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Chemical Nature of the Sheath Materials Secreted by Leafhoppers (Homoptera)

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The chemical nature of the sheath materials produced by the four species of leafhopper, *Nephotettix cincticeps* UHLER (Deltocephalidae), *Tettigella viridis* LINNÉ (Tettigellidae), *Laodelphax striatellus* FALLÉN (Delphacidae), and *Nilaparvata lugens* STÅL (Delphacidae) was studied. Histochemical tests indicated that the sheath materials of the leafhoppers were mainly lipoproteinaceous. There was also some evidence for the precence of a certain type of neutral mucosubstance, especially in the sheath materials of *N. cincticeps* and *T. viridis*. Pectic substance and chitin appeared to be absent. The results were compared with those of previous workers.

INTRODUCTION

The salivary secretions of many species of phytophagous Hemiptera are responsible for various pathological conditions on plants, otherwise they act also as vehicles for many plant viruses (CARTER, 1962). A fraction of the salivary secretions is known as "*sheath material*" which solidifies and forms so-called "*stylet sheath*" around the puncture made by the insect's mouth parts within the plant tissues. The morphological description has been made on the stylet sheaths produced by various species of Hemiptera by many investigators (e.g. DAVIDSON, 1923; SMITH, 1926; BENNETT, 1934; STOREY, 1938; PUTMAN, 1944; SORIN, 1960). Once the nature and origin of the stylet sheath were the subject of some speculation: for example, PETRI (1909) and DAVIDSON (1923) have thought that the sheaths produced by aphids are a precipitate of plant substances such as callose, calcium pectate and tannin. However, the evidence that the sheath is deposited even in artificial liquid media has offered the proof that it is solely made of the insect's oral secretion (FIFE, 1932; BENNETT, 1934; STOREY, 1939). SMITH (1933) has shown that the sheath materials of two species of leafhoppers, *Empoasca fabae* (HARRIS) and *Strictocephala festina* (SAY), are largely proteinaceous and may contain chitin, but no plant substance with a possible exception of pectose. Recently, MILES (1960) has reported that the sheath material of a plant bug, *Oncopeltus fasciatus* (DALL.) contains lipoprotein, but not chitin and carbohydrate.

The present study was undertaken in order to obtain further knowledge of the chemical properties of the sheath materials produced by four species of leafhoppers, *Nephotettix cincticeps* UHLER (Deltocephalidae), *Tettigella viridis* LINNÉ Tettigellidae), *Laodelphax striatellus* FALLÉN (Delphacidae), and *Nilaparvata lugens* STÅL (Delphacidae).

METHOD FOR COLLECTION OF THE SHEATH MATERIAL

The experiments were all carried out using the stylet sheaths which were formed on a polyethy lene membrane (18 μ in thickness) when the leafhoppers fed 2% sucrose solution penetrating the membrane. For the collection of the sheaths an apparatus illustrated in Fig. 1 was devised. A number of adult leafhoppers were confined within a feeding chamber of the apparatus, and allowed to suck the sucrose solution which filled in a sachet made of the polyethylene membrane. After several days, the membrane was removed from the apparatus, rinsed carefully in the tap water, and dried in the air.

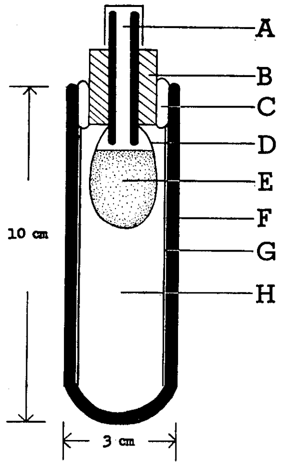

Fig. 1. The simple apparatus for collection of stylet sheaths of leafhoppers. (A) glass tube, (B) rubber plug, (C) absorbent cotton, (D) polyethylene membrane, (E) 2% sucrose solution, (F) glass vessel, (G) filter paper, (H) feeding chamber.

NOTE ON THE STYLET SHEATHS FORMED ON THE POLYETHYLENE MEMBRANE

The stylet sheaths left on the membrane were generally colorless and plastic. However, pale amber sheaths were sometimes found among the sheaths produced by *N. cincticeps* and *T. viridis*. The leafhoppers secreted the sheath material not only within the sucrose solution, but also onto the external surface of the membrane, and formed characteristic marks at the point of penetration (Fig. 2-1, 2, and 3). The mark was round in outline, its diameter was about 180 μ in *T. viridis*, about 70 μ in *N. cincticeps*, and less than 30 μ in both *L. striatellus* and *N. lugens*. The stylet sheaths formed within the sucrose solution by *N. cincticeps* and *T. viridis* were thick-walled single tube, being 30-70 μ and 70-150 μ in length respectively (Fig. 2-1). On the other hand, *L. striatellus* and *N. lugens* formed the sheaths of thin-walled elongated tube which was occasionally digitated at the proximal part and reached about 300 μ in length (Fig. 2-3 and 4). As a rule, the

