STUDIES ON THE FEEDING AND NUTRITION OF *TUBEROLACHNUS SALIGNUS* (GMELIN) (HOMOPTERA, APHIDIDAE)

II. THE NITROGEN AND SUGAR COMPOSITION OF INGESTED PHLOEM SAP AND EXCRETED HONEYDEW

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Several workers have tried to ascertain the nitrogenous and carbohydrate materials which are ingested by aphids and coccids by analysing juices, phloem exudates and extracts obtained from the insects' host plants (Evans, 1938; Michel, 1942; Lindemann, 1948; Auclair & Maltais, 1950, 1952; Gray, 1952; Hackman & Trikojus, 1952; Gray & Fraenkel, 1954; Bacon & Dickinson, 1957). It remains to be established, however, whether the insects ingest materials in the same form and at the same concentration as they occur in their host plants. The present paper gives details of a method, outlined by Kennedy & Mittler (1953), for obtaining the sap which normally enters the alimentary canal of feeding *Tuberolachnus salignus* (Gmelin). By determining the nitrogen and sugar composition of this sap and that of the honeydew excreted by the aphid, it was possible to examine the chemical relationship existing between the insect's food and its excreta.

MATERIAL AND METHODS

T. salignus was reared as previously described by Mittler (1957). Standard, 5–6 ft. tall, 2- to 4-year-old, potted *Salix acutifolia* Willd. trees were used as the experimental host plant, except where otherwise stated. Experiments were carried out in a greenhouse at a mean temperature of 20° C. under a photoperiod of 16 hr.

The stem cage. Two split annuli of cork, having an outer diameter of approximately 1·5 in., were attached 3–4 in. from each other on a willow stem. Low-melting-point wax was moulded into gaps between the cork and the willow bark, and into the radial split in each annulus. A sheet of cellophane paper was then stretched in a cylinder about the two annuli, and its slightly overlapping edges clipped together with two 2·5 in. bull-dog paper clips.

Stylet cutting technique and collection of stylet-sap. Twenty adult apterous *T. salignus* were caged on an upright willow stem. 2–3 hr. after the aphids had inserted their stylets into the stem and had excreted several droplets of honeydew, the plant was placed on its side on a bench. The soil in the pot was prevented from

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spilling and from drying out by wrapping the pot in damp sacking. The stem was gently clamped when the plant had been rotated into such a position that some of the feeding aphids were visible in profile when viewed under a binocular microscope.

As *T. salignus* normally presses its head close to the willow stem on which it is feeding, its proboscis was found to extend for at least 0·5 mm. along the surface of the willow's bark from the aphid's head to the point of entry of its stylets into a 2- — 4-year-old stem (Fig. 1 A). A fine splinter of a razor blade, secured to a slender glass rod which was held between the fingers, was brought into position over the proboscis, as indicated in Fig. 1 A, and then pressed sharply against the bark, which acted as a 'chopping-block' for the cutting operation. Resting the hand on the microscope stage or the stem steadied the blade and reduced the risk of dragging the embedded stylets from the stem before the proboscis was severed. Directly after making the cut a clear fluid, termed stylet-sap, exuded from the cut end of the proboscis stump which projected from the stem (Fig. 1 B). On carefully

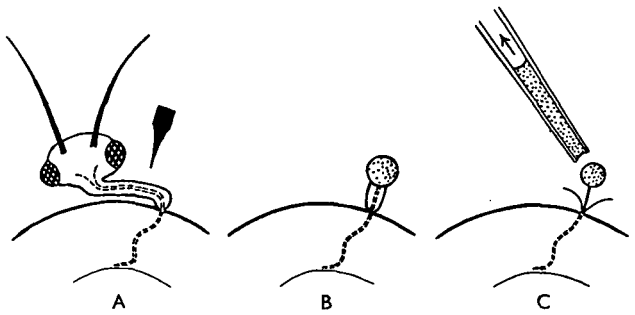

Fig. 1. Sectional views of A, the head of a feeding *T. salignus* with stylet path in host plant and blade about to sever proboscis; B, exudation of stylet-sap from proboscis stump; and C, stylet-sap being collected by a capillary pipette as it exudes from maxillary stylet stump.

