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Studies on the Salivary Glands of Rice Plant Leafhoppers

III. Salivary Phenolase

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Studies were made on the salivary phenolase of the rice plant leafhoppers. The phenolase occurred in the V-cells of the principal glands of *Nephotettix cincticeps* UHLER and *Inazuma dorsalis* MOTSCHULSKY, and in the E-follicle of *Laodelphax striatellus* FALLÉN and *Nilaparvata lugens* STÅL. The occurrence of this enzyme was also examined in the salivary glands of various homopterous insects. The phenolase oxidized *o*-diphenols, especially dopa and its derivatives, but not monophenols including tyrosine. In addition, it seemed to act on aromatic diamines and α-naphtol. Its activity was effectively inhibited by potassium cyanide, sodium dietyldithiocarbamate, sodium azide and phenylthiourea. The presence of the enzyme in the ejected sheath material was demonstrated with *N. cincticeps* and *I. dorsalis*.

INTRODUCTION

In 1926, WITHYCOMBE has first, so far as the author knows, indicated that an oxidase is secreted by a sugar-cane froghopper. In 1953, PLUMB has also shown the presence of oxidase and peroxidase in the salivary glands of an aphid, *Adelges abietis* L. Recently, a phenolase capable of oxidizing dopa and catechol has been found to occur in the salivary glands and discharged saliva of hemipterous insects, and its possible function has been discussed in relation to the stylet sheath formation (MILES, 1960, 1964, 1964a, 1965; MILES and HELLIWELL, 1961). This MILES' finding is of special interest in connection with the fact that several species of phytophagous Hemiptera induce severe growth disturbances on their host plants. It is assumed that the salivary phenolase ejected into the plant tissues during feeding of insects may disturb the plant metabolism and subsequently produce pathological conditions. It seems therefore to be valuable to re-examine and extend the MILES' investigation using different species of Hemiptera.

The present paper is concerned with the localization of the phenolase in the salivary glands of the rice plant leafhoppers and other homopterous insects, its substrate and inhibitor specificities, and the secretion of the enzyme with the sheath material. The following species were mainly used at adult stage: *Nephotettix cincticeps* UHLER (Deltocephalidae), *Inazuma dorsalis* MOTSCHULSKY (Deltocephalidae), *Laodelphax striatellus* FALLÉN (Delphacidae), and *Nilaparvata lugens* STÅL (Delphacidae).

LOCALITY OF THE PHENOLASE IN THE SALIVARY GLANDS OF RICE PLANT LEAFHOPPERS

The salivary glands were fixed in 10% formalin containing 0.72% sodium chloride for 15 min, washed in distilled water for 15 min, and incubated in 0.01 M DL-3, 4-dihydroxyphenylalanine (dopa) in 0.033 M Sörensen's phosphate buffer, pH 7.4, for 1 or 2 hr at 38°C, or in Nadi reagent (including equally 0.005 M N, N-dimethyl-*p*-phenylenediamine, 0. 005M α-naphtol, and 0.067M Sörensen's phosphate buffer, pH 7.4) for 30 min at 38°C.

The V-cells of the principal gland of *N. cincticeps* and *I. dorsalis*, and the E-follicle of the principal gland of *L. striatellus* and *N. lugens* were colored in dark brown with dopa solution and in deep blue with Nadi reagent (Figs. 1 and 2). Such colorations did not appear in the glands which were treated with boiling water before incubation, indicating that the colorations were the results of the enzymatic oxidation of dopa and Nadi reagent.

