HONEYDEW ANALYSIS AND ENZYME STUDIES OF THE GREENBUG (*SCHIZAPHIS GRAMINUM* ROND.) IN RELATION TO RESISTANCE AND SUSCEPTIBILITY IN BARLEY

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CHAPTER I

INTRODUCTION

The greenbug, Schizaphis graminum (Rond.), can cause considerable damage to cereal grain crops and during epidemics can result in complete destruction of susceptible crops. Certain varieties of cereals are somewhat resistant to greenbug infestation and damage (5). This resistance can take any or all of the three forms discussed by R. H. Painter (42): mechanical resistance, where the insect cannot penetrate the epidermis or is in some way repelled; abiotic resistance, when the insect encounters certain toxic substances upon entering the host plant; and physiological resistance, when the insect may penetrate and feed, but the host plant tolerates such feeding with few or minimal effects suffered.

The present paper explores some aspects of the third type of resistance. In this study two varieties of barley, Hordeum vulgare (L.), were used. One variety (Will) is classified as resistant and the other (Rogers) is classified as susceptible according to criteria developed by E. A. Wood at Oklahoma State University (52). The aphid has lower rates of reproduction and weight gain when feeding on Will variety barley (15). Aphid populations that the resistant Will variety barley tolerates are lethal to a stand of Rogers variety barley. The objective was to determine whether or not the greenbug was obtaining some nutrients from one variety that it could not get from the other. In order to

study this problem, several approaches might be used. One method would be to homogenize the host plants and compare them compound for compound. This would be a painstaking process which would involve identification of the compounds and then determine the significance of any similarities or differences observed. Another method which has been used in the past is to compare the constituents of honeydews produced by aphids feeding on resistant host plants and by aphids feeding on susceptible host plants. Differences in amino acid or carbohydrate composition would be two main classes of compounds that should be investigated. In this paper the amino acid and sugar compositions of honeydews collected from greenbugs feeding on a resistant variety of barley and honeydews collected from greenbugs feeding on a susceptible variety of barley were compared by thin-layer chromatographic methods. Also analyzed was the sugar composition of honeydew collected from greenbugs feeding on an artificial diet.

Several authors have shown that many plant-sucking insects penetrate the leaf or stem tissue upon which they are feeding intercellularly, while many others penetrate intracellularly. With this thought in mind, it seemed reasonable to suspect the presence of certain hydrolytic enzymes which could aid in either one or both types of penetration. The enzyme cellulase would be essential in intracellular penetration, and the enzyme pectinase would be essential in intercellular penetration. Also very important in feeding, however, not associated with penetration, would be the enzyme invertase. Invertase has been shown to catalyze the hydrolysis of sucrose, which appears to be the major sugar component in phloem sap where the insects feed (53).

CHAPTER II

LITERATURE REVIEW

Honeydew Analysis

Early in the 19th Century, many scientists became interested in the origin and composition of honeydews. They were especially interested in the very high sugar content. It was shown by several early authors (25, 27, 43) that honeydew was produced by insects feeding on plants and excreted in the form of droplets from the anus. These insects were mostly aphids and coccids. It was a mystery as to why these insects would excrete such large quantities of highly nutritious food. Hagen (24) showed that honeydew excreted by the mealybug Planococcus citri (Risso) provides an excellent diet for the adult of the fruit fly Dacus dorsalis Hendel. Many other authors have shown that honeydews collected from plant-sucking insects contain high concentrations of many sugars and amino acids (17, 18).

Auclair (5) stated that most of the early work in honeydew analysis demonstrated the presence of reducing sugars, sucrose, dextrins (usually in high concentrations), nitrogenous material, and occasional reports of melezitose.

In 1948, C. A. Mawson (32) found that honeydew of Therioaphis tilia feeding on European lime tree was composed of approximately equal amounts of glucose, fructose, and sucrose. However, Gray (21) in 1952 found that honeydew of the pineapple mealy bug Pseudococcus brevipes

(Cockerell) was more complex. In addition to the three compounds mentioned above, he found a maltose-like compound and glucose-1-phosphate. In a later paper, Gray and Fraenkel (20) determined a new trisaccharide, fructomaltose (glucosucrose), in the honeydew of Psedococcus citri (Risso) feeding on potato sprouts. Gray and Fraenkel (21) determined the carbohydrate content of the honeydew from the citrus mealybug feeding on potato sprouts, and also the carbohydrate content of the host plants, potato sprouts. Fructose, glucose, sucrose, and glucose-1-phosphate were common to both honeydew and host, but the honeydew contained, in addition, fructomaltose. They hypothesized that invertase was responsible for the formation of this trisaccharide, and that honeydew should be regarded as a digestive product and not just a mixture of excess chemicals and water.

Ewart and Metcalf (18) studied five species of coccids. In all five glucose, fructose, and sucrose were present. In three species melezitose and some unidentified oligosaccharides with Rf values less than melezitose were discovered. Baron and Guthrie (9) analyzed leaf tissue of tobacco plants and the honeydew from the peach aphid Myzus persicae which had been feeding on these leaves and found that glucose, fructose, and sucrose were common to both the leaf tissue and the honeydew. The honeydew contained, in addition, two unidentified oligosaccharides and maltose. Several other authors, (17, 28, 47), have found large concentrations of unidentified oligosaccharides in honeydew collected from various aphids and coccids. A few authors, (8, 9, 22), have also found tri- and tetrasaccharides of fructomaltose (glucosucrose), maltosucrose, and maltotrisucrose.