brushing the severed tip of the labium from the enclosed stylets, the stylet-sap was observed to exude from the cut end of the 'joined' maxillary stylets; the mandibular stylets generally curling away from the maxillary stylets (Fig. 1 C). This diagram further shows the stylet-sap being collected by means of a glass capillary pipette. For the routine collection of stylet-sap the tip of the capillary pipette was, however, so positioned that the maxillary stylet-stump entered into the lumen of the capillary; the meniscus of stylet-sap at the tip of the capillary forming a viscous seal about the stylet-stump. This position was generally maintained for 5–8 hr. and sometimes for periods of over 24 hr. by embedding the base of the capillary pipette in some modelling clay stuck to a wooden block, or by inserting the pipette into a micro-manipulator placed on the bench. Such arrangements, however, required constant attention as slight vibrations of the bench or draught on the plant's foliage frequently resulted in a displacement of the tip of the pipette and a spilling of the stylet-sap on to the bark.

With the kind co-operation of Mr A. A. Barker a light micro-manipulator was developed which could be attached directly to a willow stem, and hence maintain

the tip of the pipette in position over an exuding stylet-stump for long periods. The instrument, which was constructed by the Cambridge University Engineering Laboratory, is illustrated in Fig. 2. It consists essentially of a clamp which may be attached to stems ranging from approximately 0·3–1 in. in diameter without damaging them. The rod *r*, which carries the pipette holder, can be inserted from either end into a tube *t*, carried by the clamp. When the pipette holder is in position over an aphid colony the rod is secured within the tube by means of the screw *s*. As soon as the proboscis of an aphid has been cut a glass capillary is inserted into the pipette holder to within a few millimetres of the bark, and directed towards the exuding stylet-stump by moving the pipette holder in its ball and socket joint. The final advancement of the pipette towards an exuding stylet-stump is effected by rotating the graduated head *g*. This operates a simple screw adjustment mechanism carried by the clamp, by which the rod and pipette holder may be moved towards or away from the stem.

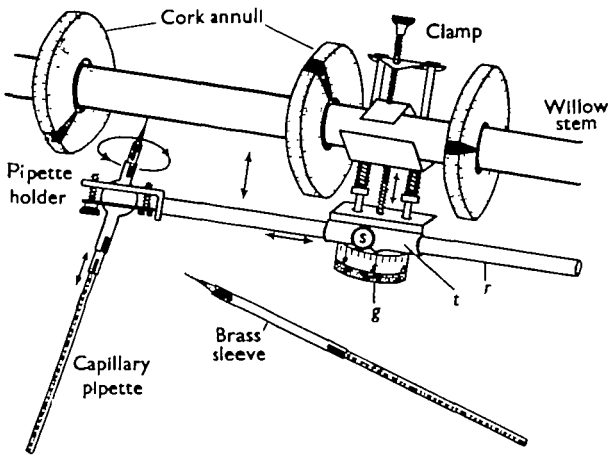

Fig. 2. Instrument used for collecting stylet-sap It is shown attached by means of the clamp to a willow stem between the cork annuli of adjacent stem cages. The directions in which it may be adjusted are indicated by arrows. For further explanation, see text.

In order to facilitate the rapid replacement of one glass capillary pipette by another during the collection of sap the capillary pipettes were each inserted into a brass sleeve before being inserted into the pipette holder. Sleeves differing in their inner diameter but of constant outer diameter were available so that capillary pipettes of different outer diameters could readily be used in one and the same pipette holder. The volume of stylet-sap collected by a capillary pipette in a certain time was subsequently determined by weighing an equal volume of mercury. The rate of exudation of stylet-sap was thereby also established.

Sap contained in the phloem tissues of pieces of bark, which were stripped from the cambial layer of some *S. triandra* L. stems was squeezed out of the tissues by means of a heavy iron roller.

In order to interrupt the normal translocation of organic solutes in the phloem

of willow stems a girdle of bark 0·25 in. wide and extending to the xylem, was removed from a number 2- to 3-year-old branches of a *S. fragilis* L. tree heavily colonized by *T. salignus*.

Collection of honeydew. Fluid samples of freshly excreted honeydew were collected for analyses by placing a waxed glass plate 1–2 in. under a colony of *T. salignus*. Honeydew droplets falling on this plate were drawn into a glass capillary pipette within a few seconds of their appearance at, and propulsion from, the anus of the aphids.