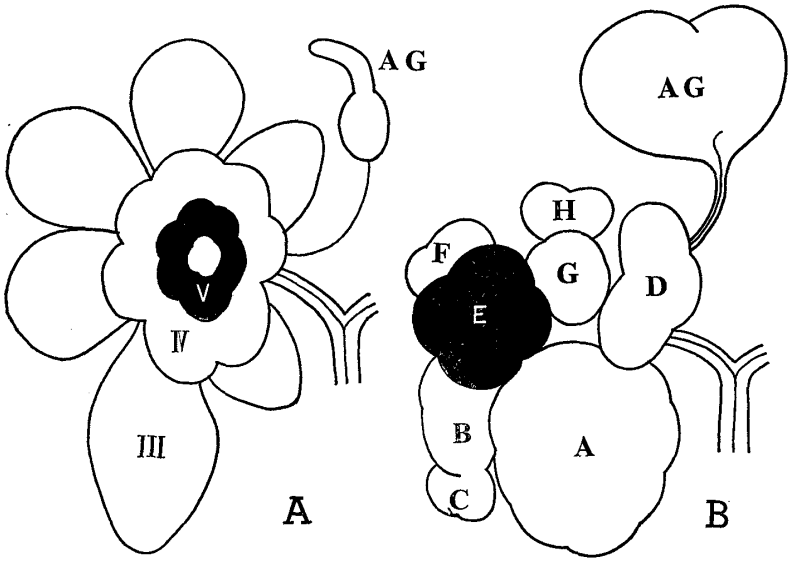

Fig. 1. The site of phenolase activity in the salivary glands of *N. cincticeps* and *I. dorsalis*(A), and in those of *L. striatellus* and *N. lugens*(B). The letters are the same as those in Sōgawa (1965).

PRESENCE OF THE PHENOLASE IN THE SALIVARY GLANDS OF VARIOUS SPECIES OF HOMOPTERA

The species given below were employed for localizing the phenolase activity in the salivary glands: *Graptopsaltria nigrotuscata* Motschulsky (Cicadidae), *Platypleura kaempferi* Fabricius (Cicadidae), *Petaphora maritima* Matsumura (Cercopidae), *Tilophora flavipes* Uhler (Cercopidae), *Erythroneura limbata* Matsumura (Cicadellidae), *Chlorita flavescens* Fabricius (Cicadellidae), *Pochazia fuscata*