Fig. 2. The stylet sheaths formed within 2% sucrose solution, and the penetration marks (M) left on the outer surface of the polyethylene membrane.
1. Single thick-walled tubular sheaths and round penetration marks produced by *N. cincticeps*, stained with methyl green-pyronin method (×185).
2. Penetration marks produced by *T. viridis* and their openings (O) made by the insect's mouth parts, stained with 1% eosin (×130).
3. Elongated thin-walled tubular sheaths and penetration marks deposited by *L. striatellus*, treated with Millon's reagent (×135).
4. Digitated sheaths formed by *N. lugens*, stained with 1% eosin (×195).

inner border of the sheaths was smooth, but the outer surface was irregularly undulated.

CHEMICAL REACTIONS OF THE SHEATH MATERIAL

To examine the chemical nature of the sheath material, various histochemical techniques were applied to the stylet sheaths deposited on the polyethylene membrane. Unless otherwise stated, details of the tests employed here can be found in Lison (1960). The results of the tests for each group of substances were summarized in Table 1 to 4.

Protein tests: The sheaths of the four species of leafhopper were colored in pink with biuret reaction, indicating the presence of peptide linkages. When subjected to ninhydrin, the sheaths of *N. cincticeps* and *T. viridis* were intensely colored in purplish blue. This indicated the occurrence of abundant amino acid residues with free carbonyl and α-amino radicals. On the other hand, those of *L. striatellus* and *N. lugens* never reacted with ninhydrin. The positive xanthoproteic reaction is due to the presence of molecules with benzene nuclei, such as tyrosine, tryptophane, phenylanine, and other aromatic compounds. Among them, the sheath materials produced by the four species contained commonly an appreciable quantity of tyrosine, this was based on a positive Millon's reaction. The result with Romieu's test indicated that the sheath materials of *N. cincticeps* and *T. viridis* might contain tryptophans, but those of *L. striatellus* and *N. lugens* did not. When Sakaguchi's test for arginine was applied, the sheaths of *N. cincticeps* and *T. viridis* showed positive coloration. However, the sheath of *L. striatellus* was negative. The positive alkaline tetrazolium test in the sheath materials of the four species was indicative of the occurence of sulphur-containing amino acids, cystein and/or cystine.

Table 1. Tests for Protein and Amino Acids in the Sheath Materials Produced by *N. cincticeps*, *T. viridis*, *L. striatellus*, and *N. lugens*

Reagent or test	*N. cincticeps*	*T. viridis*	*L. striatellus*	*N. lugens*
Biuret	●	●	●	●
Ninhydrin	●	●	○	○
Xanthoproteic[a]	●	●	●	●
Millon's	●	●	●	●
Romieu's[b]	◉	◉	○	○
Sakaguchi's	●	●	○	
Alkaline tetrazolium	●	●	●	●

● positive ◉ weak or trace ○ negative

[a] The sheath materials were treated with fuming nitric acid for several minutes, washed with distilled water, and then dipped into 10% sodium hydroxide solution.

[b] The sheath materials were mounted with sirup of concentrated phosphoric acid, and incubated at 56°C for several minutes.

Lipid tests: When treated with Sudan black B, the sheaths of the four species were stained in bluish black or black. They also absorbed definite color from saturated alcoholic solution of Sudan III. However, the sudanophilic property was not affected by ethyl alcohol, acetone, or pyridine extraction, but weakened by ethyl ether-ethyl alcohol (4 : 1 v/v) extraction in the sheath of *N. cincticeps*. With Nile blue B the sheaths were stained in blue in all cases. This coloration may indicate the absence of neutral lipids such as glycerides and cholesterides. If these lipids were sufficiently contained, the staining would give pinkish color. The sterols were detected by neither Schultz's nor Romieu's test. Also the performic acid-Schiff test for unsaturated lipids was negative in the sheaths excepting the sheath of *T. viridis* which was slightly colored. From these results, it is evident that the sudanophilic property of the sheaths indicated the presence of a certain lipid, which has no characteristics of glyceride, steroid, and probably unsaturated lipids. It is also likely that the insolubility to fat solvent is due to the stable linkage to protein.

Table 2. Tests for Lipids in the Sheath Materials Produced by *N. cincticeps*, *T. viridis*, *L. striatellus*, and *N. lugens*

Reagent or test	*N. cincticeps*	*T. viridis*	*L. striatellus*	*N. lugens*
Sudan III	●	●	●	●
Sudan black B	●	●	●	●
after acetone extraction	●	●	●	●
after pyridine extraction	●	●	●	●
after ether-alcohol (4 : 1) extraction	◉		●	
Nile blue B	blue	blue	blue	blue
after pyridine extraction	blue	blue	blue	blue
Schultz's	○	○	○	○
Romieu's[a]	○	○	○	○
PEAS	○	◉	○	○

[a] Concentrated sulfuric acid was first dropped on the sheath materials, and after a while some drops of glacial acetic acid were added on them.