Chemical methods. The paper chromatographic methods of Consden, Gordon & Martin (1944), Partridge (1948), Williams & Kirby (1948), Crumpler & Dent (1949), and Boggs, Cuendet, Ehrental, Koch & Smith (1950) were used to detect and identify the amino-acids and carbohydrates occurring in stylet-sap and in freshly excreted honeydew. Non-reducing sugars were detected by spraying chromatograms with 5% phosphoric acid in 95% ethanol, and reheating them at 100°C. for 5–10 min. after the reducing sugars had been revealed by the method of Horrocks (1949). Cubic millimetre samples of stylet-sap and of freshly excreted honeydew were applied untreated to Whatman no. 1 filter paper. Some samples were, however, heated in sealed glass-tubing with an equal volume of 6N-HCl for 24 hr. at 100°C., and the acid subsequently removed by desiccation over solid KOH, before the hydrolysate was applied to filter paper. Sap squeezed from the phloem tissues was centrifuged to remove all cell debris before being applied to filter paper.

The total sugar concentrations of stylet-sap and honeydew were estimated by the method of Morris (1948), and the total nitrogen and protein concentrations by the methods of Tompkins & Kirk (1942) and Shaw & Beadle (1949). Nessler's solution, Benedict's uric acid reagent, and the Murexide reaction were used to test for the presence of ammonia and uric acid in freshly excreted honeydew.

Applying the principle of the honeydew clock developed by Smith (1937) honeydew droplets excreted during 24 hr. by colonies of *T. salignus* feeding on intact willow stems were allowed to fall on rotating disks of filter paper. The positions of the honeydew spots on the paper disks were subsequently revealed by spraying the disks with 0·2% ninhydrin in 95% butanol, or with the benzidine reagent of Horrocks (1949), and heating them for 5–10 min. at 100°C. The colour intensity developed by the honeydew spots, furthermore, provided an index of the total amino-acid or sugar concentrations of the honeydew droplets excreted by an aphid colony within a 24 hr. period.

RESULTS

(*a*) *The exudation of stylet-sap*

Stylet-sap exuded from severed stylet-bundles provided they were embedded in turgid willow stems. Observations on the dependence of the exudation on turgor pressure have previously been recorded (Mittler, 1957). Stylet-sap failed to exude

from severed stylet-bundles if they were partially withdrawn from the stem in which they were embedded. This occasionally occurred if the aphids were disturbed before their proboscides were cut. Such failures were, however, entirely eliminated by anaesthetizing the feeding aphids with a gentle stream of carbon dioxide gas before attempting to cut their proboscides.

Although stylet-sap was generally collected from individual severed stylet-bundles for periods of only 5–8 hr. and occasionally for 24 hr., it was frequently shown that stylet-sap continued to exude for a further 2–3 days if the viscous pool of stylet-sap, which formed on the bark about an 'abandoned' severed stylet-bundle, was periodically mopped up with filter paper. On rare occasions stylet-sap ceased to exude after only a few minutes of exudation. Table 1 sets out the rate at which stylet-sap was collected continuously for 72 and 100 hr. from the severed stylet-bundle of a second- and of a third-instar nymph. It may be noted that the rate of exudation diminishes only slightly over these long periods. It has previously been shown that the rate of exudation from the severed stylet-bundles of adult apterous *T. salignus* is greater than that from the severed stylets of the nymphal instars (Mittler, 1957). Stylet-sap for chemical analyses was therefore primarily collected from the severed stylets of adult apterous aphids.

Table 1. *Stylet-sap exudation from the severed stylet bundle of a second and third instar nymph*

Instar	Period of exudation (hr.)	Total volume exuded ($mm.^3$)	Average rate of exudation ($mm.^3$/hr.)	Total sugar concentration (% w/v)
Second	0–25	18·14	0·73	8·60
	25–41	28·89	0·67	—
	41–72	49·34	0·66	8·50
Third	0–18	21·75	1·21	8·51
	18–35	19·35	1·14	8·43
	35–56	18·95	0·91	8·31
	56–76	17·80	0·89	8·38
	76–100	21·10	0·88	8·18

(*b*) *The nitrogen composition*

Aspartic acid, glutamic acid, serine, threonine, alanine, valine, leucine and/or isoleucine, phenyl alanine, asparagine, glutamine and possibly, γ-amino-butyric acid were found in stylet-sap (Mittler, 1953). The greatest concentrations of these amino-acids and amides were found in stylet-sap collected from the severed stylets of aphids which were beginning to colonize willow stems at the termination of the plants' dormancy when bud swelling was prominent; the total nitrogen concentration recorded at this time was 0·2% (w/v).

