Published in Physiological Entomology 8, issue 3, 307-314, 1983

Salicin from host plant as precursor of salicylaldehyde in defensive secretion of Chrysomeline larvae

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ABSTRACT. *Phratora vitellinae* L. and *Chrysomela tremulae* F. (Chrysomelinae, Coleoptera) feed on *Salix* or *Populus* spp. (Salicaceae). Their larvae, as well as the larvae of other chrysomelines feeding on Salicaceae, secrete salicylaldehyde. In this study, we demonstrate that salicylaldehyde is derived from salicin, a phenylglucoside present in the leaves of the host plant. The concentration of salicylaldehyde in the secretion is positively correlated with the amount of salicin in the food of the larvae. The transformation of salicin into salicylaldehyde occurs in the defence glands since the β-glucosidase activity is 4 times higher in their glands than in the gut. The larvae recover most of the glucose that results from the hydrolysis of salicin. For generalist predators, such as ants, salicylaldehyde is a more potent deterrent than saligenin or salicin.

Key words. Chrysomelidae larvae, *Phratora vitellinae, Chrysomela tremulae, C.populi, Salix, Populus,* salicin, salicylaldehyde, defensive secretion.

Introduction

All the larvae of the Chrysomelinae belonging to the tribe Phaedonini and to the genus *Phratora* in the tribe Phratorini are protected against predators and probably parasitoids by a secretion released by nine pairs of eversible glands, of which two are thoracic and seven abdominal. Most species secrete a mixture of methylcyclopentanoid monoterpenes from these glands. These chemicals are probably autogenous. The larvae producing them feed on a large range of plants, in which no obvious direct precursors of these irregular monoterpenes are known. Secondary adaptation to the chemistry of the particular host plant may explain the exceptions (Pasteels *et al.*, 1982; Pasteels, 1983). For example, the Japanese species *Gastrolina depressa* Baly feeding on *Juglans* produces juglone (Matsuda & Sugawara, 1980). The European *Chrysomela populi* L., *C.tremulae* F., *C.20-punctata* Scopoli, the American *C.scripta* F., *C.interrupta* F., the Japanese *C.20-punctata costella* (Marseul) and the European *Phratora vitellinae* L., all feeding on Salicaceae, secrete salicylaldehyde.

It has been suggested that salicylaldehyde is derived from host plant phenylglucosides such as salicin or populin (Hollande, 1909; Wain, 1943; Pavan, 1953). The first aim of this study was to verify this hypothesis experimentally for *Ph.vitellinae* and *C.tremulae*. Two different approaches are used. Newly hatched larvae of *Ph.vitellinae* were reared on *Salix* devoid of salicin. A preliminary account of some of these experiments has already been published (Rowell-Rahier & Pasteels, 1982). Larvae of *C.tremulae* were fed with labelled salicin. This last species does not develop well on salicin-free *Salix*.

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If confirmation was obtained, the second aim was to determine where in the body this transformation occurred and thirdly to establish the relationship between the quantity of precursor ingested and the quantity of defence secretion produced. The fate of the glucose moiety split from the original glucoside molecule was also examined, and the secretion of both species and of a third related species, *C.populi*, were tested for the presence of glucose. Finally, we compared the deterrency of salicin, of its aglycone saligenin and of salicylaldehyde against a potential generalist predator of chrysomeline larvae.

Materials and Methods

1. *Rearing of the larvae and collection of the secretions*

Ph.vitellinae. Larvae were reared from hatching on leaf discs (diam. 14 mm) of their normal host plants, *Salix nigricans* Sm., with or without additional salicin, and on shaved *Salix caprea* leaf discs which do not contain phenylglucosides (Hegnauer, 1973). In nature, *S.caprea* is not eaten by *Ph.vitellinae*. The undersurfaces of its leaves are covered with trichomes, but when these are removed, *S.caprea* leaves are readily accepted (Rowell-Rahier & Pasteels, 1982). Leaves of shaved *S.caprea* to which known increasing quantities of salicin were added, were also tested. The secretions of third instar larvae were collected individually, in calibrated glass capillary tubes, daily. Special care was taken to empty the glands completely. The exuviae of the third instar larvae were collected after pupation and stored in hexane. The secretions of third instar larvae collected in the field from mature (July) and old (September) leaves of *S.nigricans* were also analysed.