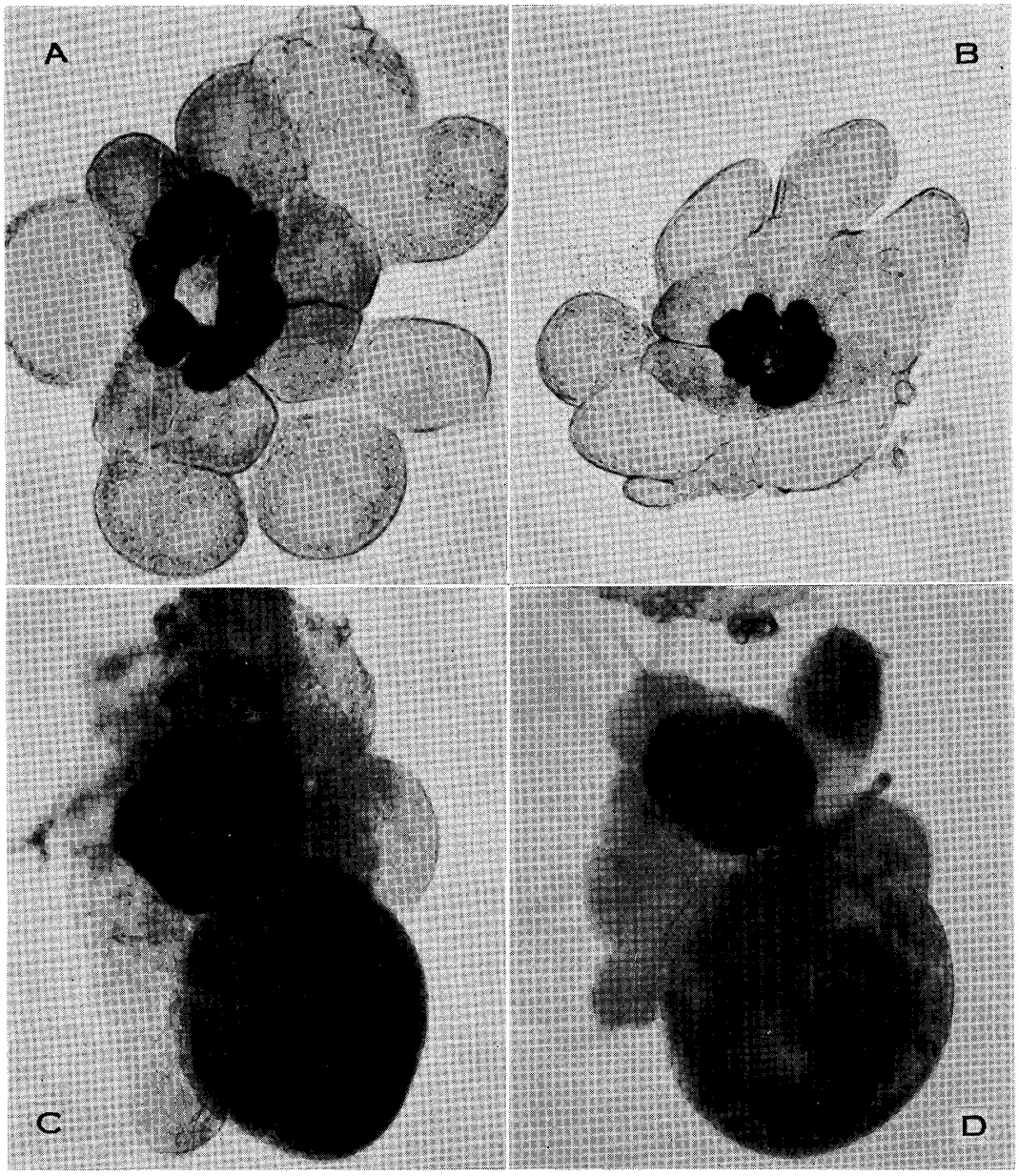

Fig. 2. Microphotographs of the principal salivary glands incubated in the dopa solution, showing the site of phenolase activity. (A) *N. cincticeps* (×100), (B) *I. dorsalis* (×100), (C) *L. striatellus* (×140), (D) *N. lugens* (×140).

albomaculata UHLER (Ricaniidae), *Geisha distinctissima* WALKER (Flatidae), *Dictyophara patruelis* STÅL (Dictyopharidae), *Oliarus apicalis* UHLER (Cixiidae), *Andes marmorata* UHLER (Cixiidae), and *Nisia atrovenosa* LETHIERRY (Meenoplidae).

The phenolase activities were found in the specified tissue of the salivary glands of all the species examined here, and the results were illustrated in Fig. 3.