During bud burst, and subsequently when the first leaves appeared on the willows' branches, the concentration of the amino-acids listed above decreased; the total nitrogen concentration of the stylet-sap falling to 0·12% (w/v). Further

growth of the foliage was accompanied by a further reduction in the concentration of all the amino-acids and amides, as well as in the concentration of total nitrogen. This is in agreement with the results of Lindemann (1948).

During leaf 'maturity' (as used by Kennedy, Ibbotson & Booth (1950) to denote the developmental condition of the foliage between its initial growth and its ultimate senescence) only small amounts of aspartic acid, glutamic acid, glutamine and traces of asparagine were detected in stylet-sap; the total nitrogen concentration was less than 0·03% (w/v) at this time.

During leaf senescence, which was marked by a yellowing of the leaves and the formation of abscission layers at the base of their petioles, the amino-acid composition of stylet-sap resembled that collected from plants in active leaf development; the total nitrogen concentration of the stylet-sap rose to 0·13% (w/v). An abundance of all the amino-acids and amides listed was also detected in stylet-sap 1–2 weeks prior to the death of some of the plants.

No change was detected chromatographically in the number and concentration of the amino-acids and amides in hourly samples of stylet-sap collected during the first 10 hr. of exudation from the cut end of a single stylet-stump. Neither had the number and concentration of the amino-acids and amides changed appreciably after 24 hr.

The amino-acid and amide composition of the honeydew excreted by *T. salignus* feeding on a willow stem was always identical with that of stylet-sap collected at the same time from the same willow stem. Each amino-acid and amide was, however, present at a lower concentration in the honeydew than in the stylet-sap; the relative reduction in the concentration of each amino-acid and amide appeared to be proportionate. The amino-acid and amide composition of the honeydew therefore invariably reflected that of the stylet-sap throughout the seasonal development of the aphids' host plant.

Quantitative total nitrogen determinations showed that *T. salignus* absorb at least 55% of the nitrogenous matter they ingest. The same number and concentration of amino-acids and amide were found to be excreted by adult and by nymphal instars of *T. salignus*. Little or no variation in the intensity of the amino-acid/ninhydrin colour reaction of honeydew droplets which had been deposited on filter paper disks by a colony of *T. salignus* during a 24 hr. period was detected by visual inspection of the paper disks. No appreciable amounts of ammonia, uric acid, proteins, peptides or their breakdown products were qualitatively or quantitatively detected in samples of stylet-sap and honeydew.

The sap squeezed from the phloem tissues of *S. triandra* stems contained the same amino-acids and amides as those found in stylet-sap collected from the same stems. The amino-acid spots on chromatograms of the expressed sap were, however, considerably less intense than those on chromatograms of the stylet-sap, and were partly obscured by peptides. It is interesting to note that the amino-acid composition of the honeydew excreted by coccids, *Eulecanium corni* (Bouché), colonizing the *S. triandra* stems closely resembled that of the honeydew excreted by *T. salignus* feeding on the same plant.

Four to five days after girdling *S. fragilis* branches on which small scattered

colonies of *T. salignus* were feeding, most of the aphids had aggregated to feed immediately above each girdle, were they formed dense colonies 3–4 in. in extent. The nitrogen concentration of the honeydew excreted by these aphids was ten times higher than that excreted by aphids remaining on the branches below the girdles or by those on intact branches of the same tree. Leaves above the girdles rapidly turned yellow and formed abscission layers, as in normal senescence.

(*c*) *The sugar composition*

While the willow trees were bearing leaves sucrose was the only sugar in stylet-sap. The honeydew excreted by *T. salignus*, on the other hand, was found to be composed of roughly equal amounts of sucrose, glucose, fructose and a non-reducing oligosaccharide. The latter was identified as the trisaccharide melezitose; its chromatographic properties being identical to those of an authentic sample of melezitose prepared by the late Dr C. S. Hudson. Glucose and fructose were the only sugars detected in acid hydrolysates of stylet-sap and honeydew.