C.tremulae. An aqueous 1% solution of labelled salicin was spread evenly (10 µl/100 (mm^2) on leaf discs (diam. 35 mm) of *Populus trichocarpa*. After the water had evaporated, the discs were offered to six separate third instar larvae. The leaf discs were renewed every 1 or 2 days. Secretions were collected daily in glass capillary tubes until the larvae died or pupated.

2. *Chemical analysis of the secretions*

Qualitative analysis of the secretions was performed by thin layer chromatography (silica gel; eluent CH_2Cl_2 or $CH_2Cl_2/CH_3OH/H_2O$ 80:19:1; spray reagent $CeSO_4$ in H_2O/H_2SO_4) or gas chromotography (10% Carbowax 20M at 125°C and 3% OV1 at 160°C after silylation).

The volume of each individual's secretion was found by measuring the height of the liquid in the calibrated capillary tubes under a binocular microscope, with stage micrometer. The secretions were then pooled for each treatment and dissolved in known volumes of solvent (methanol or hexane). The amount of salicylaldehyde in the secretion was determined by quantitative gas chromatography analysis of the solutions and by comparison with standard curves derived from reference solutions of salicylaldehyde. The amounts given in Tables 1 and 2 were calculated from two different gas chromatography injections.

Quantitative analysis for glucose was performed by evaporating known amounts of crude secretions to dryness under reduced pressure and the solid residues dissolved in 0.5 ml of Tri-sil (Pierce chemical). After standing at room temperature for 2 h, the amount of glucose was evaluated by gas chromatography analysis (3% OV1 at 160°C) and by comparison of the area of the peaks attributable to silylated α and β glucose with those obtained with a standard solution.

3. *Detection of β-glucosidase activity in the larvae of C.tremulae*

Three third instar larvae were dissected in Taylor's physiological fluid and the different parts of the body (Table 3) were immediately put in 2 ml of buffer solution at pH 6 (Na_2HPO_4/CH_3COOH). They were then ground, homogenized and centrifugated. 0.5 ml of an aqueous solution of [7-^{14}C] salicin (50 mg in 50 ml, specific activity (S.A.) 0.25 mCi/mM) was added to 1.5 ml of each of the cleared solutions of the different parts of the larvae. The resulting solutions were left at 36°C for 3 h. They were then extracted twice with 3 ml of dichloromethane. The organic layers were combined, filtered first on a filter paper and then on a small silica gel column.

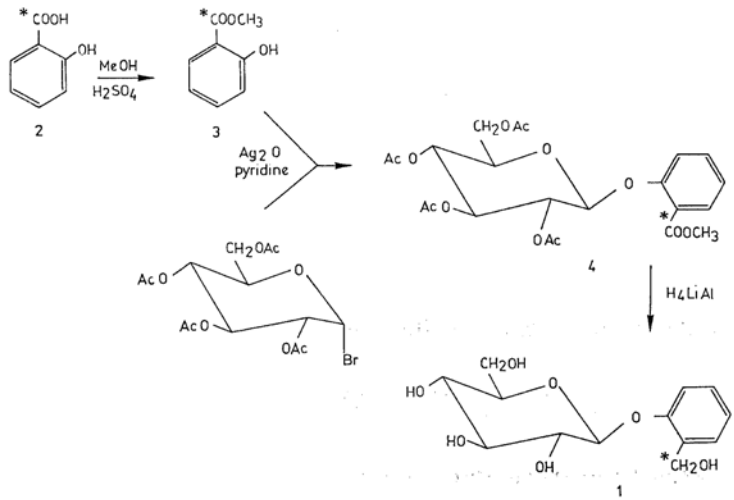

FIG. 1. Scheme of synthesis of [7-^{14}C] salicin.

