Composition of Honeydew Excreted by Pineapple Mealybugs[1]

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THE EXCRETION OF THE PLANT-SUCKING INSECTS, aphids and coccids, is a clear, syrupy liquid and contains much unabsorbed organic material. This honeydew contains a high percentage of sugars and is much sought after as food by ants and other insects. In some cases, the excreted liquid, on drying, leaves a white deposit on the leaves. Manna is such an excretion from the coccid *Trabutina mannipara* feeding on tamarisk in Sinai, and according to Weber (*1*) it contains 55 per cent cane sugar, 25 per cent invert sugar, and 19.3 per cent dextrin, making a total of 99.3 per cent carbohydrates.

The reason for this apparently wasteful method of feeding is generally thought to be a deficiency of organic nitrogenous compounds in the plant juice. The insect must therefore take in large quantities of plant juice in order to get sufficient amino acids and proteins, with the result that it has a large excess of carbohydrates to dispose of (*2*). In the work reported here, this was not found to be the case with pineapple mealybugs (*Pseudococcus brevipes* Ckll.), since large quantities of free amino acids were found to be present in their honeydew, as well as large quantities of sugars.

The pineapple mealybug is the well-known insect that has been shown by Carter (*3*) to be responsible for "mealybug wilt" of pineapples, a unique, systemic disease that seems to be due to a nonreproducing toxin secreted by the mealybugs. Our work came about as a by-product of studies in which the salivary secretions of mealybugs were being examined for toxins. In some of the experiments, mealybugs were allowed to feed through a membrane into a nutrient medium, and it became apparent that the salivary secretions might be contaminated with the honeydew excreted by the bugs. Therefore, an examination of the constituents of the honeydew was undertaken.

One- and two-dimensional filter paper chromatograms were prepared, using the ascending method described by Williams and Kirby (*4*). The solvent for the one-dimensional chromatograms was fresh *n*-butanol-acetic acid-water (4:1:1 v/v). For the two-dimensional chromatograms, honeydew was collected with a capillary, diluted 1:5 with water in most cases and 4 µl of this solution was applied in the corner, 3 cm from each edge, on a sheet of Whatman No. 1 filter paper (11″×14″). Phenol-water (8:2 w/w) was used as the first solvent and *n*-butanol-acetic acid-water (4:1:1 v/v) as the second; this solvent combination seemed to be very satisfactory because of its versatility in separating different types of compounds. After drying at room temperature, the chromatograms were dried at 60° C for an hour and then sprayed with the appropriate reagent. It was found that several different types of compounds could be detected on the same chromatogram by using successive reagents.

To detect amino compounds, after drying the chromatograms were sprayed with 0.25 per cent ninhydrin in *n*-butanol saturated with water (*4*), and heated 20 minutes at 80° C. To detect sugars, dried chromatograms were sprayed with 1 per cent potassium permanganate in 2 per cent aqueous sodium carbonate (*5*). This spray could be applied after ninhydrin, and the sugars appeared as yellow spots. Sugars were also identified with the benzidine-trichloroacetic reagent (*6*), which could also be applied with some usefulness after the paper had already been treated for amino acids.

A new reagent was found to be specific in differentiating ketoses from aldoses. The paper is sprayed with a saturated solution of 2,4-dinitrophenylhydrazine in 95 per cent ethanol containing 1 per cent concentrated HCl, and heated a few minutes at 70° C. This spray gives an orange spot on a light-yellow background for fructose and compounds containing fructose, such as sucrose, raffinose, and fructose-6-phosphate. Other sugars do not give any color that can be distinguished from the light-yellow background. As little as 3 µg of fructose can be detected by this method after chromatographing with the butanol-acetic acid solvent.

Malic acid, citric acid, and partially neutralized citric acid were detected with 0.1 per cent bromocresol-green in 95 per cent ethanol (*7*). Citric acid appears yellow, and disodium citrate forms two spots, one yellow and one blue, when phenol is the developing solvent. It was found that partially neutralized citric acid formed four spots when chromatographed in two dimensions with the solvents described above.