Mittler (36), in comparing the sugar composition of ingested phloem

sap and excreted honeydew of Tuberolachnus salignus feeding on willow stems, found sucrose to be the only sugar in phloem sap, but the honeydew contained roughly equal amounts of sucrose, fructose, glucose, melezitose, and oligosaccharides. Two authors (11, 5) have reported the complete hydrolysis of sucrose from the ingested phloem sap with high amounts of glucose and fructose present.

As early as 1894, Raumer (43) reported 3.2% nitrogenous compounds in honeydew. However, the majority of workers continued to regard honeydew as essentially a sugar mixture until in 1952 Maltais and Auclair (29) reported twenty-two free amino acids and amides present in the honeydew of Myzus circumflexis feeding on Katahdin potato. Gray (22) reported relatively large amounts of 19 free amino acids and amides present in the honeydew of Pseudococcus brevipes (Cockerell) feeding on immature pineapple fruit. Maltais and Auclair (30) suggested that the amino acid composition of honeydew could be of importance in studying the causes of plant resistance to aphids. Gray (22), however, came to the conclusion that the insects were not excreting large amounts of usable nutrients in order to obtain some amino acids and amides present in only small quantities in the plant sap, since there were so many amino acids and amides present in high concentration in the honeydew. Gray (22) also reported that five amino acids present in the honeydew were not present in the plant sap.

Contrary to this, Mittler (38) reported that the honeydew collected from the aphid Tuberolachnus salignus feeding on Salix spp. contained the same number and relative amounts of amino acids and amides as were present in the phloem sap exuding from cut stylet stumps and also sap collected from crushed bark. He also stated that the concentration of

each amino acid and amide was 55% greater in phloem sap than in honeydew. Expressed sap was also less concentrated. Aspartate, glutamate, glutamine, and asparagine were present in higher concentrations than the other amino acids.

In studying the relationship of amino acid composition of honeydew to resistance, Auclair, Maltais, and Cartier (3) determined that three susceptible varieties of cultivated peas, Pisum sativum L., contained a higher concentration of free amino acids than did the three resistant varieties. Aphids (Acyrthosiphon pisum Harr.) feeding on resistant varieties of cultivated peas have lower growth and reproductive rates.

In a later paper, Auclair (4) also determined that honeydew collected from pea aphids feeding on susceptible varieties of peas contained slightly higher concentrations of amino acids and amides than did honeydew collected from pea aphids feeding on resistant varieties. Also, a greater portion of the ingested material was excreted and the rate of excretion was greater on susceptible varieties.

A most exhaustive study of this problem was done by Ortman (40). He studied the free amino acids in the spotted alfalfa aphid and the pea aphid and their honeydews in relation to feeding on a range of alfalfa selections varying in their degrees of resistance to these two aphids. He found differences of only one to two types of amino acids in the honeydew compositions of the two aphids feeding on the same alfalfa selection, but there were significant differences in concentrations of certain amino acids. Ortman concluded that the high concentration of solutes in the honeydew would be due to the fact that some factor was in short supply. Bragdon and Mittler (10) showed that the aphid Myzus persicae feeding on artificial diets exhibited definite differential ab-

sorption of six amino acids put into the diet. However, Maltais and Auclair (30) using pea aphids feeding on pea leaves which had been supplied with additional exogenous concentrations of amino acids showed no such differential absorption, i.e., the composition of the honeydew reflected the composition of what had been added. Srivastava (47) and von Dehn (16) both demonstrated the possibility that a peptidase could possibly account for additional amino acids being present in honeydew. Peptides have been found in phloem sap (5).

Enzymes in Aphid Extracts and Honeydew

The enzyme invertase has been demonstrated by Auclair (5) and Duspiva (17) to be present in homegenates of six species of aphids. No activity was found in salivary gland extracts. Bacon and Edelman (6) using commercial invertase and invertase prepared from potatoes demonstrated a new role for invertase when a trisaccharide was formed upon incubating the enzyme preparations with 45% sucrose. This type of activity was termed transcarbohydrase activity. Wolf and Ewart (51) have demonstrated two types of transcarbohydrases occurring in the honeydews of two coccids, Icerya purchasi Mash. and Coccus hesperidum L. The former has melezitose as the principal trisaccharide in its honeydew, the latter has glucosucrose. The extracts from the honeydews, using sucrose as substrate, produced the corresponding trisaccharides in vitro.

Srivastava and Auclair (46) extracted invertase from the alimentary canal of the pea aphid, Acyrthosiphon pisum (Harr.) and found that it hydrolyzed sucrose, trehalose, melezitose, turanose, and maltose. It is therefore an alpha-glucosidase. In one case, traces of oligosaccharides were detected. Saxena and Bhatnagar (45) using gut invertase extracted

from Oxycarenus hyalinipennis (Costa.) could not form oligosaccharides *in vitro*. They concluded that glucose is needed in excess of fructose in order to obtain transglucosidation. The aphid honeydew contains oligosaccharides.

