

Studies on the Salivary Glands of Rice Plant Leafhoppers

II. Origins of the Structural Precursors of the Sheath Material

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Localization of the structural precursors of sheath material in the salivary glands of *Nephotettix cincticeps* UHLER and *Laodelphax striatellus* FALLÉN was investigated. In *N. cincticeps*, the sheath material originates as two distinct secretions, protein and probable mucolipid, produced respectively in the IV- and V- cells in the principal gland. In *L. striatellus*, the precursors, protein and unsaturated lipid, elaborated in the A- and coupled G- and H-follicles of the principal gland respectively. In addition, a certain mucosubstance in the accessory gland may enter into the sheath material.

INTRODUCTION

It has previously been found that the salivary glands of leafhoppers are complicated in structure and consist of several kinds of secretory cells; and the probability of a complex of secretory products has been suggested (DOBROSKY, 1931; NASU, 1963; SŌGAWA, 1965). This seems to be connected with the fact that hemipterous insects eject at least two types of secretion from the stylet bundle, viz. sheath material and watery saliva (STOREY, 1939; DAY et al., 1952; MILES, 1959, 1959a). However, the precise functions of the salivary glands, and the nature of their secretions have not satisfactorily resolved hitherto, excepting the glands of a plant-sucking bug, *Oncopeltus fasciatus* (DALLAS) (MILES, 1960a). These subjects are of considerable importance for the further investigations on the feeding mechanism of sucking insects with special relationship to the production of plant diseases, and on the transmission of virus diseases by these insects.

The present study relates the histochemical properties of the different secretory cells of the salivary glands of leafhoppers to the origin of the sheath material, the chemical nature of the latter has already been studied (SMITH, 1933; MILES, 1960; SŌGAWA, 1967).

MATERIAL AND METHOD

The adults of *Nephotettix cincticeps* UHLER (Deltocephalidae) and *Laodelphax striatellus* FALLÉN (Delphacidae), which had successively been reared on rice seedlings in a constant temperature cabinet, were used for the present study. Unless otherwise stated, histochemical tests were performed upon the whole salivary glands fixed briefly in 10% formalin containing 0.8% sodium chloride.

The detailed procedures for almost all histochemical tests use herefollowed those of LISON (1960).

RESULTS

The results were assembled in Table 1 to 3 and Figs. 1 and 2. The anatomical terms are the same as those used in the earlier paper (SŌGAWA, 1965).

Protein tests. When the salivary glands were treated with 0.2% boiling ninhydrin solution for some minutes, the II- and IV-cells of *N. cincticeps* and the A-follicle of *L. striatellus* were colored violet blue. Of them the IV-cells and A-follicle were tanned to light brown by means of treatment with 0.1M benzoquinone solution for 1 hr at 37°C. With Millon's reagent these tissues were also intensely stained pink or red within 3 hr at room temperature. A positive Ehrlich's reaction was obtained in the IV-cells and A-follicle. In addition, the IV-cells gave strongly positive rosindole and xanthydroxol reactions, giving violet shades. These reactions in the A-follicle was apparently weaker than those in the IV-cells. Moreover, the IV-cells and A-follicle were able to reduce neotetrazolium in alkaline condition (pH 8.5–8.8) after treatment with thioglycerol. In this test the III-cells of *N. cincticeps* and the G- and H-follicle of *L. striatellus* reacted to a reduced extent. When the DDD (2, 2'-dihydroxy-6, 6'-dinaphtyldisulfide) method was applied alone on the salivary glands fixed in 5% trichloroacetic acid for 15 min, the IV-cells and A-follicle gave yellowish orange coloration. This reaction was not affected by pretreatments with iodine-iodide solution (0.0038% I+0.0033% KI) and 0.1 M ethylmaleimide solution for 4 hr at 37°C, nor with 10% potassium cyanide solution for 4 hr at room temperature.