A pineapple leaf, 18 inches long, was made radioactive by allowing it to photosynthesize for 24 hours in an atmosphere containing $C^{14}O_2$, similar to the method described by Calvin (*8*). The capacity of the

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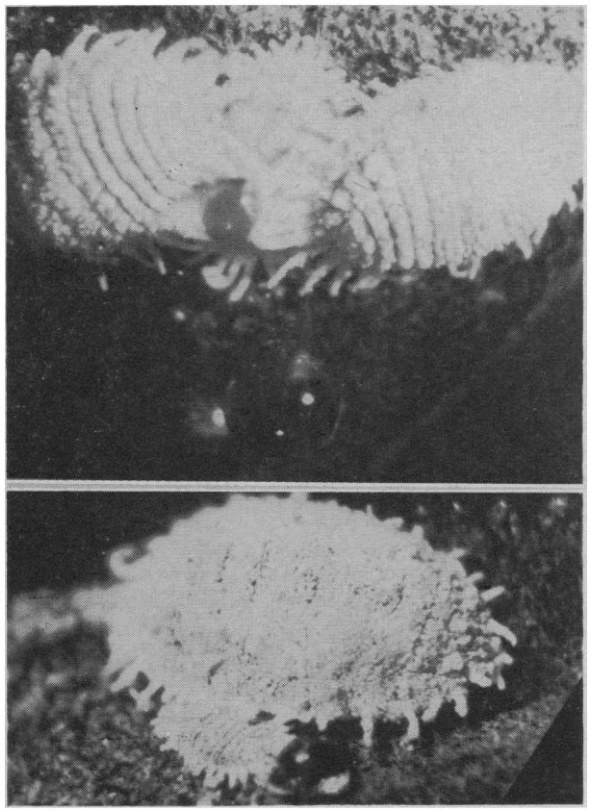

FIG. 1. Pineapple mealybugs (*Pseudococcus brevipes*, Cockerell) feeding on immature pineapple fruit and showing the clear globules of excreted honeydew.

photosynthetic chamber was 900 ml, and 900 mg $BaCO_3$[3] containing 0.5 mc C^{14} were used.

The radiograms were prepared by placing the developed chromatograms in close contact with Eastman No-Screen X-Ray film for 7 days, as described by Benson *et al.* (*9*). The radioactivity of the spots was determined by using a Geiger tube (1.75 mg/cm² mica window) placed on a 4-mm-thick plastic spacer with a hole in it, 1 inch in diameter. A smaller hole, 1 cm in diameter, in the same plastic sheet was used for localizing smaller spots on one-dimensional strips.

Preliminary experiments. Pineapple mealybugs excrete small droplets of a perfectly clear colorless fluid (Fig. 1). Under normal conditions the mealybugs are attended by ants, which eagerly seek the honeydew, but if the ants are kept away the excretions sometimes accumulate as droplets on the backs of the mealybugs near the posterior end. Honeydew is excreted from the anus and must not be confused with secretions from several different types of glands, such as wax or a liquid which is extruded from several pores when the bug is touched, and which solidifies quickly.

A few of the honeydew droplets were gathered with a capillary from the backs of several bugs and chromatographed in one dimension. One strip showed four well-defined spots of sugars with the permanganate spray. Two of the spots were identified as fructose and sucrose with the new spray test described above. The other sugars were identified later by using two-dimensional paper chromatograms and radioautography. One strip was sprayed with ninhydrin for amino acids. Since it was expected to find only sugars in the honeydew, it was a great surprise to find 12 amino-acid spots appearing on the paper strip.

Carbohydrate and other radioactive compounds. In the second experiment, medium-sized mealybugs were removed from green pineapple fruit, starved one day, and then caged on a pineapple leaf that had photosynthesized in an atmosphere containing $C^{14}O_2$ for 24 hours. After the bugs had fed on the radioactive leaf for 48 hours, some of their clear honeydew excreta had collected on the clean plastic top of the cage opposite the bugs. It was observed later that the mealybugs were able to squirt out tiny droplets of this honeydew with enough force to carry it upward for a distance of 5 mm, where it adhered to the top of the cage. The honeydew was collected, chromatographed in two dimensions, and sprayed with ninhydrin. Only 3 amino acids appeared; they were identified as cystine, aspartic acid, and glutamic acid from their R_f values and their relative positions as obtained from chromatograms of artificial mixtures of pure amino acids. The same chromatogram was sprayed on the reverse side with alkaline permanganate. The three distinct yellow spots that appeared were identified as