Carbohydrate tests: The sheaths of *N. cincticeps* and *T. viridis* gave clear positive reaction to Molish test for carbohydrates, and those of *L. striatellus* and *N. lugens* were weakly positive. The sheaths used in this test were washed thoroughly with both water and ethyl alcohol in order to remove the adventitiously contaminated sugar from dietary liquid. The sheath of *N. cincticeps* reacted with Anthrone reagent, but that of *L. striatellus* did not. Also in UV-tungstate test for polysaccharides, the sheaths of *N. cincticeps* and *T. viridis* showed weak but definite positive coloration, but those of *L. striatellus* and *N. lugens* were quite negative. With various techniques employing Schiff's reagent after oxidation, such as PAS, Bauer's, and Casella's reactions, the sheaths of both *N. cincticeps* and *T. viridis* reacted always positively, while those of *L. striatellus* and *N. lugens* were weakly positive or frequently negative. To ascertain whether the positive reactions indicated carbohydrate or not, the acetylation procedure was used. If the PAS-positive reaction of the sheaths is due to carbohydrates, it becomes negative after acetylation, and then becomes positive again with subsequent saponification. The PAS-positive reactivity of the sheaths was not affected with acetylation, and therefore the confirmation for carbohydrate was not obtained. The PAS-positive reaction was also unaffected by pyridine extraction procedure. When the sheaths of the four species were placed in 0.05% toluidine blue solutions buffered at pH 2.5, 4.1, and 7.0, they did not show metachromasis, indicating the absence of acid mucopolysaccharides. On the basis of the above-mentioned reactions of the sheaths, it was indicated the presence of carbohydrate, possibly a certain neutral mucosubstance, in the sheath materials of *N. cincticeps* and *T. viridis*. Such substance may be also contained in the sheath materials of *L. striatellus* and *N. lugens*.

Table 3. Tests for Carbohydrates in the Sheath Materials Produced by *N. cincticeps T. viridis L. striatellus*, and *N. lugens*

Reagent or test	*N. cincticeps*	*T. viridis*	*L. striatellus*	*N. lugens*
Molish[a]	●	●	◉	◉
UV-tungstate	●	●	○	○
Anthrone[b]	●		○	
Toluidine blue (pH 2.5, 4.1, 7.0)	blue	blue	blue	blue
PAS	●	●	◉	◉
after acetylation	●	●	◉	◉
Bauer's	●	●	○	○
Casella's	●	●	●	●

[a] The sheath materials were covered with a drop of 4% alpha-naphtol in ethanol and concentrated sulfuric acid, and warmed by steam for 3 minutes.

[b] The sheath materials were covered with a drop of 2% Anthrone reagent in 75% sulfuric acid, and warmed by steam for several minutes.

Other tests: The sheaths of *N. cincticeps* and *T. viridis* were stained with 0.02% ruthenium red solution, but this staining property was not destroyed by the pretreatment with 1N hydrochloric acid at 60°C for 30min. Therefore, the affinity of these sheaths for ruthenium red was not an indication of the presence of pectic substances. The sheaths of *L. striatellus* and *N. lugens* were not stained with this dye. The sheath materials of the four species showed negative result with iodine-zinc chloride test for chitin, and dissolved in 1N potassium hydroxide at 60°C within 24 hours. The sheaths of *N. cincticeps* and *T. viridis* gave positive Feulgen reaction for deoxyribonucleic acid, but not stained in blue with methyl green-pyronin method. Both argentaffin and nitrosophenol reaction for polyphenols were negative in the sheaths of the four species. The sheaths were also never reacted with Schiff's reagent without prior oxidation treatment, indicating the absence of free aldehyde groups. From these results, it is apparent that the sheath materials produced by the four species do not contain pectic substances, chitin, nucleic acids, polyphenols and free aldehyde groups.

Table 4. TESTS FOR CHITIN, PECTIC SUBSTANCES, NUCLEIC ACIDS, POLYPHENOLS, AND ALDEHYDE GROUPS IN THE SHEATH MATERIALS PRODUCED BY *N. cincticeps*, *T. viridis*, *L. striatellus*, AND *N. lugens*

Reagent or test	*N. cincticeps*	*T. viridis*	*L. striatellus*	*N. lugens*
Iodine-zinc chloridae	○	○	○	○
Ruthenium red[a]	○	○	○	○
Feulgen	◉	●	○	○
Methyl green-pyronine	red	red	red	red
Argentaffin	○	○	○	○
Nitrosophenol[b]	○	○	○	○
Schiff	○	○	○	○

[a] The sheath materials were stained with 0.02% ruthenium red in water befour and after treatment with 1N hydrochloric acid at 60°C for 30 minutes.