Lipid tests. The V-cells of *N. cincticeps* were deeply stained in black with Sudan black B, and the III-cells in various degree of blue. When the fresh glands were immediately treated with 1% mercuric chloride solution for 3 min and followed by Schiff's reagent for 15 min, a reddish color developed in the V-cells alone (plasmal reaction). The III-cells were occasionally gave a purplish shade with Nile blue B (chloride), and showed Schiff positive reaction with prior treatment of performic acid reagent or after irradiation with ultraviolet rays for 5 hr. In *L. striatellus* the G- and H-follicle showed definite affinity for Sudan black B, usually the latter being more intense. These follicles rarely took up purplish color after staining with Nile blue B, and gave positive performic acid-Schiff and ultraviolet rays-Schiff reactions. When treated with 20% ferric chloride solution for about 20 min and mounted with a drop of solution prepared by mixing equal volumes of glacial acetic acid and concentrated sulfuric acid (Schultz's test), no characteristic coloration occurred in any part of the glands of both the species.

Carbohydrate tests. In the salivary glands of *N. cincticeps*, an intense PAS-reaction was found in the V-cells, and weaker one in the II- and III-cells. By extraction with boiling methanol-chloroform mixture (1:1 v/v) for 3 hr, the PAS-positivity of the III-cells was abolished, but that of the II- and V-cells was retained. After acetylation procedure (60°C, 48 hr), the V-cells still maintained the reactivity, while the II-cells became negative. Also the III-cells exhibited stronger Schiff-positive reactions after treatment with 4% chromic acid for 1 hr (Bauer's reaction) and 0.5% potassium permanganate for 5 min (Casella's

Table 1. HISTOCHEMICAL REACTIONS FOR PROTEINS AND AMINO ACIDS IN THE SALIVARY GLANDS OF *N. cincticeps* AND *L. striatellus*

Reagent or test	<i>N. cincticeps</i>						<i>L. striatellus</i>									
	I	II	III	IV	V	VI	A	B	C	D	E	F	G	H	A	G
Ninhydrin	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Benzoquinone	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Millon's	-	±	-	##	-	-	##	±	-	-	-	-	-	-	-	-
Ehrlich's	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-
Rosindole	-	-	-	##	-	-	+	-	-	-	-	-	-	-	-	-
Xanthidrol	-	-	-	##	-	-	+	-	-	-	-	-	-	-	-	-
Alkaline tetrazolium	-	-	+	+	-	-	+	-	-	-	-	-	-	-	+	+
DDD	-	-	-	±	-	-	±	-	-	-	-	-	-	-	-	-
DDD after iodine treatment	-	-	-	±	-	-	±	-	-	-	-	-	-	-	-	-
DDD after ethylmaleimide treatment	-	-	-	±	-	-	±	-	-	-	-	-	-	-	-	-
DDD after potassium cyanide treatment	-	-	-	±	-	-	±	-	-	-	-	-	-	-	-	-

+ Positive, ± Trace, - Negative. Number of positive signs indicates relative intensity of reaction.

Table 2. HISTOCHEMICAL REACTIONS FOR LIPIDS IN THE SALIVARY GLANDS OF *N. cincticeps* AND *L. striatellus*

Reagent or test	Substance detected	<i>N. cincticeps</i>						<i>L. striatellus</i>								
		I	II	III	IV	V	VI	A	B	C	D	E	F	G	H	AG
Sudan black B	Lipids	-	-	+	-	##	-	-	-	-	-	-	-	##	##	-
Nile blue B (pink staining)	Neutral lipids	-	-	±	-	-	-	-	-	-	-	-	-	-(±)	-(±)	-
Performic acid-Schiff	Unsaturated lipids	-	-	+	-	-	-	-	-	-	-	-	-	##	##	-
UV-Schiff	Unsaturated lipids	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Plasmal	Plasmalogen	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Schultz's	Sterols	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. HISTOCHEMICAL REACTIONS FOR CARBOHYDRATES IN THE SALIVARY GLANDS OF *N. cincticeps* AND *L. striatellus*