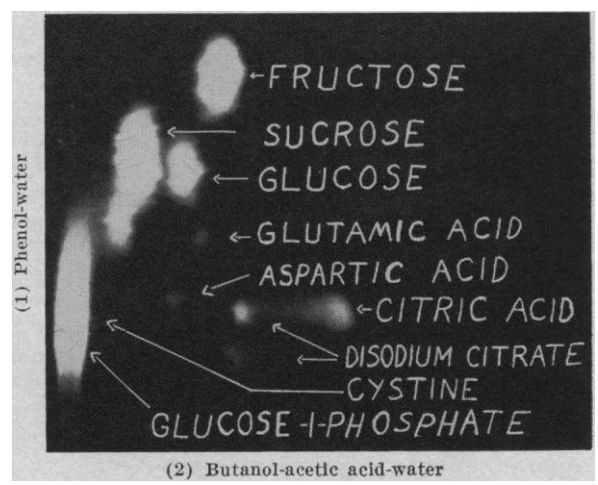

FIG. 2. Radiogram of honeydew excreted by pineapple mealybugs while feeding for 48 hr on a radioactive pineapple leaf. The leaf had photosynthesized in an atmosphere containing $C^{14}O_2$ for 24 hr.

sucrose, glucose, and fructose. A radioautograph was made with the same chromatogram (Fig. 2). The radioactivity of each spot is given in Table 1.

The purpose of the radioactive carbon was to detect small amounts of material or those not easily detected by the usual tests. The elongated spot that did not move in the butanol-acetic solvent was not readily identified by the various chemical sprays. The lower,

[3] The $BaC^{14}O_3$ was purchased from the Oak Ridge National Laboratories on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

TABLE 1

RADIOACTIVITY OF SPOTS SHOWN IN FIG. 2

Spot	cpm	Spot	cpm
Fructose	2524	Cystine	Not determined
Glucose	776	Aspartic acid	74
Sucrose	2836	Glutamic acid	60
Glucose-1-phosphate	3420	Citric acid	261

more intense part (*arrow*) gave a positive test for phosphates (*9*) and is the position that pure glucose-1-phosphate occupies when run in two dimensions in these solvents. It was not established that the entire spot was glucose-1-phosphate (the upper half of the spot was probably dextrins), but proof that this elongated spot contained glucose and phosphate was obtained as follows:

Several one-dimensional chromatograms of the radioactive honeydew were run in the *n*-butanol-acetic acid-water solvent. One strip was sprayed for phosphate (*9*), and two blue spots containing phosphate appeared—one at the origin and the other at R_f 0.19. The latter spot was not radioactive and thus did not show up in the radiogram. The spot at the origin of another strip was cut out and eluted with hot water. After concentration, this material was partially hydrolyzed by heating for a few minutes in 2 *N* HCl and then evaporating the water and acid. The residue was taken up with a few drops of sugar solution containing fructose, glucose, and sucrose as carriers. The resulting solution was then chromatographed with the same solvent, and the sugar spots were located with the permanganate spray. The spots were counted as before, except that the small hole, 1 cm in diameter, was placed over the spots. The counts for one minute for each spot after correction for background were: fructose, 8; sucrose, 12; and glucose, 168. This shows that only glucose is produced on hydrolysis; however, over half the activity remained near the origin, since it probably was not completely hydrolyzed.

The citric-acid and disodium-citrate spots were located with indicator, and the reagent could be applied after treating the paper with ninhydrin. The spot just below sucrose in Fig. 2 was not identified, but its R_f values correspond with maltose. The concentration was evidently too low to be chemically identified. Two black spots were formed with mercuric nitrate reagent (*10*) for purines and pyrimidines, but one of the spots coincided with cystine, and the other was very near aspartic acid.

