Wilde de, J. Breeding the Colorado potato beetle under controlled conditions. Zeitschrift fur Pflanzenkranpheiten (Pflanzenpathologie) und Pflanzenschatz. 1957.