The residue obtained after evaporation of the solvent under reduced pressure was dissolved in 10 ml of scintillation fluid (insta-gel). The level of radioactivity was measured by liquid scintillation counting. Two controls were performed, either with 1.5 ml of buffer solution plus 0.5 ml of [7-^{14}C] salicin solution or with 1.5 ml of a buffered solution of β-glucosidase from sweet almond (Serva) (15 mg in 30 ml buffer solution at pH 6) plus 0.5 ml of [7-^{14}C] salicin solution.

4. *Synthesis of* [7-^{14}C] *salicin* (Fig. 1)

[7-^{14}C] salicin ((1) Fig. 1) was prepared starting from [7-^{14}C] salicylic acid (2) (S.A. 27 mCi/mM) according to the sequence of reactions described in Fig. 1. Conversion of (2) (100 mg; 0.1 mCi) into the methyl ester (3) was accomplished by treatment with methanol/sulphuric acid 10% (Vogel, 1956), from which 81 mg of [7-^{14}C] methylsalicylate was obtained. Formation of the O-glucosidic bond was produced using Robertson & Waters' (1930) procedure for the synthesis of phenol β-glucosides. Accordingly, 248 mg of 1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-[7-^{14}C] methylsalicylate (4) were obtained from 81 mg of (3), 610 mg of 2,3,4,5-tetra-O-acetyl-β-D-glucopyranosyl bromid (Hudson & Johnson, 1915), 0.5 ml of quinoline and 325 mg of dry Ag_2O. Finally, lithium aluminium hydride reduction of (4) (248 mg) dissolved in dioxane and refluxed for 4 h, followed by decomposition of the excess of H_4LiAl with methanol, evaporation of the solvent, filtration over mixed bed resin (Dowex 1-X8 and 50 W-X8) and silica gel chromatography (eluent: $CHCl_3/CH_3OH$ 8:2), furnished 107 mg of [7-^{14}C] salicin (1); S.A. 0.25 mCi/mM.

5. *Deterrent activity of salicin, saligenin and salicylaldehyde against ants*

Aqueous solutions of salicin or saligenin and emulsions of salicylaldehyde were prepared in 10^{-1} M sucrose. A binary choice between 50 µl of pure sucrose solution and of a test solution or emulsion was given to a laboratory culture of ants, *Myrmica rubra*, on their foraging area. The drops of liquid were

deposited in depressions (diam. 7 mm) in a paraffin surface on a glass slide. The ants were allowed to drink for 10 min. Different concentrations, ranging from 1 M to 10^{-3} M, were tested for each compound and in each case at least nine replicates were made. Consumption by the ants was determined by weighing the solutions before and after the experiment. After correcting the evaporation, the results were analysed for significance by the Walsh test (one-tailed).

Parro (1981) showed that two identical sources of food are usually unevenly exploited by ants, due to differential recruitments resulting from random discovery of the sources. A large variability between replicates was thus expected, especially at low concentrations. The data, therefore, were pooled for calculation of a deterrency index for each dilution. Deterrent activity is given by $(C - E)/(C + E) \times 100$, where C is the consumption of the sucrose solution and E the consumption of the tested solution. In this deterrency index, the difference between the consumption of the control sucrose solution and of the test solution is expressed as a percentage of the total consumption by the ants during the experiment to avoid bias due to variation in motivation or physiological state between different groups of ants.