It was rather puzzling to find 12 amino acids in the honeydew in the first experiment and only 3 in the second. After the mealybugs had fed 4 days on the radioactive leaf, the honeydew was again collected and chromatographed. It was then found to contain 7 amino compounds (cystine, aspartic acid, glutamic acid, serine, asparagine, glutamine, and one unidentified spot). All amino compounds that were not masked by sugars contained some radioactive carbon. Malic acid was also found in the 4-day excretion by indicator sprays and a radioautogram.

Amino components and their increase with feeding period. It appeared that the longer the period of feeding by the mealybugs, the more numerous the amino acids excreted in the honeydew. This view was substantiated by a third experiment carried out on a much larger scale. Two hundred mealybugs were caged on each of 20 leaves from different pineapple plants. The cages were removed, the excretions collected, and new, clean cages put on at regular intervals of 24 hours for the first 4 days, and then intervals of 48 hours for the next 6 days. The excretions were chromatographed in fresh *n*-butanol-acetic acid solvent for 40 hours, dried, and sprayed with ninhydrin. The average number of amino acids that appeared in the one-dimensional chromatograms for each sample of bugs that produced enough honeydew to collect is shown in Table 2, along with the number of replicates and the standard error. The results show conclusively that the number of amino acids excreted in the honeydew increases with the period of feeding of the bugs up to about 10 days. After 10 days no further increases were noticed in the one-dimensional chromatograms. As the period of feeding increased, the amount of excretion decreased, and after 10 days only two of the lots of bugs excreted enough honeydew to collect. After 6 or 8 days, one tenth the amount of honeydew (diluted 1:10) gave a higher concentration of amino acids (based on intensity and size of the spots on the chromatogram) than the undiluted honeydew collected during the first few days. Differences in composition were noted which could have been due to differences in the mealybugs or the leaves, or starvation from the time they were removed from the green fruit until they were put on the leaves. For instance, after 24 hours' feeding and using the same volume of honeydew, three samples contained only 1 amino acid, whereas several samples contained 7 amino acids in each. The two-dimensional chromatograms of Fig. 3 show the differences between two samples collected after feeding 1 day and then again during the 8–10-day feeding period. Fig. 3 also illustrates the increase in number of amino acids with the increase in feeding time.

TABLE 2

RELATION OF MEALYBUGS' FEEDING PERIOD TO NUMBER OF AMINO ACIDS FOUND IN THEIR EXCRETION

Period of mealybug feeding (days)	1	2	3	4	6	8	10
No. samples	15	17	14	9	12	7	2
Av no. amino acids/sample in excretion	4.0	7.3	8.4	9.8	11.3	12.0	13.0
Standard error	0.62	0.56	0.89	0.62	0.80	0.57	0

Source of the amino acids. Where do the amino acids found in the honeydew excreted by the mealybugs come from? That this increase of amino acids was not due to nitrogen fixation outside the mealybugs was shown by allowing an aliquot of honeydew to