Reagent or test	Substance detected	<i>N. cincticeps</i>						<i>L. striatellus</i>									
		I	II	III	IV	V	VI	A	B	C	D	E	F	G	H	AG	
PAS	Carbohydrates (unsaturated lipids)	-	+	+	-	##	-	-	-	-	-	-	+	-	##	##	##
PAS after lipid extraction		-	+	-	-	##	-	-	-	-	-	-	+	-	-	-	##
PAS after acetylation		-	-	+	-	##	-	-	-	-	-	-	+	-	-	-	##
Bauer's	Carbohydrates	-	-	+	-	-	-	-	-	-	-	-	-	-	##	##	-
Casella's	Carbohydrates (unsaturated lipids)	±	±	+	±	±	±	±	±	±	±	±	±	±	##	##	±
Toluidine blue (metachromasia)	Acid mucopolysaccharides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alcian blue 8GS	Acid mucopolysaccharides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

reaction). The G- and H-follicle and the accessory gland of *L. striatellus* were intensely PAS-positive. The E-follicle was also weakly positive. The reaction of the accessory gland and E-follicle was not removed by acetylation nor by methanol-chloroform extraction, whereas that of the G- and H-follicle disappeared completely by both the treatments. Intense positive reactions occurred in the G- and H-follicle with the Bauer's and Casella's techniques. The aldehyde blocking agents, aniline and dimethylcyclohexanedion, which were employed between periodic acid and Schiff's reagent destroyed all of the PAS-reaction. No metachromasia was demonstrated on the salivary glands of both the species with 0.1 % toluidine blue solutions buffered to pH 2.4 and 4.1. Besides, no positive staining occurred with 1 % Alcian blue 8GS solution at pH 2.4.

DISCUSSION

DOBROSKY(1931) has first found that the principal salivary gland of a leafhopper, *Cicadela sexnotata* (FALLEN), consists of five kinds of secretory cells; recently NASU (1963) has confirmed the same fact in the gland of *Nephotettix* sp. (later identified as *N. apicalis* MOTSCHULSKY). These investigators have also described that some kinds of secretory cells of the gland serve as a mucous gland, and others as a serous one. This information is noteworthy with regard to the evidence that hemipterous insects secrete two types of saliva, namely the sheath material and the watery saliva (STOREY, 1939; DAY et al., 1952; MILES, 1959, 1959a). In the previous paper (SŌGAWA, 1965), an attempt has been made to find out which kinds of cells or follicle in the salivary glands of rice plant leafhoppers produce the sheath material; and on the basis of cytological appearance the V-cells of the principal gland of *N. cincticeps* and the accessory gland of *L. striatellus* have been postulated as probable sources of the sheath material. However, this suggestion is lacking the chemical evidence. For this reason, it was thought advisable to investigate the histochemical properties of the salivary glands for determining the exact source of the sheath material. During the course of investigation, a parallel study which has been made of the chemical nature of the emitted sheath material (SŌGAWA, 1967) afforded available information.

In the salivary glands of *N. cincticeps*, the V-cells were strongly stained with Sudan black B and gave the positive plasmal reaction, although the Schultz's reaction for sterols and the performic acid-Schiff and UV-Schiff reactions for unsaturated lipids were all negative. These results suggest that the V-cells contain a certain lipid substance. Furthermore, the PAS-positive color appeared to be located in the same cells even after lipid extraction with methanol-chloroform. The PAS-positive reaction was resistant to blockade by acetylation procedure. The similar evidence was observed in the salivary products of *Oncopeltus fasciatus* (DALLAS) (MILES, 1960; SALKELD, 1960) and in the sheath material (MILES, 1960; SŌGAWA, 1967). According to GOMORI (1954), some types of polysaccharide, mainly mucins, have this property. However, the V-cells did not exhibit metachromasia with toluidine blue, nor affinity for Alcian blue 8GS; suggesting the absence of acid mucopolysaccharides. From these staining reactions, the PAS-positive material involved in the V-cells was considered as a certain neutral mucosubstance. Both the mucosubstance and lipid in these cells are thought to be present as a mucolipid by conjugating with each other. In view of