Chatters and Schlehuber (13) while studying the mode of penetration of the stylet of the greenbug *Schizaphis graminum* (Rond.) determined that penetration was largely intercellular and terminated in the phloem tissue. Saran, (44) working with both biotype "A" and biotype "B" greenbugs, showed that penetration could be either inter- or intracellular and could terminate either in the phloem tissue or in leaf mesophyll. Auclair (5) states that aphid feeding is mostly in the sieve tubes of the phloem tissue. Since the greenbug, biotype "B", had been shown by several workers to penetrate barley plants both intercellularly and intracellularly, it seemed reasonable to suspect that its saliva would contain certain hydrolytic enzymes to aid in penetration. In 1956, McAllan and Adams (35) found a pectin polygalacturonase present in both whole aphid extracts and in saliva from four species of aphids. This was the first report of pectinase in aphids. In a later paper in 1961, McAllan and Adams (33) discussed the significance of pectinase in intercellular penetration. By histological studies, they determined that of 19 species of aphids, sixteen were intercellular penetrators and had pectinase activity. Those not having pectinase (polygalacturonase) activity were intracellular penetrators.

In 1963, Adams and Drew (2), adapting some concepts and work done with fungi and bacteria, discovered a cellulose hydrolyzing factor in a total of twelve aphids feeding on a variety of host species. In a later paper (1), they examined 58 species of aphids from a wide range of host

plants and found that most had the ability to convert carboxymethyl cellulose to glucose and cellobiose.

CHAPTER III

METHODS AND MATERIALS

Collection of Honeydew

Plant Material

Ten-day to 2-week old plants of barley, Hordeum vulgare L., varieties Will (resistant) and Rogers (susceptible) were used to rear the greenbugs. The plants were grown in pots of soil under greenhouse conditions with an average daytime temperature of 85 F.

Rearing of Aphids and Collection of Honeydew

When the barley plants were ten days old, greenbugs, Schizaphis graminum (Rond.), biotype "B", were placed on the leaves and allowed to begin feeding. The aphids were mostly apterous instars. When aphids were continuously in the feeding position, a rectangular plastic cage was clamped onto the leaf caging the aphids inside. The plastic cage was made of a clear, non-toxic plastic with dimensions of $1\frac{1}{2}$" X 3" X $\frac{1}{2}$" when closed and the ends had been ground out slightly so as not to damage the leaf or interfere with translocation when clamped onto the leaf.

Honeydew droplets adhered to the inside of the plastic cage as they were expelled from the aphids. Collection of honeydew droplets was continued in this manner for several days at the end of which time the cages were removed and frozen in a deepfreeze. The honeydew droplets which had been inside the cage for several days were almost completely dried out. All amino acid and sugar analyses were done using these

honeydew samples.

Thin-Layer Chromatographic Techniques

Thin-Layer Chromatography of Sugars

The method of Walker and Ricci (49), developed for use with sugar-beet carbohydrates, was used for honeydew analysis. They used a one-dimensional chromatogram with a double development in two solvents. The first solvent system consisted of 65% ethyl acetate, 23% isopropanol, and 12% water. The second solvent system consisted of 55% ethyl acetate, 30% isopropanol, and 15% water. Plates were developed in solvent "A", air dried, and developed in the same direction in solvent "B".

Plate material consisted of 10 grams Celite mixed with 10 grams $CaSO_4$ in 50 ml of 0.02 M sodium acetate for 5 min. in a Lourdes blendor with the rheostat set at 60%. Five plates were spread immediately to avoid settling. Plates were then air-dried and activated at 125 C for 30 min. Resolution was fastest and best developed at 35 C in covered tanks which had been previously lined with filter paper to allow saturation of the atmosphere.

The spray reagent consisted of 5% conc. sulfuric acid, 5% anisaldehyde, and 90% ethanol. Chromatograms were sprayed evenly and heated at 125 C for 15 min. until bluish balck spots developed for all carbohydrates.

A variation of this method consisted of using a different plate material and spray reagent. The plate material was 18 grams of MN 300 cellulose mixed with 108 ml of water in the blendor for 10 min. at a setting of 65% on the rheostat. The spray reagent used was a 2% solution of $AgNO_3$ in water. The developing solvents and technique, the

spreading technique, and spraying technique remained the same.

Thin-Layer Chromatography of Amino Acids

The technique developed by Heathcote and Jones (26) for rapid resolution of naturally occurring amino acids by thin-layer chromatography was used to determine the amino acid content of honeydew. Plate material was 18 grams of MN 300 cellulose mixed with 108 ml of water in an omnimixer for 10 min. at a setting of 60% on the rheostat. The resulting slurry was spread on glass plates at a thickness of $\frac{1}{4}$ mm. Plates were then air dried and activated for 10 min. at 105 C.

After spotting, the plates were developed in a two-dimensional solvent system with the first system consisting of isopropanol : formic acid : water in the proportions 40 : 2 : 10 and the second solvent system consisting of methyl-ethyl ketone (2-butanone) : tertiary butanol : 30% ammonium hydroxide : water in the proportions 30 : 50 : 10 : 10. The solvents were mixed outside the tank and then poured down the side, not allowing for equilibration before plates were placed inside.