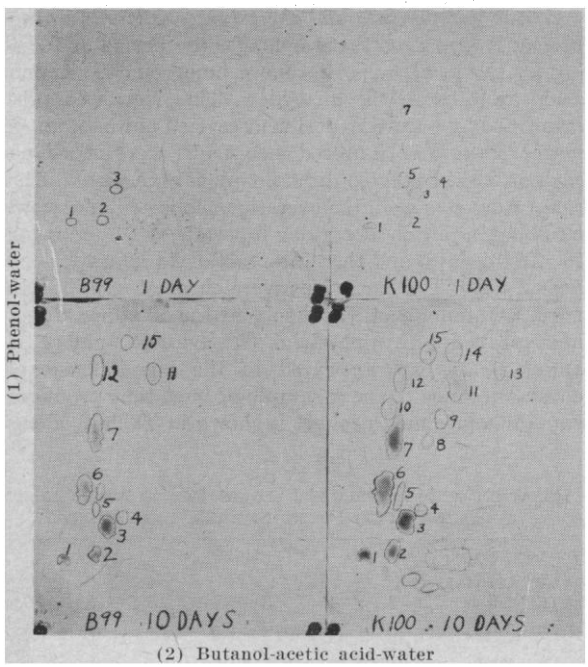

FIG. 3. Two-dimensional filter paper chromatograms of honeydew excreted by two lots of mealybugs feeding on two different leaves, and showing increase in number and amount of amino acids with time of feeding The two samples of honeydew for the upper chromatograms were collected after the mealybugs had fed on the leaves for 1 day; honeydew for the lower chromatograms was collected from the same two lots of bugs during an 8–10-day period on the same leaves. Spots outlined with tangential lines were fluorescent in ultraviolet light. The compounds reacting with ninhydrin are outlined in pencil and indicated by numbers; the spots that have been identified are: (1) cystine, (2) aspartic acid, (3) glutamic acid, (5) serine and glycine, (6) asparagine, (7) glutamine, (8) alanine, (9) tyrosine, (10) histidine, (11) tryptophane, (13) phenylalanine, and (15) proline.

stand 3 days, and comparing it with the fresh honeydew. No increase in amino acids was noticed. Also, in some cases, the excretion was removed from the backs of several mealybugs shortly after it was excreted, and it still contained as many as 12 amino acids. It cannot be said that the amino acids come entirely from unabsorbed amino acids in the plant juices, since 6 or 7 of the amino acids that appear in the honeydew do not appear in the juice pressed from frozen pineapple leaves on which the mealybugs fed. This was shown by an experiment in which the honeydew collected from mealybugs during the 8–10-day period on a pineapple leaf was compared chromatographically with the juice pressed from the same leaf after freezing and thawing it. The excretion of mealybugs feeding on a small green pineapple fruit was also compared with the juice of the same fruit. These chromatographs are shown in Fig. 4.

It can be seen from Fig. 4 that 7 amino acids appeared in the honeydew that did not appear as free amino acids in the leaf juice; these spots are marked with the letters *A–G*. One of the most striking of these was proline (spot *G*), which gives the characteristic yellow color with ninhydrin. Not all these 7 amino acids were found in bound form in the proteins of the leaf juice. This was shown on an aliquot of the same juice as used in chromatograph No. 1 of Fig. 4, by precipitating the proteins with 10 per cent trichloroacetic acid, and hydrolyzing the precipitate in 6 *N* HCl for 24 hours at 105°–110° C in a sealed tube. The acid was removed by evaporating in a vacuum desiccator over solid NaOH, adding water, and repeating the evaporation several times. After applying the hydrolysate to the paper, the spots were treated with NH_3 vapor, dried, and chromatographed in two dimensions. The amino acids in the proteins of the juice were thus compared with the free amino acids in the juice. Spots *F* and *G* were found in small amounts in the protein hydrolysate, leaving 5 spots (*A, B, C, D,* and *E*) which were found in the honeydew but not in the juice or in the hydrolyzed proteins of the juice.