During the first few days after the aphids had begun to feed on willows whose dormancy had been broken and whose buds were swelling, traces of four non-reducing oligosaccharides were detected in the stylet-sap in addition to sucrose. The honeydew excreted by the aphids at this time contained these oligosaccharides apparently unchanged in quantity and quality, in addition to sucrose, glucose, fructose and melezitose.

The total sugar concentration of stylet-sap obtained from willows kept in the greenhouse was found to lie between 5 and 10 % (w/v) and to be at least 90 % (w/w). The total sugar concentration of stylet-sap obtained from willows which were kept in a room with only a weak illumination and which were colonized by several hundred *T. salignus*, however, gradually fell to almost 1 % (w/v) during 2–3 weeks. No attempt was made to correlate the total sugar concentration of the stylet-sap with the seasonal development of the host plant. The total sugar concentration of the honeydew did not differ by more than 5 % from that of the stylet-sap. The total sugar concentration of the stylet-sap changed only very slightly during its continuous exudation from one and the same stylet-stump (Table 1). No variation in the intensity of the benzidine/sugar colour reaction on filter paper disks of honeydew droplets which had been excreted during a 24 hr. period by a colony of *T. salignus* was detected by visual inspection of the paper disks.

DISCUSSION

Tóth (1946) claimed that the symbiotic micro-organisms of aphids fix atmospheric nitrogen, and that aphids consequently excrete larger amounts of nitrogenous matter than they ingest. If this were the case aphid honeydew should contain nitrogenous materials differing from those ingested. The results of the present investigation indicate, however, that the amino-acids and amides excreted by *T. salignus* are not the products of atmospheric nitrogen fixation, but that each of the amino-acids and amides is ingested in its free form and at a higher concentration

than that at which it is excreted. As these findings did not entirely rule out the possibility that the aphids are fixing atmospheric nitrogen through the agency of their symbionts, a closer examination of this subject was undertaken and will be reported elsewhere (Mittler, in preparation).

A melezitose content of 46·3% (w/w), in the honeydew of *Lachnus roboris* L. was reported by Michel (1942), who did not detect the trisaccharide in phloem sap obtained from the aphid's host plant, and who concluded that the trisaccharide is a by-product of the aphid's digestive processes. The occurrence of considerable amounts of melezitose in the freshly excreted honeydew of *T. salignus*, and its absence in stylet-sap, is further evidence that this trisaccharide is synthesized within the bodies of these lachnids. Gray & Fraenkel (1953) have suggested that the trisaccharide fructomaltose may be expected to arise in the digestive system of any animal that possesses invertase and utilizes sucrose in its diet. Duspiva (1953), Bacon & Dickinson (1957) and Wolf & Ewart (1955) have, furthermore, demonstrated that enzymes, present within the bodies of aphids and coccids, and in their honeydew, are capable of an *in vitro* synthesis of melezitose and other oligosaccharides from sucrose. The fact that considerable amounts of oligosaccharides are present in honeydew excreted by aphids and coccids indicates that these compounds are not merely transitory intermediate products formed during the hydrolysis of dietary sucrose, but that they are purposefully synthesized within the insects' bodies. The significance of these chemical changes has, however, remained obscure. The possibility that intracellular micro-organisms, which occur in the mid-gut epithelium of aphids (Schanderl, Lauff & Becker, 1949), or that enzymes, which occur in phloem sap (Wanner, 1953*b*; Zimmermann, 1954), are involved in these carbohydrate changes should not be overlooked.

Yust & Fulton (1943) claimed that the sap which exudes from the broken ends of the embedded stylet-bundles of *Aonidiella aurantii* (Mask.) is undoubtedly the coccid's food. The results of the present and previous investigation (Mittler, 1957) leave little doubt that stylet-sap is identical with the sap normally ingested by *T. salignus*. The question has been raised, however, whether stylet-sap is identical with the unchanged phloem sieve-tube sap of the aphid's host plant (Duspiva, 1954). As this question is of considerable importance for plant physiological investigations as well as for further aphid nutritional studies, which may make use of the stylet cutting technique, the chemical evidence related to this question will briefly be discussed.