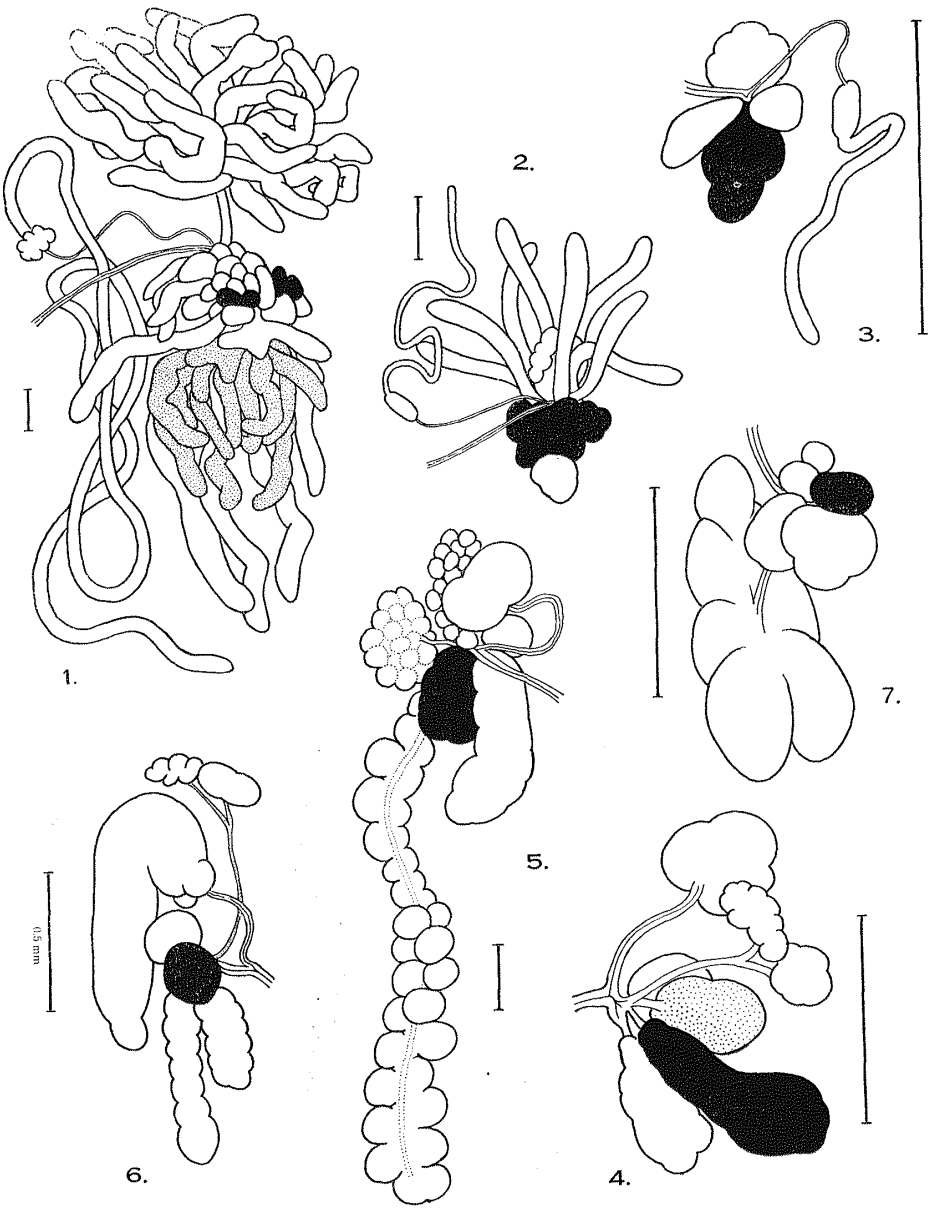

Fig. 3. The salivary glands of various Homoptera, showing the sites of phenolase activity (blacked or shaded). 1. *Graptopsaltria nigrotuscata* Motschulsky (Cicadidae), 2. *Tilophora flavipes* Uhler (Cercopidae), 3. *Chlorita flavescens* Fabricius (Cicadellidae), 4. *Geisha distinctissima* Walker (Flatidae), 5. *Dictyophara patruelis* Stål (Dictyopharidae), 6. *Oliarus apicalis* Uhler (Cixidiae) and 7. *Nisia strovenosa* Lethierry (Meenoplidae). Scale : 0.5 mm.

SUBSTRATE SPECIFICITY OF THE SALIVARY PHENOLASE

In most cases, the salivary glands were lightly fixed in the formol-saline solution, and incubated in 0.01M solutions of various phenolic compounds, listed in Table 1, in 0.033M Sörensen's phosphate buffer, pH 7.4, for 1 or 2 hr at 38°C.

Saturated solutions were used when the solubility of the compounds was less than 0.01M. The action of the phenolase toward aromatic diamines and naphtols was studied using 1 % or saturated solutions buffered to pH 6.5 by 0.067M Sörensen's phosphate buffer. The intensity of the activities was determined by relative density of color produced by the oxidation products in the cells containing the enzyme. The results were summarized in Table 1.

The salivary phenolases of the four species of rice plant leafhopper were similar in substrate specificity, but somewhat different in intensity of the activities; the

Table 1. Substrate Specificity of the Phenolase in the Salivary Glands of *N. cincticeps, I. dorsalis, L. striatellus,* and *N. lugens*

Substrate	*N. cincticeps*	*I. dorsalis*	*L. striatellns*	*N. lugens*
o-Cresol	±	±	±	±
p-Hydroxybenzoic acid	−	−	−	−
Tyrosine	−	−	−	−
Tyramine	−	−	−	−
Catechol	++	++	++	+
Dopa	+++	+++	+++	+++
Dopamine	+++	+++	+++	+++
Noradrenaline	+++	+++	++	+
Adrenaline	+	+	±	±
Protocatechuic acid	+	+	+	+
Caffeic acid	+	+	+	+
Vanillic acid	−	−	−	−
Ferulic acid	−	−	−	−
Resorcinol	±	±	±	±
Hydroquinone	−	−	−	−
Pyrogallol	++	++	++	+
Gallic acid	+	+	+	±
Rutin	0	0	+	±
p-Phenylenediamine	+++	+++	+++	+++
N, N-Dimethyl-*p*-phenylenediamine	+++	+++	±	±
N-Phenyl-*p*-phenylenediamine	+++	+++	+++	+++
Benzidine	+	0	+	±
Tolidine	++	0	++	+
α-Naphtol	0	0	+++	+
β-Naphtol	0	0	−	−

\+ Positive (Number of sign indicates relative intensity of reaction.)
± Trace
− Negative
0 Not tested

phenolase of *I. dorsalis* was the most active, and that of *N. lugens* was the least. The salivary phenolase acted preferably on *o*-diphenols, particularly those with amino group in their side chain such as dopa, dopamine, and noradrenaline; although catechol and pyrogallol were considerably oxidized. Phenolic carboxylic acids such as protocatechuic, caffeic, and gallic acids were also but slowly oxidized. However, methoxyl derivatives, ferulic and vanilic acids, were not utilized as substrates for the salivary phenolase. A flavonoid with *o*-diphenol unit, rutin, was weakly oxidized by the phenolase of *L. striatellus* and *N. lugens*. Resorcinol was slightly oxidized, but hydroquinone was not. Monophenols excepting *o*-cresol were not attacked at all by the salivary phenolase. The salivary glands gave trace reaction in 0.1M *o*-cresol solution.