Results

1. *Secretion of Ph.vitellinae larvae*

Larvae fed on *S.nigricans* produced copious amounts of secretion, which was shown by gas chromatography to contain salicylaldehyde. Larvae fed shaved *S.caprea* extruded their glands normally but no secretion could be collected. When salicin was added to the shaved leaves of *S.caprea*, the secretion was partly restored (Table 1). Thus salicylaldehyde appears to be derived from salicin. The concentration of salicylaldehyde in the secretion is positively correlated with the amount of salicin in the food of the larvae (Table 1). The amount of salicin in the discs of *S.nigricans* was calculated from the mean weight of the discs and the salicin content of the leaves determined by quantitative high performance liquid chromatography (HPLC) analysis. Qualititative HPLC analysis of *S.nigricans* leaves showed no significant quantity of phenylglucosides other than salicin to be present (Rowell-Rahier, in preparation). When the larvae were fed on their normal host plant, and the secretion collected daily, most of the ingested salicin was excreted as salicylaldehyde (Table 1). The incorporation of salicin into the defensive secretion was less efficient when added to shaved leaves of

TABLE 1. Quantitative analysis of the secretion of the third instar larvae of *Ph.vitellinae*.

Food plant:	*S.caprea* shaved + increasing quantities of salicin				*S.nigricans*			*S.nigricans* Wild larvae in:	
					Young leaves	Old leaves	Additional salicin	July	September
Salicin ingested /day (μmol)	0	0.17	0.27	0.35	0.43*	0.46*	2.52*	?	?
Volume individual secretion (10^{-3} μl) ($\bar{x} \pm$ SD)	0	17 ± 4	58 ± 22	46 ± 4	48 ± 19	84 ± 41	41 ± 20	104 ± 33	92 ± 25
n		17	30	13	9	30	15	6	13
Amount of salicylaldehyde in pooled secretion (μM/μl)	0	0.23	1.33	1.53	2.95	5.61	6.20	3.13	7.70
Mean quantity of salicylaldehyde per larva after 24 h (μM)	0	0.004	0.08	0.07	0.14	0.46	0.25	–	–

* The quantity of salicin in the *S.nigricans* leaves was determined by quantitative HPLC analysis.

TABLE 2. Amount of salicylaldehyde and glucose in the secretions of *C.tremulae*, *C.populi* and *Ph.vitellinae*.

Secretion of:	Amount of salicylaldehyde (μM/μl)	Amount of glucose (μM/μl)
C.tremulae (on *P.trichocarpa*)	1.13	0.25
C.populi (on *P.trichocarpa*)	2.16	0.21
Ph.vitellinae Wild larvae on *S.nigricans*	7.70	0.02

n = pooled secretion of several tens of larvae.

S.caprea or to leaves of *S.nigricans*. The volume of secretion collected daily in the laboratory was lower than the volume of the secretion obtained from larvae in the field (Table 1).

The exuviae of the third instar larvae reared on *S.nigricans* old leaves or on *S.caprea* shaved leaves plus salicin (150 μg/disc) also contained salicylaldehyde (107.2 and 15.9 μg/exuviae). There was no salicylaldehyde in the exuviae of the third instar larvae reared on shaved leaves of *S.caprea* alone.

Quantitative gas chromatography analysis showed that there is a small quantity of glucose in the secretions of *Ph.vitellinae* larvae (Table 2).

2. *Secretion of C.tremulae and C.populi larvae*

The crude secretion of *C.tremulae* larvae fed on discs of *P.trichocarpa* covered with [7-^{14}C]salicin exhibited a high level of radioactivity (85,919 d/min/mg). Radio-gas-chromatography (1% SE 30 at 75°C) showed all the radioactivity to be associated with a peak having the same retention time as salicylaldehyde.

Thin layer chromatography and gas chromatography showed glucose and salicylaldehyde to be the main constituents of the secretions of *C.tremulae* and *C.populi* (Table 2). The concentrations of glucose and salicylaldehyde in the secretions were, however, far from being equimolar, indicating that the glucose formed by salicin hydrolysis was in great part recovered by the larvae.

3. *β-glucosidase activity in different tissues of C.tremulae larvae*

β-Glucosidase activity was detected mainly in the gut and glandular tissues of the larvae (Table 3). Similar activity was found for the thoracic glands and the gut (experiment 3). The body wall, including the defence glands showed activity 4 times greater than that found in the larval gut (experiment 2). Since the four thoracic glands are more developed than the

TABLE 3. β-glucosidase activity in parts of the body of *C.tremulae* larvae.