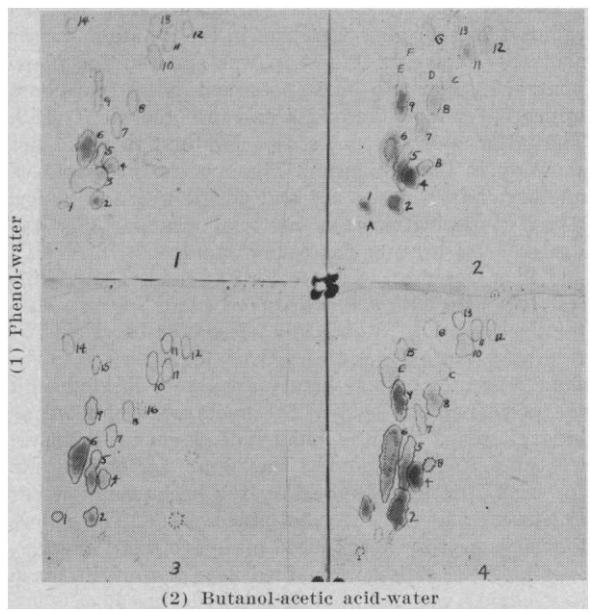

FIG. 4. Two-dimensional filter paper chromatographs sprayed with ninhydrin and showing the difference in composition between (1) juice of pineapple leaf fed on by mealybugs for 10 days, and (2) honeydew excreted by mealybugs during the 8–10-day feeding period on the same leaf, and also the difference in composition between (3) juice of a green pineapple fruit and (4) honeydew from mealybugs fed on the same fruit. Compounds reacting with ninhydrin appear as dark spots in the photograph and are outlined in pencil. Tangential lines mark spots that fluoresced in ultraviolet light. The spots that have been identified are: (1) cystine, (2) aspartic acid, (3) glutathione, (4) glutamic acid, (5) serine (lower half of spot) and glycine (upper half), (6) asparagine, (7) threonine, (8) alanine, (9) glutamine, (10) tryptophane, (11) valine, (12) phenylalanine, (*C*) tyrosine, (*E*) histidine, and (*G*) proline.

From Fig. 4 it can be seen that several amino acid spots appeared in the pineapple leaf which did not appear in the honeydew; they are spots 3, 10, and 14. Spot 3 was identified as glutathione. Similar results were observed with the honeydew from bugs feeding on the green fruits and the juice of the fruits. Usually more amino acids were found in the excreta of mealybugs feeding on pineapple leaves than in the excreta of those feeding on pineapple fruit. Numerous amino

acids were also found in the honeydew excreted by mealybugs feeding on yucca leaves.

Some of the amino acids found in the honeydew excreted by the mealybugs are evidently unabsorbed amino acids from the plant juices, as are some of the main sugar components. The presence of sucrose indicates a lack of invertase activity in the intestinal tract. The fact that the 3 amino acids excreted by mealybugs feeding on a radioactive leaf contained C^{14} does not mean incontestably that they were unabsorbed amino acids from the juice, but one would think they were.

The amino acids present in the excretion but not in the food source could be metabolic waste products for nitrogen excretion by the mealybugs, or they could be produced by symbionts within the insects. Toth, Walsky, and Batori (*11*) have reported nitrogen fixation by macerated aphids in a solution of oxalacetic acid and glucose. The possibility that nitrogen fixation occurs in mealybugs is being investigated. An experiment was carried out in which 1000 mealybugs were fed for one week on agar containing 0.1 per cent oxyquinoline benzoate, a powerful fungicide and moderate bactericide. The mealybugs were then transferred to pineapple leaves and their excreta collected. As before, many amino acids were found in the honeydew. Evidently, the antiseptic did not impair the mealybugs' ability to excrete amino acids in their honeydew. Symbionts of several forms have been found enclosed within the mycetomes of two strains of pineapple mealybug by Carter (*12*), and a relationship might be found between the form (species) of symbionts present and the different amino acids found in the excretion of the bugs.

The increase of amino acids in the honeydew with the increase in feeding period by the mealybugs may have some relationship to their ability to produce wilt in pineapples, since an increase in toxicity of the mealybugs with an increase in feeding period has been found to occur (*13*). The toxicity of the mealybugs is also governed by the nutrition of the insect (*14*).

The presence of these important nutrients in the honeydew certainly seems to be a wasteful method of feeding, but it may be necessary for the mealybugs to suck in excessive quantities of juice in order to get sufficient quantities of other substances that occur in very small amounts. These findings tend to confirm the theory that the availability of these excretions as food for ants has undoubtedly been a factor in the evolution of the close relationship between these two groups of insects.

The general view that plant-sucking insects, which excrete copious quantities of excess carbohydrates in their honeydew, must take in large amounts of plant juice in order to get sufficient amounts of amino acids and proteins, was not found to be the case with pineapple mealybugs. Relatively large amounts of as many as 19 different amino acids and amides have been found in the honeydew excreted by pineapple mealybugs (*Pseudococcus brevipes,* Ckll.) by the method of paper chromatography. The number of amino acids excreted was shown to increase with the period of feeding. The 16 amino components of the honeydew that have been identified from their R_f values are cystine, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, tryptophane, valine, phenylalanine, histidine, and proline. Three ninhydrin spots have not been identified. At least 5 amino acids were found in the honeydew which were not found in the food source.

The carbohydrate components identified by different sprays and radiograms of radioactive honeydew were fructose, glucose, sucrose, glucose-1-phosphate, and possibly maltose. Malic acid, citric acid, and salts of citric acid were also found.

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