[b] The sheath materials were covered with a drop of glacial acetic acid and 10% sodium nitrite solution. After several minutes, the solutions were removed with filter paper, and the sheath materials were then treated with 10% sodium hydroxide solution.

SOME COMPARATIVE ASPECTS OF THE CHEMICAL NATURE OF SHEATH MATERIALS

The results obtained in the present study were compaired mainly with those by SMITH (1933) and MILES (1960). They were summarized in Table 5.

The proteinaceous nature of the sheath materials has been demonstrated in various species of Hemiptera. BÜSGEN (1891) has reported a positive biuret and Millon's reaction in the stylet sheaths of *Aphis brassicae* and *Coccus cacti.* HORSFALL (1923) has also reported that the sheaths produced by certain aphids give a positive xanthoproteic reaction. In the sheaths of leafhoppers, *Empoasca fabae* and *Strictocephala festina,* SMITH (1933) has obtained positive biuret, ninhydrin, and xanthoproteic reactions. In addition, he demonstrated the presence of tryptophane, tyrosine, and arginine using several color reactions characteristic of such amino acids. DAY et al. (1952) have applied the Millon's reaction in order to find out the stylet sheaths formed in the plant tissues by six species of leafhopper. Moreover, MILES (1960) has shown that the sheath material of a plant bug, *Oncopeltus fasciatus,* contains protein which is rich in tyrosine and contains trace of tryptophane. In the present study, the occurrance of protein was confirmed in the sheaths of the four species of leafhopper.

SMITH (1933) has reported that sheaths produced by *E. fabae* and *S. festina* are stained by neither of the dyes Sudan III and Scharlach R, nor osmic acid; but in the present experiments, those of the four species of leafhopper were all stained with Sudan black B and Sudan III. Miles (1960) has also indicated the presence of lipid in the sheath material of *O. fasciatus* by the evidence that when heated gently in concentrated nitric acid saturated with potassium chlorate, the material dissolves and unmistakable sudanophilic globules appears to be produced, although he has been unable to obtain definite positive result with Sudan dyes. Further he has considered that the lipid contained in the sheath material is a steroid on the basis of the positive Liebermann-Burchard's test. Miles (1961) has also extracted phospholipid from the precursor of sheath material of *Eumecops australasiae*.

Table 5. Chemical Composition of the Sheath Materials Secreted by Several Species of Hemiptera

Species examined	Protein	Lipid	Chitin	Pectic substance	Muco-substance
Empoasca fabae (Harris) (Cicadellidae)[a] *Strictocephala festina* (Say) (Membracidae)[a]	●	○	◉	◉	○
Nephotettix cincticeps Uhler (Deltocephalidae) *Tettigella viridis* Linné (Tettigellidae)	●	●	○	○	●
Laodelphax striatellus Fallén (Delphacidae) *Nilapalvate lugens* Stål (Delphacidae)	●	●	○	○	◉
Oncopeltus fasciatus (Dall.) (Lygaeidae)[b]	●	●	○	○	○

● Presence, ◉ Possible presence, ○ Absence. [a] Smith (1933), [b] Miles (1960).

In the present study, Molish test for carbohydrates was positive in the sheaths of the four species of leafhopper, and there were also some indicative evidence that a certain type of neutral mucosubstance was contained in the sheaths, particularly in those of *N. cincticeps* and *T. viridis*. In the sheath material of *O. fasciatus*, Miles (1960) has concluded that carbohydrate is absent on the basis of negative Molish test and anomalous PAS reaction. This conclusion, however, may arise some question, because Miles (1960 a) has detected carbohydrate in the contents of the anterior lobe of the principal salivary gland, which has been considered as a precursor of the sheath material. Salkeld (1960) has also suggested that the secretion of the anterior lobe of *O. fasciatus* is composed of muco- or glyco-protein and neutral mucopolysaccharide. These conflicts may be reconciled by the following explanation. Bronskill et al. (1958), Miles (1960 a), and Salkeld (1960) have shown that the contents of the anterior lobe of *O. fasciatus* is composed of two fractions; and Miles (1960 a) has noted that the one fraction is water-dispersible. Therefore, if carbohydrate is contained in this fraction, it will be eliminated from the sheath material in the process of coagulation. However, the anomalous PAS reaction in the sheath materials of the leafhoppers examined here remains still problematic.

Pectic substance and chitin which had been suggested to be present in the sheaths of leafhopper by Smith (1933) could not be detected in the present study. Miles (1960) has also shown the absence of such substances in the sheath of *O. fasciatus*. He has further demonstrated that the pectic substance does not diffuse into the stylet sheath deposited within the plant tissue.

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