Zweigelt (1914), Davidson (1923) and Bramstädt (1948), have suggested that aphids inject carbohydrases into their host plant to hydrolyse starch or other insoluble carbohydrate matter, and that the breakdown products pass up their stylets. The absence of maltose or other reducing sugar in stylet-sap indicates that its sugar content is not the result of such digestive processes. The almost exclusive occurrence of sucrose in stylet-sap indicates that sucrose is a natural constituent of the phloem sap of willow stems. Sucrose has in fact been shown to predominate in the phloem sap of large numbers of other plants (Wanner, 1953*a*; Ziegler, 1956; Zimmermann, 1957) and has also been shown to occur in the exudate from the

broken stylet-bundles of *Aonidiella aurantii* (Yust & Fulton, 1943). As the sucrose concentration and the rate of exudation of stylet-sap falls off only slowly during 3 days of continuous exudation from a single severed stylet-bundle which is embedded in a turgid willow stem, the exudation cannot be the result of temporary physico-chemical changes which aphids have been supposed to induce within their host plant (Zweigelt, 1914). It has previously been suggested (Mittler, 1957) that the normal turgor pressure of the sieve-tube sap and the large capacity of the phloem is responsible for maintaining the exudation.

The fact that the amino-acid and amide composition of the stylet-sap does not change appreciably during 24 hr. of continuous exudation from a single severed stylet-bundle also shows that proteolytic enzymes, which Bramstädt (1948) has demonstrated in aphid salivary glands, are not hydrolysing plant proteins to give rise to the amino-acids found in stylet-sap. The excretion by *Eulecanium corni* and *T. salignus* of the same amino-acids, suggests that these insects are ingesting sap of the same composition. The results of chromatographic analyses of the sap squeezed from the phloem tissues of willow stems, indicate that the amino-acids and amides found in stylet-sap are in fact present in solution in their free form in the plants' phloem tissues. The fact that the concentrations of amino-acids and amides as well as of sucrose in stylet-sap is maintained over long periods of exudation indicates that water, amino-acids, amides and sucrose pass *en masse* through the sieve-tubes of a willow stem towards the sieve-tube tapped by the stylets.

It is interesting to note that a single *T. salignus* ingesting 10–40 mm.^{3} per day of a phloem sap having a sucrose concentration of 10% (w/v) imposes a sucrose drain of 1–4 mg. per day on its host plant. As 2 mg. of sucrose may be the approximate photosynthetic product of 100 cm.^{2} of leaf per hr. (Spoehr, 1926) the drain imposed on the plant by one aphid per day is equivalent to the amount of carbohydrate a leaf area of 50–200 cm.^{2} may photosynthesize per hr. If photosynthesis were to take place for 10 hr. per day the aphid would ingest the photosynthetic product of 5–20 cm.^{2} of leaf per day.

The seasonal fluctuations in the amino-acid composition and total nitrogen content of the stylet-sap strongly suggest that stylet-sap is identical with the sieve-tube sap of an aphid-free plant. These fluctuations are, moreover, in accordance with plant physiological concepts of the mobilization and conservation of nitrogenous matter by a plant during the growth and senescence of its foliage (Kostytschew, 1931). Kennedy *et al.* (1950) stated that 'it is through a study of the developmental physiology of plants, with special reference to the phloem sap which is the aphids' food, that we may eventually hope to explain aphid distribution.' One may speculate that the recorded fluctuations in the amino-acid composition of stylet-sap are partly responsible for the changing suitability to aphids of plants, or parts of plants, in different developmental conditions.

SUMMARY

1. The aim of this investigation has been to determine the sugar and nitrogen composition of the phloem sieve-tube sap ingested by *Tuberolachnus salignus* (Gmelin) feeding on *Salix acutifolia* stems, and to compare it with that of the honeydew excreted by the aphids.

2. A cage suitable for confining *T. salignus* on the willow stems is described.

3. Details are given of a technique, outlined by Kennedy & Mittler (1953), for collecting the fluid, termed stylet-sap, which exudes from the cut end of severed embedded stylet-bundles.

4. A method is described for collecting honeydew droplets immediately they are excreted by feeding *T. salignus*.

5. The nitrogenous matter ingested by *T. salignus* is in the form of free amino-acids and amides. The same amino-acids and amides are ingested but in greater amounts than they are excreted.

6. The number and concentration of the amino-acids and amides in stylet-sap and honeydew fluctuate with the seasonal development of the host plant.

7. The honeydew contains sucrose, fructose, glucose and melezitose. These sugars are derived from sucrose, the only sugar normally ingested.

8. The evidence for the identity of stylet-sap with the unchanged sieve-tube sap of the host plant is discussed.

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