Experiment	Part of the body used	d/min	Fraction of the total activity found in the different parts of the body (%)
I	Three whole larvae	30,407	100
II	Three larvae dissected in:		
	Body walls + defence glands and traces of fat body	42,945	80
	Fat body	344	1
	Digestive system + Malpighian tubules	9,948	19
III	Three larvae dissected in:		
	Metathoracic glands	14,645	52
	Digestive system	13,647	48
Control	Buffer solution only	36	–
	Buffered solution of β-glucosidase	156,297	–

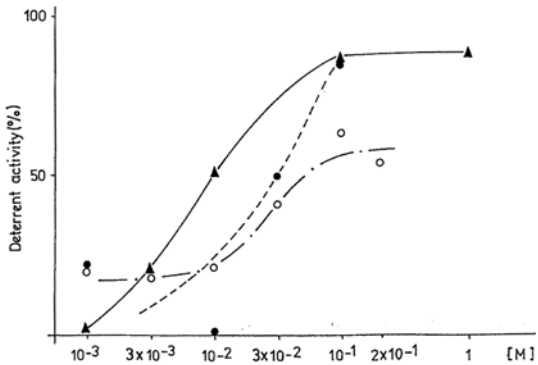

FIG. 2. Deterrent activity of salicin (o, –·–), saligenin (•, – – – –) and salicylaldehyde (▲, ———). Calculation of the deterrency index is explained in Material and Methods, section 5.

fourteen abdominal glands, this suggests that the β-glucosidase activity detected in the body wall is restricted to the defence glands.

4. *Deterrent activity of salicin, saligenin and salicylaldehyde against ants*

The results are summarized in Fig. 2. The test solutions significantly deterred ants ($P < 0.01$) at concentrations of 10^{-2} M and above for salicin and salicylaldehyde and of 3×10^{-2} M and above for saligenin.

Salicylaldehyde was a more effective deterrent against *M.rubra* than saligenin and salicin, especially at the lower concentration (10^{-2} M). Salicylaldehyde and saligenin were both very active deterrents (activity above 80%) at the higher concentrations tested (10^{-1} M, 1 M). At these concentrations deterrency was probably total, even though the index did not reach 100%. Excited ants bumping into the test solutions during the experiment caused some loss of weight, thus weight loss reflects more than consumption. The deterrent activity of salicin, was low within the range of concentrations tested.

Discussions and Conclusion

Larvae of both *C.tremulae* and *Ph.vitellinae* use salicin, found in leaves of their host plant, as the precursor of salicylaldehyde which is then released from their defence glands as an aqueous emulsion. In *Ph.vitellinae*, there is no autogenous source of salicylaldehyde since no secretion at all is produced on a salicin-free diet. Salicin was the only source of salicylaldehyde for larvae feeding on leaves of *S.nigricans* since these leaves did not contain other phenylglucosides. However, it is possible that different phenylglucosides such as populin, present in the leaves of other food plants, may also be used as precursors of salicylaldehyde by some chrysomeline larvae. This hypothesis has yet to be tested.

The high β-glucosidase activity found in the dorsal glands of *C.tremulae* indicates that the biosynthesis of salicylaldehyde from salicin probably occurs in the defence glands themselves. The lower β-glucosidase activity in the digestive tract is, in itself, not surprising, since β-glucosidases are common in many phytophagous insects (Morgan, 1976).

The larvae of *Ph.vitellinae* appear to utilize salicin for their defence secretion with great efficiency. They excrete most of the salicin ingested as salicylaldehyde when fed on their normal host plant, *S.nigricans*, and 'milked' every day. In nature, secretion is not likely to be renewed daily. Indeed, when the larvae are disturbed, the secretion appears at the tip of the reservoirs, but most of it can be withdrawn again into the reservoir during the retraction

of the latter into the body (Garb, 1915; Hollande, 1909). The fate of the salicin ingested when the glands are already full remains to be investigated.