Among the aromatic diamines tested, *p*-phenylenediamine and N-phenyl-*p*-phenylenediamine were readily oxidized and gave strong coloration at the sites of the phenolase activity in the glands of the four species. In dimethyl-*p*-phenylenediamine solution, the V-cells of *N. cincticeps* and *I. dorsalis* were immediately colored in deep blue, while the E-follicles of *L. striatellus* and *N. lugens* were little done. When the salivary glands were kept in the solutions of the above-mentioned three diamines for prolonged time, the tissues other than the phenolase-containing tissues, especially the epitherial cells of the conducting parts, gave positive reaction. This reaction seemed to be attributed to the action of cytochrome oxidase since the three aromatic diamines used here were oxidizable by cytochrome oxidase system (BURSTONE, 1960). Diaminodiphenyl compounds, benzidine and tolidine, may be oxidized in the V-cells of *N. cincticeps* and in the E-follicle of *L. striatellus*, although the results were more or less variable and not always positive. The E-follicle of *N. lugens* was slightly colored in tolidine solution, but not in benzidine. α-Naphtol but not β-naphtol was oxidized in the E-follicle of *L. striatellus* and *N. lugens*. Regarding the oxidation of aromatic diamines, it should be noted that no peroxidase activity was detected in the salivary glands of the four species of rice plant leafhopper by means of zinc-leucos method of LISON (1960).

INHIBITION OF THE SALIVARY PHENOLASE ACTIVITY

The effect of phenylthiourea (PTU) known as a specific inhibitor of phenolases on the oxidation of catechol and dopa by the salivary phenolase of *N. cincticeps* was preliminarily examined altering the concentration and pH of the substrate solutions. The results showed that the inhibitory effect of 0.005M PTU was the most clearly recognized when 0.005M dopa solution adjusted to pH 6.5 was used (Table 2). Therefore, the tests on inhibition of the salivary phenolase activities were all carried out in this way: the glands which were briefly fixed in the formol-saline solution were incubated in 0.005M dopa with 0.005M inhibitor in 0.033M Sörensen's phosphate buffer, pH 6.5 for 1 hr at 38°C.

As shown in Table 3, the salivary phenolases of the four species of rice plant leafhopper were effectively inhibited by pottassium cyanide, sodium diethyldithiocarbamate (DIECA), PTU, and sodium azide.

Table 2. Inhibitory Effect of PTU on the Salivary Phenolase of *N. cincticeps*

Substrate	Concentration	pH	Inhibitor	Relative intensity of coloration in the V-cells					
				10	20	30	40	50	60
				(min)					
Catechol	0.01 M	7.4	None	+	++	+++	++++	++++	++++
			PTU	±	+	++	++	+++	+++
Catechol	0.01	6.5	None	+	++	+++	+++	++++	++++
			PTU	±	+	++	++	+++	+++
Catechol	0.005	7.5	None	+	++	+++	+++	+++	+++
			PTU	±	±	+	++	++	++
Catechol	0.005	6.5	None	+	+	+++	+++	+++	+++
			PTU	±	±	+	+	+	++
Dopa	0.01	7.4	None	+	++	+++	+++	+++	+++
			PTU	+	+	++	+++	+++	+++
Dopa	0.005	7.4	None	+	++	++	+++	+++	+++
			PTU	±	±	±	+	+	+
Dopa	0.005	6.5	None	±	+	++	+++	+++	+++
			PTU	−	−	−	±	±	±

Table 3. Phenolase Activity[a] in the Salivaty Glands of the Rice Plant Leafhoppers in the Presence of Inhibitors

Inhibitor	*N. cincticeps*	*I. dorsalis*	*L. striatellus*	*N. lugens*
None	+++	+++	+++	+++
KCN	−	−	−	−
DIECA	−	−	−	−
NaN_3	±	±	−	−
PTU	±	±	−	−

[a] Dopa was used as a substrate.

PHENOLASE ACTIVITY IN THE EMITTED SHEATH MATERIAL

The following experiments were undertaken in order to demonstrate the secretion of the salivary phenolase during feeding of the rice plant leafhoppers. The apparatus used were shown in Fig. 4. The leafhoppers were allowed to probe into 1 % aqueous sucrose solution containing 0.1 % dopa, catechol or protocatechuic acid through a stretched Parafilm M® membrane. After about two hours, the stylet sheaths deposited on the membrane were observed under binocular. In another experiment, 2 % agar gel containing 1 % sucrose and 0.1 % dopa was directly exposed to probing of the leafhoppers. Some typical results were shown in Fig. 5.