After development in both solvent systems the chromatograms were sprayed with a 0.01% solution of ninhydrin in acetone and heated at 60 C until spots were fully developed.

Methods for Quantitating Sugars and Amino Acids

Quantitation of Amino Acids

Two different ways of quantitating separated amino acids were used. One method was to scrape off spots from chromatograms which had been previously developed with the ninhydrin spray mentioned above. The spots were scraped off after they were fully developed and the color was eluted from plate material in a 50% solution of n-propanol and the ab-

sorbance was determined at 570 nm. The other method was to develop identical chromatograms and spray one and from this chromatogram determine where the spots were located on the other undeveloped chromatogram. The area of approximate location was then scraped off and the amino acid was eluted from the plate material with water and ethanol. The elutants were combined and the ninhydrin method of Fels and Veatch (19) and Moore and Stein (39) was used to quantitate the amino acid.

Quantitation of Sugars

In order to quantitate carbohydrates on chromatograms, identical chromatograms were developed and one was sprayed to locate the carbohydrates and the other was left unsprayed. Areas corresponding to the developed spots on the sprayed chromatogram were scraped off of the unsprayed chromatogram, placed in centrifuge tubes and the sugars were then extracted with first water and then 95% ethanol. The two washes were combined and quantitated according to the ferricyanide method for determining reducing sugars developed by Park and Johnson (42). Nonreducing sugars first were hydrolyzed with 1 N H_2SO_4 at 100 C for 20 min. (14).

Hydrolysis of Honeydew Samples

Honeydew samples were hydrolyzed by two methods prior to chromatography. The first method used was a 20 µl sample mixed with 1 ml of 1 N H_2SO_4 in boiling water for 30 min. with the addition of 1 ml of 1 N $Ba(OH)_2$ after cooling in order to neutralize and desalt the solution. The solution was then centrifuged at 10,000 x g for 20 min. in a bucket centrifuge and the precipitate was discarded. In the other method, 1 N HCl was mixed with the honeydew in place of 1 N H_2SO_4 and the mixture was heated in boiling water for 30 min. and the resulting solution evap-

orated to dryness with a rotary evaporator. The solutions were then chromatographed by the thin-layer method previously described for sugars.

Chromatography of Oligosaccharides

Honeydew samples were spotted on thin-layer chromatograms prepared according to the method previously described for thin-layer chromatography of sugars. The chromatograms were then developed five times in solvent "B" only in order to separate the oligosaccharides present in the honeydew.

Sugar Standards

Standard sugar solutions were dissolved in 80% ethanol in a concentration of 1 mg. / ml. Only reagent grade materials were used. Sugars used were sucrose, glucose, fructose, galactose, raffinose, maltose, trehalose, glucuronic acid, galacturonic acid, mannose, sedoheptulose, and melezitose.

Amino Acid Standards

Stock solutions of amino acids and amides were made up in distilled and deionized water in a concentration of 0.05 M. Amino acids and amides used were alanine, asparagine, cystine, glutamic acid, glutamine, aspartic acid, glycine, histidine, isoleucine, lysine, phenylalanine, proline, threonine, tyrosine, valine, methionine, serine, cysteine, leucine, arginine, and tryptophan.

Method for Spotting Honeydew and Standards

Approximately 50 dried honeydew droplets were dissolved in 0.1 ml of distilled, deionized water for the honeydew from greenbugs fed on Will barley and for the honeydew from greenbugs fed on Rogers barley. Five µl were then taken up in a 10 µl syringe from each honeydew sample and spotted on the chromatograms. Five µl were used for both amino acid

and carbohydrate analysis. Two µl of each sugar standard and each amino acid standard were used.

Enzyme Studies

Protein Extraction from Greenbugs

Three grams of greenbugs were ground in a mortar and pestle in 25 ml of 0.05 M phosphate buffer pH 7.0. The resultant liquid was decanted into a centrifuge tube and the residual solids were washed with an additional 25 ml of buffer. Extraction was made with ice cold buffer. The homogenate was further ground with a hand homogenizer and centrifuged at 0 C for 20 min. at 10,000 x g and the precipitate was discarded. Ammonium sulfate was added to the supernatant to make it an 85% saturated solution and the resulting precipitate was removed by centrifugation at 25,000 x g for 30 min. The precipitate was resuspended in 25 ml of the cold phosphate buffer and dialyzed against distilled water for 24 hours at 3 C. The dialyzed protein was then assayed by the Folin-Ciocalteau method (14) and yielded 5 mg protein / ml.

Pectinase Assays

Stock enzyme solution was made up with commercial pectinase of fungal origin at a concentration of 0.1 mg / ml. Substrate for pectinase assays was prepared by dissolving pectic acid to a final concentration of 2% in 0.1 N NaOH which was then neutralized with an equal volume of 0.1 N HCl. Fifty ug / ml of streptomycin was added to the liquid and several drops of toluene were spread on top of the liquid to retard bacterial activity (35). In all pectinase assays, 0.015 M acetate buffer, pH 4.6 was used (34). Incubation was carried out at room temperature for periods of from 15 min. to 2 hours. In assays for pectinase activity by

extracts from greenbugs, 1 and 2 ml of the extract was mixed with 1 ml of substrate. Ten and 20 µl of the incubation mixtures were spotted on chromatograms and developed according to the method previously described for thin-layer chromatography of sugars.