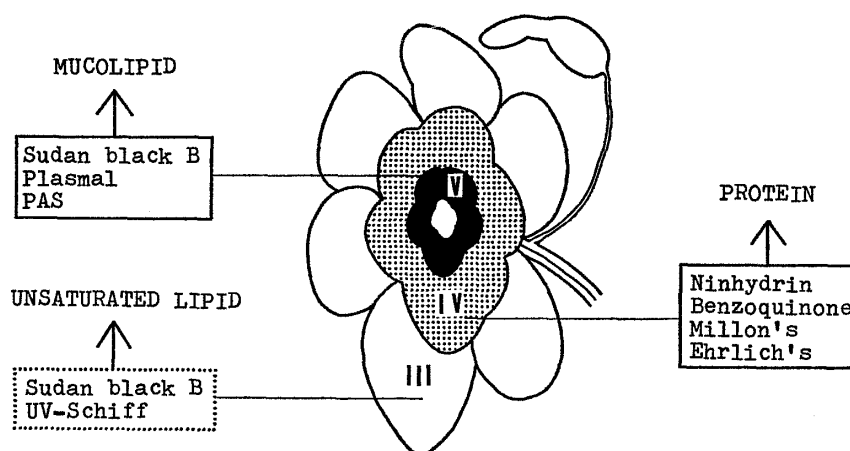


Fig. 1. The main histochemical reactions and probable nature of the secretory products of the III-, IV- and V-cells in the principal salivary gland of *N. cincticeps*. The IV- and V-cells were considered as sources of the structural precursors of sheath material.

the fact that lipid and neutral mucosubstance are the components of sheath material of leafhoppers (SŌGAWA, 1967), it is clear that the products of the V-cells enter into the sheath material. It is, however, unlikely that the sheath material is secreted by the V-cells alone, because there was no evidence that protein which was the other main component of the sheath material was produced in these cells. The IV-cells, which were filled with conspicuous granules (SŌGAWA, 1965), gave positive ninhydrin and benzoquinone reactions, indicating the presence of protein. The occurrence of tyrosine and tryptophan was also demonstrated here by the Millon's reaction and the Ehrlich's, rosindole and xanthidrol reactions respectively. According to MILES (1964, 1965), the sulphhydryl groups play an important role in coagulation of the discharged sheath material. To test this aspect, the alkaline tetrazolium and DDD methods were adopted here. Although the alkaline tetrazolium was positive, the DDD test did not give a clear indication for the presence of sulphhydryl groups; only a yellowish orange color occurred in the V-cells with this test both before and after blocking sulphhydryl groups with iodine and ethylmaleimide and reducing disulphide bonds to free sulphhydryl groups with potassium cyanide. The yellowish coloration observed here is probably due to the combination of the tyrosyl residues, being rich in the V-cells, with naphthanil diazo blue B (azoreaction) contained in the medium for the DDD reaction, because the similar coloration developed with only the diazonium reagent. Apart from the equivocal and negative reactions for sulphhydryl groups, it can be at least concluded that the IV-cells contain highly a proteinaceous substance, and seems more reasonable to assume that the proteinaceous substance is a precursor of the sheath material. In this regard, the previous opinion that the IV-cells secreted digestive enzymes (SŌGAWA, 1965) must be revised. On the basis of the above consideration, it came to the conclusion that in the salivary glands of *N. cincticeps* protein and both lipid and mucosubstance of the sheath materials were respectively secreted from the IV- and V-cells of the principal gland. In the present study, the cytoplasm of the III-cells showed weak but definite sudanophilic

nature and gave the positive performic acid-Schiff and UV-Schiff reactions, indicative of unsaturated lipids. However, the significance of this is uncertain. The parts of the principal gland consisted of both the IV-cells coincide generally with a "mucous gland" in the salivary glands of *N. apicalis* described by NASU (1963). On the other hand, the mucous gland, so-called by DOBROSKY (1931), of *C. sexnotata* includes the secretory cells corresponding to the III-cells of *N. cincticeps* in addition to the above-mentioned two types of cells.