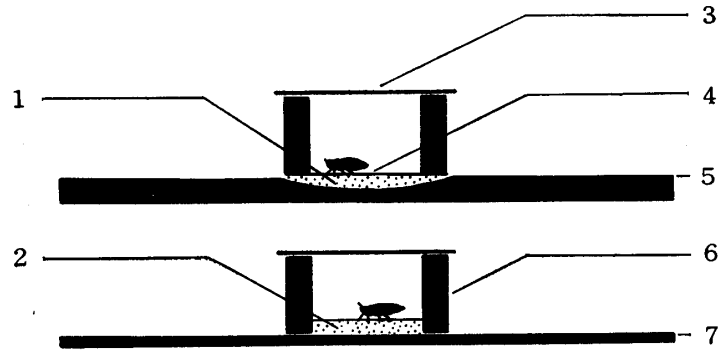

Fig. 4. Two types of simple apparatus used to examine the secretion of the salivary phenolase by the rice plant leafhoppers.
(1) 1% aqueous sucrose solution containing 0.1% dopa, catechol, or protocatechuic acid, (2) 2% agar gel containing 1% sucrose and 0.1% dopa, (3) cover glass, (4) stretched Parafilm M®, (5) hollow glass, (6) glass ring, (7) slide glass.

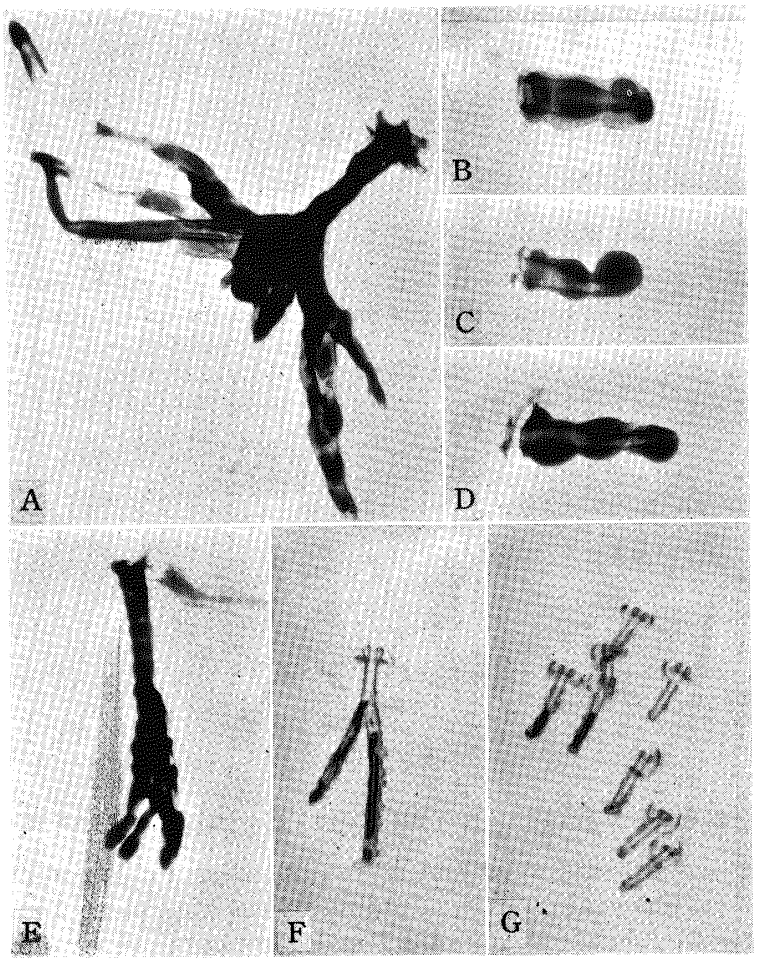

Fig. 5. Microphotographs of the stylet sheaths formed within the solution of agar gel containing sucrose and *o*-diphenols (×150). (A) The sheath of *N. cincticeps*, blacked within the agar gel containing dopa. (B, C, & D) The sheaths of *N. cinticeps*, blacked in the dopa solution. (E) The sheath of *I. dorsalis*, blacked within the agar gel containing dopa. (F) The colorless sheath of *N. lugens*, formed in the protocatechuic acid solution. (G) The colorless sheaths of *L. striatellus*, formed in the catechol solution.