Another method of analyzing incubation mixtures for pectinase activity was to measure the increase in reducing power of the mixture by use of the 3,5-dinitrosalicylic acid reagent. After the alloted time period, 1 ml portions were removed and added to 1 ml of the 3,5-dinitrosalicylic acid, heated for 15 min., cooled, and diluted with ten ml of 0.1 N NaOH and absorbance determined at 570 nm (14).

Cellulase Assays

Commercial cellulase of fungal origin was used to make a stock solution of concentration 0.1 grams / ml. A 1% solution of MN 300 cellulose was used as the substrate for assays of commercial cellulase and protein extract from greenbugs. All reactions contained 1 ml of substrate + 1 to 2 ml of the enzyme to be tested and 5 ml of 0.05 M acetate buffer, pH 5.0, and were carried out at room temperature at periods up to 5 hours while being constantly shaken. At the end of the alloted time periods, 10 and 20 µl portions were spotted on thin-layer chromatograms along with standards of glucose and cellulose and developed according to method previously described for thin-layer chromatography of sugars.

Invertase Assays

Protein extracts from the greenbug were assayed by incubating 1 ml portions with 5 ml of 0.05 M acetate buffer, pH 5.6 containing 6% sucrose. After one hour a 1 ml aliquot of the mixture was assayed for reducing sugars by the 3,5-dinitrosalicylic acid reagent method (14).

Absorbancies of solutions were determined at 570 nm. Also, 5 µl were spotted on a thin-layer chromatogram along with glucose and sucrose standards and developed according to the method for thin-layer chromatography of sugars previously described.

Assays for Pectinase in Saliva

To assay pectinase activity in greenbug saliva, two approaches were used. In one approach, 50 greenbugs were caged on Whatman #1 filter paper which had been impregnated previously with 2% pectic acid, made up as described in the section under pectinase assays. Cages were of the type described for collection of honeydew. After the insects were caged for 24 hours, the filter paper was sequentially washed with 10 ml of cold acetone, 10 ml cold 70% ethanol and 5 ml distilled water. Washings were combined and evaporated to dryness with a rotary evaporator and taken up in 1 ml distilled water. Fifty greenbugs were also caged on filter paper which had not been impregnated with 2% pectic acid, and also on filter paper which had been kept moist for the 24 hour period. These filter papers were eluted as the one described above. Five µl were then spotted on chromatograms along with pectic acid and galacturonic acid standards and developed according to the method previously described for thin-layer chromatography of sugars.

In the other approach, 5 greenbugs were caged on sachets containing artificial diet and 2% pectic acid, on sachets containing only artificial diets, and on sachets containing artificial diet with 2% pectic acid and 50 µg / ml streptomycin. After 24 hours, 5 µl of the diet solutions were spotted on chromatograms along with pectic acid and galacturonic acid standards and developed according to the method previously described for thin-layer chromatography of sugars.

For a complete description of the construction of sachets and artificial diets see D. C. Cress (15). These sachets also served for the collection of honeydew from greenbugs feeding on artificial diets. The honeydew droplets adhered to the inside of the plastic stopper and could be easily stored.

CHAPTER IV

RESULTS AND DISCUSSION

Carbohydrate Content of Honeydew

From the results of several thin-layer chromatograms given in Table I, it can be seen that the monosaccharides glucose, fructose, and galactose were present in all honeydew samples as was the disaccharide maltose (maltose-like). Raffinose was the only trisaccharide to be identified, and it was also present in all three honeydew samples. By special chromatographic methods the oligosaccharides were separated into six distinct spots which remain unidentified. Commercial standards of oligosaccharides were unavailable and several attempts at acid hydrolysis of honeydew samples produced a single compound tentatively identified as a furfuryl.

Several methods were utilized in an attempt to quantitate the carbohydrate components. However, these methods gave inconsistent results. Visual comparison of the chromatograms showed that equal numbers of honeydew droplets gave darker spots for the Rogers honeydew samples and the artificial diet honeydew samples.

Perhaps the most significant observation was the complete absence of sucrose in any of the honeydew samples. The greenbugs feeding on the plants might have had access to other sugars besides sucrose since it is not known what the exact carbohydrate composition of barley plant sap is. However, in the artificial diet the only source of carbohydrate was

TABLE I

CARBOHYDRATE COMPOSITION OF HONEYDEWS FROM THE

GREENBUG, (*SCHIZAPHIS GRAMINUM* ROND.)

