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UPTAKE OF LINAMARIN AND LOTAUSTRALIN FROM THEIR FOODPLANT BY LARVAE OF *ZYGAENA TRIFOLII*

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Key Word Index—*Lotus corniculatus*; Fabaceae; *Zygaena trifolii*; Lepidoptera; cyanogenic glucosides; linamarin; lotaustralin; sequestration.

Abstract—Experiments in which unlabelled and [aglycone ^{14}C]-labelled cyanogenic glycosides, linamarin and lotaustralin, were fed to larvae of the moth *Zygaena trifolii* on leaves of an acyanogenic strain of their food plant, *Lotus corniculatus*, showed that the larvae retained about 20–45% of the glucosides consumed. The larvae in nature usually feed on plants of *L. corniculatus* which themselves contain linamarin and lotaustralin. Earlier experiments had shown that the larvae of *Zygaena* spp. are able to synthesize these glucosides from valine and isoleucine and so both sequestration and biosynthesis of the same compounds can occur. This is the only such occurrence yet known in the relationships between plants and insects.

INTRODUCTION

Larvae of *Zygaena trifolii* and many related species of the Zygaenidae contain the cyanogenic glucosides linamarin (2-β-D-glucopyranosyloxy-2-methylpropionitrile) and lotaustralin (2-β-D-glucopyranosyloxy-2-methyl-2*R*-butyronitrile) [1]. The larvae of several species (e.g. *Z. filipendulae*, *Z. trifolii*) feed on *Lotus corniculatus* (Fabaceae) which itself contains linamarin and lotaustralin in varying ratios [2]; thus both larvae and foodplants contain the same cyanoglucosides [3, 4]. Nevertheless, it has been shown by incorporation of ^{14}C-labelled and ^{13}C-enriched valine and isoleucine that the larvae of *Z. filipendulae* synthesize linamarin and lotaustralin themselves [1, 5; Nahrstedt, A. and Davis, R. H, in preparation], by specific incorporation of the respective amino acid into the aglycone part of the respective glucoside, using the intact carbon skeleton of the amino acids except for the carboxyl carbon [5]. In this respect the incorporation is identical to biosynthetic pathways of cyanogenic glucosides established in plants [6]. However, several observations during estimation of quantitative data on the distribution and ratio of both glucosides in larvae and their individual foodplants made it worthwhile to investigate whether the larvae of *Zygaena* spp. are able use the cyanoglucosides of their foodplants in addition to their own biosynthetic products.

RESULTS AND DISCUSSION

Comparison of the linamarin:lotaustralin ratio of individual foodplants from natural populations with that of larvae of *Z. trifolii* or *Z. filipendulae* feeding upon them indicates a similarity of the glucoside composition of host and herbivore (Fig. 1). The larvae feeding on plants which had a low linamarin portion also had a low linamarin portion in their total cyanoglucoside mixture (i.e. population 'Flamborough'), and vice versa (i.e. population 'Birmingham'). Only the 'Rubery' population does not fit. These values suggest an influence of the glucoside composition of the foodplant on the glucoside composition of the larvae, although variations in the biochemical potency of the different larval populations cannot be ruled out. If the foodplant influences the glucoside mixture of the larvae two explanations are possible.

First, the ratio of glucosides in the foodplant resembles its ratio of biogenetic precursors, valine and isoleucine; a foodplant low in linamarin is also low in valine, thus resulting in a poor linamarin synthesis in the larvae from poor supply of valine from their food. This hypothesis has not been tested in the present experiments but some indications have previously been obtained for imagines of *Heliconius melpomone* (Nymphalidae) that show the influence of the precursor availability on the glucoside ratio [7].

Second, the larvae are able to use the cyanoglucosides of their foodplant directly and in addition to their own capacity for synthesis. This hypothesis has been tested and proved by the following feeding experiments, using *Z. trifolii* and an acyanogenic strain of *L. corniculatus* (C-27) which is nearly free of cyanoglucosides and the corresponding β-glucosidase which is able to hydrolyse linamarin and lotaustralin.

Two sets of five final instar larvae of *Z. trifolii* were fed with either linamarin (18 mg) or lotaustralin (21 mg) on leaves of acyanogenic *L. corniculatus* over 70 hr, followed by starvation for 15 hr. During this time the first set consumed 14 mg (57 μmol) linamarin, the second 16 mg (61 μmol) lotaustralin. Table 1 shows that the larvae fed with linamarin possess a higher linamarin : lotaustralin ratio (65:35) when compared to the control (55:45), whereas those fed with lotaustralin show a higher proportion of lotaustralin (38:62). To eliminate developmental variation the values were calculated on the basis of 1 g dry weight: the values for lotaustralin of the control (82 μmol) and of the linamarin fed larvae (100 μmol) are