When *N. cincticeps* and *I. dorsalis* produced the stylet sheaths in the sucrose solution containing dopa, their stylet sheaths were nearly black; and in the solutions containing catechol or protocatechuic acid, they were reddish brown. Generally the stylet sheaths were uniformly colored, but in some cases, the outer zone of the sheaths remained colorless (see Fig. 5-B). The stylet sheaths formed in the agar gel containing dopa by *N. cincticeps* and *I. dorsalis* were also intensely darkened. No diffusion of the color into the agar gel was recognized. On the other hand, the stylet sheaths produced by *L. striatellus* and *N. lugens* did not take any coloration in the solutions nor in the agar gel.

DISCUSSION

In insects the phenolases occur most generally in the cuticle and blood. Their various functions have been reviewed by SUSSMAN (1949). In addition to the cuticle and blood, they are also contained in the collecterial gland (BRUNET and KENT, 1955) tracheal gland (ROTH and STAY, 1958), and extraembryonic fluid in the eggs (JONES, 1958). Recently MILES (1960) and MILES and HELLIWELL (1961) have found that an oxidase other than cytochrome oxidase occurs in the accessory salivary gland and in the ejected saliva of plant bugs, *Oncopeltus fasciatus* (DALL.) and *Elasmolomus sordidus* (F.). MILES (1964) has further examined the nature of the oxidase with four species of plant bug, *Eumecopus australasiae* DON., *Tectocoris lineola* F., *Elasmolomus sordidus* (F.) and *Dysdercus sidae* MONTS; and showed that the oxidase catalyzed the oxidation of dopa and catechol, but not cresol, and its activity was inhibited by potassium cyanide and phenylthiourea, and to a lesser extent by sodium azide. On the basis of these results, the oxidase in the salivary glands has been indicated to be a polyphenol oxidase. In the same paper, MILES has also reported that such phenolase generally occurs in the salivary glands of representatives from various families of Hemiptera.

The site of the phenolase activity in the salivary glands can be easily detected by the appearance of dark coloration when the glands are incubated with suitable substrates such as dopa and catechol or with Nadi reagent. In the salivary glands of *N. cincticeps* and *I. dorsalis*, the phenolase activity was recognized in the cluster of V-cells. In the other species of leafhopper, *Stenocotis depressa* WOLK and *Eurymeloides punchra* SIGN., the same part of the glands has been shown to contain the enzyme by MILES (1964). The V-cells of leafhoppers have another function, secretion of lipid and mucosubstance which are the components of the sheath material (SŌGAWA, 1967a). This evidence, the simultaneous occurence of the phenolase with the structural precursors of the sheath material in the same cells, indicates naturally the involvement of the phenolase in the process of the stylet sheath formation. It was, in fact, shown that the phenolase activity occurred in the emitted sheath material of *N. cincticeps* and *I. dorsalis*. On the other hand, in the salivary glands of *L. striatellus* and *N. lugens*, the phenolase was present in the E-follicle of the principal gland independently of the precursors of the sheath material.

The cuticular phenolase, frequently refered to as tyrosinase, has long been known to possess two different catalytic activities, i.e. cresolase and catecholase activity (e.g. NELSON and DAWSON, 1944; MASON, 1955). DRESSLER and DAWSON