Honeydew collected from greenbugs feeding on resistant Will barley			Honeydew collected from greenbugs feeding on susceptible Rogers barley			Honeydew collected from greenbugs feeding on an artificial diet		
Rg Value	Probable Identity	Concentration relative to Std. at 2 µg	Rg Value	Probable Identity	Concentration relative to Std. at 2 µg	Rg Value	Probable Identity	Concentration relative to Std. at 2 µg
1.18	Fructose	[a] +	1.14	Fructose	++	1.15	Fructose	++
1.00	Glucose	+	1.00	Glucose	++	1.00	Glucose	++
.95	Galactose	+	.93	Galactose	++	.93	Galactose	++
.76	Sucrose	0	.75	Sucrose	0	.76	Sucrose	0
.56	[b] Maltose-like	+	.56	Maltose-like	++	.56	Maltose-like	++
.40	Raffinose	++	.39	Raffinose	++	.39	Raffinose	++

a) + means greater than; ++ means much greater than; 0 means compound not present.

b) Maltose, mannose, and trehalose all gave the same Rg values.

TABLE I (Continued)

Rg of Oligosaccharides

Honeydew sample as described above		Honeydew sample as described above		Honeydew sample as described above	
Rg Value	Relative Amounts Present	Rg Value	Relative Amounts Present	Rg Value	Relative Amounts Present
[a].72	[b]++	.72	+++	.72	+++
.64	++	.64	+++	.63	+++
.57	++	.53	++	.56	++
.26	++	.26	++	.27	++
.17	+	.19	+	.17	+
.14	++	.15	++	.14	++
.12	+	.12	+	.12	+

a) These Rg values were calculated after honeydew samples had been developed five times in solvent "B". The only standard was raffinose which gave an Rg value of .72 in this developing system.

b) + means barely visible; ++ means substantial amount present; +++ means large amount present.

30% sucrose. In this case the appearance of galactose and raffinose seems rather remarkable. The physiological significance of these compounds is not clear at this time.

Even though sucrose was absent, there were large amounts of glucose and fructose found in the samples. This finding does not seem to be universal among plant-sucking insects or even among the aphids. Gray (22), using the pineapple mealybug, Pseudococcus brevipes, feeding on green pineapple fruit, found the honeydew to contain approximately equal amounts of fructose, glucose, sucrose, and maltose. Mittler (36), using the aphid Tuberolachnus salignus feeding on young willow stems, found that the honeydew contained roughly equal amounts of sucrose, glucose, fructose, and melezitose. However, Burns, et al (11), using the tulip-tree scale, Toumeyella liriodendri, feeding on yellow-poplar twigs, found the honeydew to be completely devoid of sucrose.

Reports of high amounts of oligosaccharides present in aphid honeydew seem to be the rule rather than the exception. Auclair (5), states in a review article that in the analysis of twenty-eight honeydew samples of homopterous insect species, only eight failed to show any oligosaccharides. Of those species not showing any oligosaccharides, four showed compounds of the gluco-, malto-, maltotrio-, and maltotetro-sucrose series. Since sucrose was the only sugar available to the insect in many cases it was obvious that the varied carbohydrates present in the honeydew samples were synthesized from sucrose. Gray and Fraenkel (20) were among the first to hypothesize that invertase was the enzyme responsible for such digestive action. Later, White and Maher (50) and Wolf and Ewart (51) provided experimental evidence that invertase did indeed possess transglucosidation and transfructosidation activity.

Since McAllan and Adams (35) showed the presence of an enzyme, pectin polygalacturonase, in whole-body homogenates of four aphid species, and a pectinase in the saliva of twenty-three species of aphids, the presence of galacturonic acid was suspected in the honeydew analysis. This hypothesis is further supported by the fact that Saran, (44), has shown the greenbug, biotype "B", to be both a parenchyma and a phloem feeder. When it is a phloem feeder, it penetrates intercellularly and must hydrolyze the pectin of the middle lamella. This would release galacturonic acid units which might appear in the honeydew. The honeydew was analyzed for these units. In no case was there any evidence for the presence of galacturonic acid.

Amino Acids

Table II shows the results of three thin-layer chromatograms of each honeydew sample. From these results it is seen that the amino acids glutamic acid, aspartic acid, isoleucine, and alanine are common to honeydew samples collected from aphids feeding on either Will or Rogers varieties of barley. The Rogers honeydew sample contained two additional amino acids, methionine and threonine. Visual comparisons of the chromatograms indicated that the spots common to both the honeydew samples analyzed were of approximately the same intensity. Two methods of quantitating amino acid spots removed from the chromatograms were utilized. Both gave inconsistent results.

The presence of glutamic acid, aspartic acid, and alanine in honeydew is not too surprising since these amino acids are present in largest amounts in the translocatory stream in the phloem where the aphid is known to feed (53). However, the biotype "B" can also feed in the

TABLE II

AMINO ACID COMPOSITION OF HONEYDEWS FROM THE GREENBUG, (*SCHIZAPHIS GRAMINUM* ROND.)