The low incorporation by *Ph.vitellinae* of salicin painted on leaf discs of shaved *S.caprea* into the secretion may be explained in various ways. Larvae fed with *S.caprea*, which is not their normal food plant, may be in a suboptimal physiological state. This may affect the efficiency of the defence glands. Similarly, salicin concentration may reach a suboptimal toxic level when added to the physiological amount already present in *S.nigricans*. The glandular β-glucosidases may also be saturated and unable to cope with the extra amount of salicin.

The variation in volume of secretions found between individual third instar *Ph.vitellinae* larvae is too large to show a clear relationship between the volume of the secretion and the quantity of precursor. This may in part be due to difficulty in completely emptying the glands during the collection of the secretion. It is noteworthy that the volume of the secretion of the larvae collected 'in nature' was larger than the volume of the secretion of the larvae 'milked' daily in the laboratory. This suggests that the volume of the secretion cannot be totally restored in 24 h.

The low concentration of glucose found in the secretion of *C.tremulae*, *C.populi* and *Ph.vitellinae* suggests that glucose is recovered after hydrolysis of salicin. Approximate calculations (based on published metabolic rate of similar insects (Keister & Buck, 1974), the quantity of salicin ingested and the proportion of glucose in the final secretion) show that when the secretion is collected daily, the reabsorbed glucose may cover as much as 31.6% of the daily calorific requirement of a *Ph.vitellinae* larva.

The use of salicin as a precursor of salicylaldehyde in the defensive secretion may have two advantages to chrysomeline larvae. First, it provides them with relatively cheap defence compared to that of their relatives producing monoterpenes. Secondly, it enables them to mobilize an otherwise unexploited nutritional resource. This may be particularly relevant for folivorous insects. Leaves, because of their high fibre and cellulose content, have a low available caloric density (McNab, 1978). The evolution of high β-glucosidase activity in the gut, allowing the complete hydrolysis of plant phenylglucosides, may have been prevented by the potential toxicity of the resulting aglycone or its derivatives. Exocrine glands, using the aglycone in a defence secretion, are , on the other hand, highly suitable as a site for a high β-glucosidase enzymatic activity.

The transformation of salicin to salicylaldehyde is also advantageous because salicylaldehyde is a stronger ant deterrent than salicin. Its higher volatility makes salicylaldehyde a more suitable compound to repel enemies at a distance than saligenin or salicin; at the concentration found in the glands, salicylaldehyde is liquid, the others are solids. To our knowledge there is no example of solid chemical secretions being used for defence other than mechanically. Salicylaldehyde is also present in the defence secretion of various carabids and of the anthophorid bee, *Pithitis smaragdula* (Blum, 1981). Pasteels *et al.* (1983) suggested that 'volatile irritants' such as the aldehydes, frequently encountered in defensive secretions of Arthropods, are primarily aimed at small predators and parasitoids.

The repellancy test described above using sugar solutions admittedly does not demonstrate that salicylaldehyde acts at a distance or on simple contact, as opposed to acting as a feeding deterrent. Optimal defence against predators would, however, exclude a simple feeding deterrent; further work is required to establish this unequivocally.

Thus the secretion of salicylaldehyde, derived from salicin of the host plant, appears advantageous to the chysomeline larvae feeding on Salicaceae for many reasons. It is therefore not surprising that it has evolved at least three times, in the genus *Chrysomela*, *Phratora*, and in one population of *Plagiodera versicolora* (Pasteels, 1983). A parallel evolution may have occurred in the Japanese species *G.depressa* feeding on *Juglans* (Matsuda & Sugawara, 1980).

Acknowledgments

We are grateful to the 'National Fund for Scientific Research' (Belgium) and Geigy Jubiläum Stiftung for financial support. Many thanks are also due to H. F. Rowell, D. B. McKey and D. Daloze for valuable discussions and critical reading of the manuscript.

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