(1960) and AERTS and VERCAUTEREN (1964) have represented the available experimental evidence for the explanation of the dual activities of this type of phenolase. A partially purified phenolase from larvae of a mealworm, *Tenebrio molitor,* has been demonstrated to bear two types of active center, one for the cresolase activity, and one for the catecholase activity. As mentioned already, the salivary phenolase of plant bugs acts on dopa and catechol, but not on cresol (MILES, 1964). This suggests that the salivary phenolase is lacking in the cresolase activity. If it is true, the salivary phenolase seems to be a distinct phenolase from the cuticular enzyme. In order to verify this the substrate specificity of the salivary phenolase of leafhoppers was examined. As a result of the experiment it was elucidated that (a) the most effective substrates were dopa and dopamine among the compounds tested here, (b) *o*-diphenols with acidic side chain were weakly oxidized, and (c) monophenols, methoxyl derivatives of *o*-diphenols, resorcinol, and hydroquinone were little, or not at all oxidized. From these results, it is apparent that the salivary phenolase is distinct from the cuticular phenolase in the substrate specificity, although both the enzymes are similar in the susceptibility to copper enzyme inhibitors such as PTU and DIECA. It was also supposed that the affinity of the salivary phenolase for substrates depended on the position of the phenolic hydroxy groups on the benzene ring and the structure of the side chain of the substrate molecule. Although it was not clear whether or not the results were due to the true enzymatic oxidation, several aromatic diamines were oxidized at the site of phenolase activity in the salivary glands. With regard to this fact, it is worth mentioning that OHNISHI (1954) has shown that a phenolase from the cuticle of white prepupae of *Drosophila viridis* differs from the blood phenolase of the same insect in being able to oxidize dimethyl-*p*-phenylenediamine. ITO (1953) has noted that the isolated larval and pupal cuticles of *Bombyx mori* are capable of oxidizing *p*-phenylenediamine. Similarly WHITEHEAD et al. (1960) has shown that the phenolase from the colleterial gland of the cockroach, *Periplaneta americana*, promotes the oxidation of not only several *o*- and *p*-diphenols but also *p*-phenylenediamine. Although PLUMB (1953) has shown an oxidase and peroxidase activities in the salivary glands of the aphid, *Adelges abietis,* using benzidine and *p*-phenylenediamine as substrates, it is most probable that these activities are due to the phenolase demonstrated by MILES (1964, 1965). Generally, the salivary phenolase of the leafhoppers is similar in the substrate specificity to a true *o*-dihydric phenolase which has been isolated in crystalline form from larvae of the blowfly, *Calliphora erythrocephala* (KARLSON and LIEBAU, 1961), and considered to take part in the formation of puparium. N-Acetyldopamine has been regarded as a natural substrate for this enzyme (KARLSON and SEKERIS, 1962). According to MILES ((1964a) and MILES and LLOYD (1967), a natural substrate of the salivary phenolase is dopa. The salivary phenolase is, however, quite different from a laccase-like phenolase prepared from the colleterial gland of the cockroach, *Periplaneta americana,* which is involved in the hardening and darkening of the oötheca (WHITEHEAD et al., 1960) in the following points: (a) the phenolase from the colleterial gland can not act on dopa and dopamine which are preferentially attacked by the salivary phenolase, (b) conversely, it promotes rapid oxidation of *o*-diphenols with acidic side chain which seem not to be suitable substrates for the salivary phenolase, and (c) it is capable of oxidizing

hydroquinone. The natural substrate of the laccase-like phenolase has been demonstrated to be protocatechuic acid (BRUNET and KENT, 1955).

It has been well known that the sheath material is a main salivary product, which is discharged when the stylet is inserted into the plant tissues and forms the stylet sheath. In the present experiment it was shown that the salivary phenolases of *N. cincticeps* and *I. dorsalis* were secreted along with the sheath material. Also aphids have been found to secrete the phenolase with it (MILES, 1965). However, in the cases of heteropterous bugs the enzyme is secreted with a watery saliva independently of the sheath material (MILES and HELLIWELL, 1961; MILES, 1964). These evidences suggest the involvement of this enzyme in the process of the stylet sheath formation. According to the observations by BENNETT (1934), STOREY (1939), and DAY et al. (1952), the sheath material of leafhoppers is secreted as a highly viscous solution when the stylet bundle is protruded into the feeding medium, and gels rapidly to form the stylet sheath even within the aqueous solutions. This rapid coagulation of the sheath material is surmised to be a result of the condensation of protein molecules, which have been demonstrated to a main constituent of the sheath material (SMITH, 1933; MILES, 1960; SŌGAWA, 1967). In the insect cuticle, it is a decisive evidence that the tanning of the cuticular protein, which is mediated by the action of the phenolase, results in the hardening and darkening of the cuticle. There the cuticular phenolase oxidized polyphenols to quinones which conbine with free amino groups of protein molecules, causing extensive cross bondings of protein chains, which are thereby converted into a continuous, closely knit structure. Although there is, at present, no direct evidence, it is conceivable that the sheath material ejected with the phenolase set to gel by an analogous process to that of the cuticular tanning. In the present experiments, no evidence that *L. striatellus* and *N. lugens* secreted the phenolase during their feeding was obtained, in spite of the presence of this enzyme in the salivary glands. In this respect, it may be thinkable that both the species secrete a small amount of quinones, instead of the enzyme, which are produced in the E-follicle of the principal salivary gland where the phenolase exists. However, MILES (1964a; 1965) has emphasized that the formation of disulphide and hydrogen bonds is an essential event in the process of coagulation of the sheath material, and he has considered that the phenolase serves to prevent the swelling of the deposited stylet sheath.

The salivary phenolase may be attributable to some plant diseasee caused by hemipterous insects' feeding. WITHYCOMB (1926) has suggested that an oxydase in the saliva discharged by a froghopper is at least partially connected with supernormal oxidation with formation of red pigment in the infected sugar-cane by this insect. MILES (1965) and MILES and LLOYD (1967) have also described that the salivary phenolase is concerned with the synthesis of auxin and implicated in the formation of plant galls.

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