Honeydew collected from greenbugs feeding on resistant Will barley				Honeydew collected from greenbugs feeding on susceptible Rogers barley			
Amino Acid Identity	Rf Solvent 1	Rf Solvent 2	Concentration relative to Std. at 8 µg	Amino Acid Identity	Rf Solvent 1	Rf Solvent 2	Concentration relative to Std. at 8 µg
Alanine	.55	.11	=[a]	Alanine	.54	.10	=
Isoleucine	.73	.47	+	Isoleucine	.74	.46	+
Aspartic Acid	.41	.00	++	Aspartic Acid	.43	.01	++
Glutamic Acid	.51	.00	++	Glutamic Acid	.51	.00	++
				Methionine	.64	.36	+
				Threonine	.45	.47	+

a) = means approximately equal to; + means greater than; ++ means much greater than.

parenchyma where it would have access to a greater variety of nitrogen sources. Whole plant extracts of both Will and Rogers barley have indicated few quantitative or qualitative differences between the two varieties (12). Auclair (5) reported in a review article that in eight species studied, not including Schizaphis graminum (Rond.), all eight honeydew samples had the same number of amino acids as were present in the sap of their respective hosts. However, Burns, et al (11), found two amino acids in the honeydew of the tuliptree scale, Toumeyella liriodendri, feeding on yellow-poplar stems that were not found in the host sap. He labeled these waste products. Gray (22), reported five amino acids in the honeydew of pineapple mealy bugs feeding on young pineapple plants which were not present in the plant sap.

In the case of most aphids, then, the mechanism of nitrogenous digestion seems to be that part of what goes in must come out unchanged. Analysis of Will and Rogers barley leaves (12) for free amino acids shows twenty-three amino acids in common, with Rogers extract having three extra unidentified amino acids. Quantitatively the extracts were similar except for significantly greater amounts of aspartic acid and asparagine in the Rogers extract. Due to great difficulties in perfecting a technique, no data on organic constituents of barley phloem tissue has yet been reported. In the light of these results it can be postulated that the greenbug, when feeding on susceptible Rogers variety barley, can obtain two extra amino acids which are identifiable in the honeydew. As an alternate hypothesis it is conceivable that methionine and threonine could occur in dipeptide form in Rogers, be taken up during feeding and be digested to the corresponding amino acids. Von Dehn (16) has shown that there is a dipeptide present in Cirsium arvense and

that glycine is present in the honeydew of Aphis acanthi feeding on that plant. Glycine is not, however, present in the plant sap and this suggested to von Dehn that a peptidase could be active in the aphid's gut. Srivastava, et al (47), have also shown the possibility of a peptidase in gut extracts of Acyrthosiphon pisum.

Since the physiological significance of the amino acids found in the honeydew samples is not known, it cannot be said whether or not the greenbug derives any special benefit from feeding on one plant or the other.

Enzyme Studies

The chromatographic results of the commercial pectinase assay given in Table III show that the activity is linear with time, with an enzyme concentration of 0.1 mg / ml. At the end of one hour incubation time, approximately 4 µg of galacturonic acid were formed. To assay for polygalacturonase activity, one ml of a dialyzed protein extract made from greenbugs, biotype "B", was used in place of the commercial pectinase. Results of chromatography of the reaction mixtures showed no pectinase activity in any of the protein extracts from greenbugs.

Another method of following the breakdown of pectic acid to galacturonic acid was to measure the increase in reducing power of the reaction mixture with a 3,5-dinitrosalicylic acid reagent. However, the pectic acid blank gave inconsistent readings. The readings were variable enough to completely overshadow any enzyme activity which might have been present.

The commercial cellulase failed to hydrolyze the MN 300 cellulose (Table IV). Also, there was no activity with the protein extract from

TABLE III

ENZYMATIC ACTIVITY OF COMMERCIAL PECTINASE AS ASSAYED BY THIN-LAYER CHROMATOGRAPHY

Reaction Mixture	Compounds Identified in Substrate Reaction Mixture in Comparison with Standard Containing 2 ug			
	Pectic Acid		Galacturonic Acid	
	Rf	Amount Present	Rf	Amount Present
PA[a] + 1 ml H_2O	.05	[b] ++	.20	0
" + " " Comm. Pectinase for 15 min.	"	++	"	-
" + " " " " " 30 "	"	++	"	=
" + " " " " " 1 hour	"	++	"	=
" + " " " " " 2 hours	"	++	"	+
" + " " Protein Extract	"	++	"	0
1 ml H_2O + 1 ml Protein Extract	"	0	"	0

a) PA is an abbreviation for pectic acid.

b) 0 means compound not present; - means less than standard; = means approximately equal to standard; + means greater than standard; ++ means much greater than standard at 2 ug.

greenbugs (Table V). No other substrates were tested for cellulase.

TABLE IV

ENZYMATIC ACTIVITY OF COMMERCIAL CELLULASE AS ASSAYED BY THIN-LAYER CHROMATOGRAPHY

Reaction Mixture	Cellulose, Rg .00 Amount Present	Glucose, Rg 1.00 Amount Present
Cellulose + 1 ml Comm. Cellulase	[a] ++	0
" + 1.5 ml Comm. "	++	0
" + 1 ml Comm. "	++	0
" + 1 ml H_2O "	++	0
Comm. Cellulase + 1 ml H_2O	0	0

a) 0 means compound not present; ++ means much greater than standard.

Assays for pectinase and cellulase in greenbug saliva consisted of caging the aphids on filter paper (dry) and filter paper which had been impregnated with 1% pectic acid and kept slightly moist. Chromatography showed that there was no evidence for either cellulase or pectinase activity in the saliva of greenbugs. There was also no evidence of hydrolysis of pectic acid when it was added to the artificial diet and greenbugs were allowed to feed on it for 24 and 48 hours.