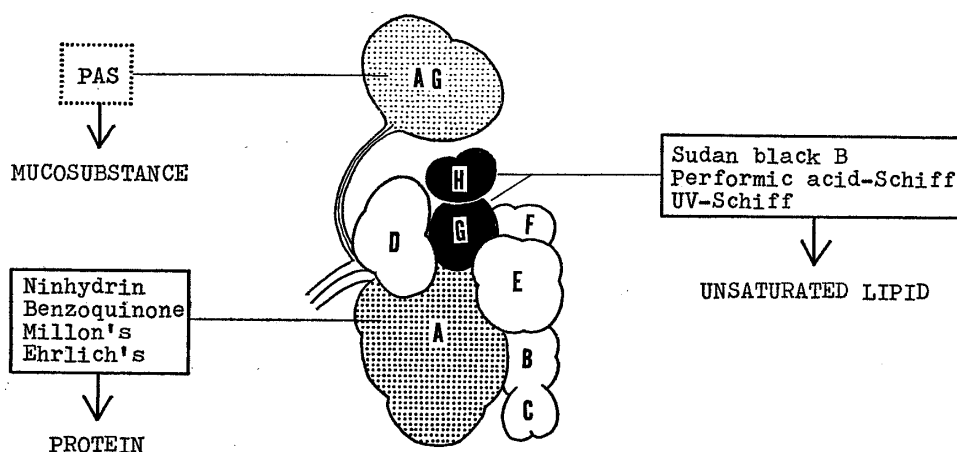


Fig. 2. The main histochemical reactions and probable nature of the secretory products of the A-, G- and H-follicles in the principal salivary gland and the accessory gland of *L. striatellus*. These tissues were considered as sources of the structural precursors of sheath material.

In the salivary glands of *L. striatellus*, lipid substance was found to be localized in the coupled G- and H-follicles by Sudan black B staining. The positive performic acid-Schiff and UV-Schiff reactions suggested the unsaturated nature of the lipid substance. The PAS-positivity of these follicles is possibly due to the unsaturated lipid, since it was lost by lipid extraction with methanol-chloroform. It is worth to note here that PEARSE (1954) has described that unsaturated lipids are PAS-positive. The intensive positive reactions occurred in both the follicles with the Bauer's and Casella's methods are also attributable to the unsaturated lipid. It is almost likely that the G- and H-follicles are the source of the lipid moiety of the sheath material since there is no other follicle containing lipid substance. The A-follicle, which was quite similar to the IV-cells of *N. cincticeps* in the cytological appearance and stainability (SOGAWA, 1965), was characterized by the positive ninhydrin, Millon's, and Ehrlich's reactions and the rapid tanning with benzoquinone. These reactions indicate undoubtedly the presence of protein being rich in tyrosine and containing tryptophan appreciably. However, the presence of sulphhydryl groups was not proved successfully with the DDD methods. Like the IV-cells of *N. cincticeps*, it is almost certain that the A-follicle contributes to the production of a proteinaceous precursor of the sheath material, although this follicle has preliminary supposed to elaborate digestive enzymes (SOGAWA, 1965). The accessory gland was indicated to contain some neutral mucosubstance by the positive PAS-reaction which was not abolished by prior acetylation nor

lipid extraction with methanol-chloroform, resembling to the staining behavior of the sheath material. In conclusion, it is evident that in the salivary glands of *L. striatellus* the components of sheath material, lipid and protein, are produced by the coupled G- and H-follicles and the A-follicle respectively. The mucous substance in the accessory gland may enter into the sheath material.

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