In all of these cases negative results do not prove that pectinase

or cellulase enzymes assayed for are in reality not present. It is possible that the assays described were not sensitive enough to detect them.

TABLE V

THIN-LAYER CHROMATOGRAPHIC ASSAYS FOR PECTINASE, CELLULASE, AND INVERTASE ACTIVITY IN A PROTEIN EXTRACT FROM GREENBUGS, (SCHIZAPHIS GRAMINUM ROND.)

Breakdown Products in Reaction Mixture in Comparison with Standard Containing 2 ug

	Amounts Present of		
Reaction Mixture	Glucose (Rg 1.00)	Fructose (Rg 1.10)	Galacturonic Acid (Rg .20)
Enzyme Extract + 1 ml H_2O	[a]0	0	0
Cellulose + 1 ml Enzyme Extract	0		
Sucrose + 1 ml Enzyme Extract	++	++	
Pectic Acid + 1 ml Enzyme Ext.			0
Cellulose + 1 ml H_2O	0		
Pectic Acid + 1 ml H_2O			0
Sucrose + 1 ml H_2O	0	0	

a) 0 means compound expected but not observed; ++ means much greater than standard.

Table V also shows that there was a high amount of invertase activity present as demonstrated by the dense spots of glucose and fruc-

tose and also by the large amount of reducing sugar produced upon incubation. These results show two things. First, that the protein extract from the greenbugs contained at least one active enzyme, and secondly that the extremely large amounts of oligosaccharides present in the honeydew could be the direct result of this enzyme's activity. However, upon chromatographing the resulting reaction mixtures, only glucose, fructose, and sucrose were identified. There was no indication of the presence of any of the oligosaccharides in the honeydew. Similar results were obtained by Srivastava and Auclair (46) when they extracted invertase from the alimentary canal of the pea aphid, Acyrthosiphon pisum (Harr.) and tested its activity with a variety of substrates, including sucrose. It hydrolyzed sucrose, but only in one isolated instance did the enzyme show transglucosidative activity. However, this aphid also has a considerable amount of oligosaccharides present in the honeydew. Bacon and Edelman (6), while studying the action of invertase preparations, discovered that oligosaccharides resulted when high concentrations of sucrose were used (30-45%). These concentrations are similar to what occurs in natural feeding conditions.

CHAPTER V

CONCLUSION

There was only a slight quantitative difference between the carbohydrate content of honeydew collected from greenbugs feeding on susceptible Rogers barley and honeydew collected from greenbugs feeding on resistant Will variety barley. In addition, the carbohydrate content of honeydew collected from greenbugs feeding on an artificial diet consisting of 30% sucrose as its only carbohydrate source was quantitatively and qualitatively similar to the honeydew from Rogers barley. These results indicate that sucrose is the only sugar directly required by the greenbug from any source and that other carbohydrates may be obtained via hydrolysis and isomerization of sucrose. The presence of galactose and raffinose in the honeydew from the insects feeding on the artificial diet which contains sucrose as the sole sugar are indicative of this process.

The absence of sucrose and the high concentrations of oligosaccharides in the honeydew samples could be related due to the activity of the enzyme invertase. Invertase activity was found in whole aphid homogenates; however, no transgluco- or transfructosidase activity was found. It has been pointed out, however, that in order to observe oligosaccharide synthesis with invertase, a concentration of 30-45% sucrose needs to be present in the incubation mixture (51).

For the amino acids common to both honeydew samples, the respective

concentrations were very similar. The two additional amino acids identified in the honeydew from the susceptible Rogers barley also were of similar concentrations to the other amino acids. Bragdon (10) demonstrated that certain aphids possess the ability to differentially absorb certain amino acids. The preferential absorption serves to increase reproduction and longevity of that particular aphid. Since the greenbug, biotype "B", apparently is a parenchyma feeder as well as a phloem feeder, it could conceivably have access to all the free amino acids found in whole plant extracts (12). In this respect, the greenbug differs from other aphids in that the honeydew does not reflect the amino acid content of the host plant upon which it is feeding. However, the possibility that the aphid may possess a peptidase enzyme which would hydrolyze a peptide to its corresponding amino acids cannot be ruled out, provided that a peptide was found in the susceptible variety.

The results of assays for the enzymes pectinase and cellulase were negative. In this case, negative results can mean that if these enzymes are present, they are not present in the concentration that the enzyme invertase is, or that the assay conditions were inadequate to detect the presence of the enzyme. The cellulose used as a substrate for cellulase may not have been suitable, since the fungal cellulase did not hydrolyze it. The alternate hypothesis is that the enzymes are in reality not present.

In conclusion, the carbohydrate content of the various honeydew samples seems to have no direct bearing upon the resistance or susceptibility of the host plant upon which the greenbug is feeding. Sucrose is apparently the only carbohydrate requirement. Absorption of sucrose seems to be a passive phenomenon, whereas the absorption of amino acids

could be an active process due to the preference exhibited for certain amino acids found in the honeydew samples. The example of preferential absorption of amino acids could possibly be related to the resistance or susceptibility of a particular host species.

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