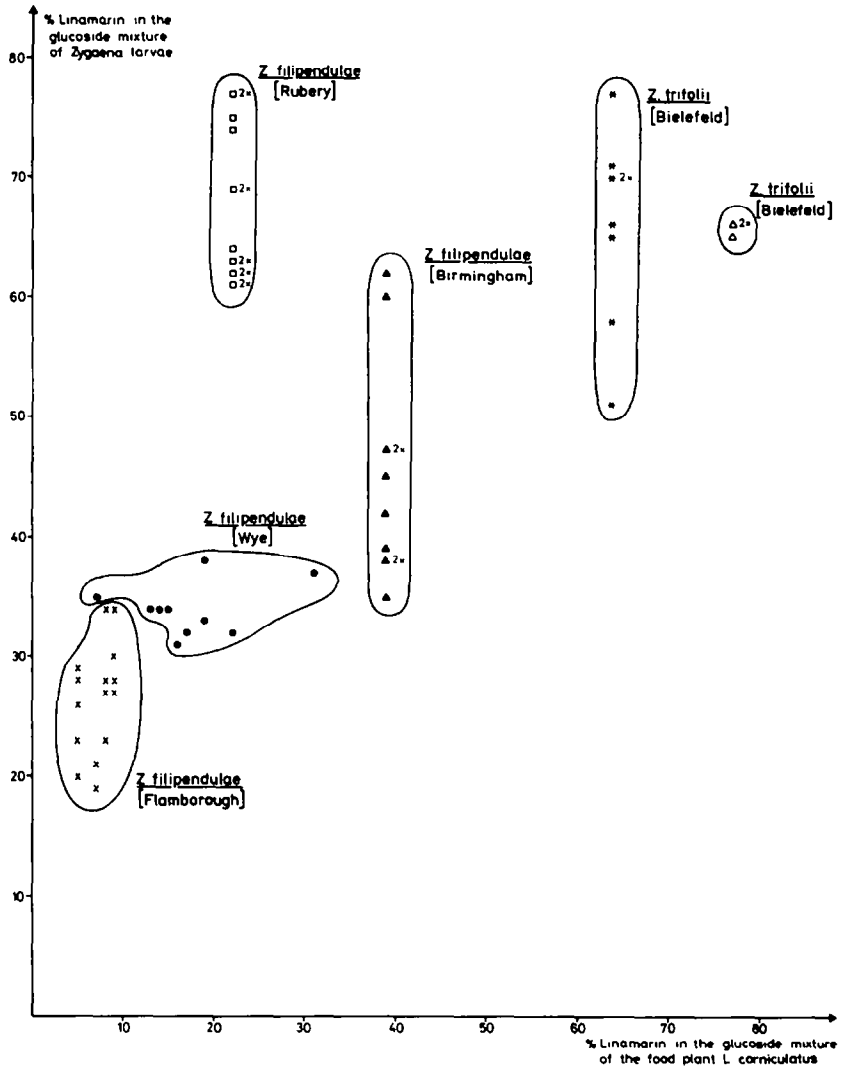

Fig. 1. Correlation between the composition of the cyanoglucoside mixture (linamarin and lotaustralin) of the foodplant *L. corniculatus* and the larvae of *Zygaena* spp. Each column of marks in the population 'Flamborough' and 'Wye' represent larvae feeding on one individual plant of the same population; from the populations 'Rubery', 'Birmingham' and 'Bielefeld' only combined plant material was available. Each mark represents one and the sign 2 × two larvae.

Table 1. Contents of linamarin and lotaustralin in three sets of larvae of *Zygaena trifolii*

Sample	Dry wt (mg)	per set (μmol)			per 1g dry wt (μmol)	
		CN^-	lin	lot	lin	lot
Five larvae consumed 57 μmol linamarin	139	40.0	26.0 (65%)	14.0 (35%)	187	100
Five larvae consumed 61 μmol lotaustralin	155	39.5	15.0 (38%)	24.5 (62%)	97	158
Four larvae consumed leaves (control)	70	12.8	7.0 (55%)	5.8 (45%)	100	82

Two sets were fed with either linamarin or lotaustralin on leaves of *Lotus corniculatus* (strain C-27); the third set was fed with untreated leaves.

similar, the values for linamarin of the control (100 μmol) and of the lotaustralin fed larvae (97 μmol) are nearly identical; the linamarin content in the linamarin fed larvae was higher by 87% (187 μmol) in comparison to the control (100 μmol) and the lotaustralin content in the lotaustralin fed larvae by 92% (158 μmol) in comparison to the control (82 μmol). These data allow the calculation of the amounts of glucoside apparently retained after feeding, on the basis of the actual dry matter of each set, which is *ca* 12 μmol (21% of 57 μmol consumed) for

linamarin and *ca* 11.5 μmol (19% of 61 μmol consumed) for lotaustralin. The faeces of the larvae were tested for cyanoglucosides and contained 0.5% (linamarin set) and 0.8% (lotaustralin set) of the consumed glucosides indicating that the remainder, *ca* 80%, must have been metabolized. Thus, consumption of a glucoside enhanced the tissue content of that particular glucoside, providing strong indications that food composition influences the tissue content and that sequestration occurs.

These results and indications were clearly confirmed by a feeding experiment with [aglycone ^{14}C]glucosides. In the second experiment one set of six and one of seven final instar larvae were fed with either 30 μmol [aglycone ^{14}C]linamarin or 16 μmol [aglycone ^{14}C]lotaustralin, respectively, on leaves of *L. corniculatus* (C-27 strain) over 120 hr followed by starvation for 15 hr. The results are displayed in Table 2. The percentage ratio of the glucosides in the sets fed labelled glucosides changed in the expected direction but not as drastically as in the first experiment. On the basis of 1 g dry matter the increase for linamarin was *ca* 50%, that for lotaustralin was *ca* 25% when compared to the control. Again the linamarin content of lotaustralin-fed larvae (161 μmol) and of the controls (148 μmol) and the lotaustralin content of the linamarin-fed larvae (114 μmol) and of the controls (98 μmol) were fairly close. On the basis of the dry weight of each set and the mean control values of linamarin (~ 155 μmol) and lotaustralin (~ 105 μmol) the uptake of linamarin was *ca* 45%, that of lotaustralin *ca* 36%. The faeces of the larvae was tested for radioactivity and contained 5.5% (linamarin set) and 2.5% (lotaustralin set) of the consumed glucoside indicating that about 50–60% must have been metabolized. The differences between these values and those from the first experiment probably are due to the larger number of larvae and the smaller amount of glucosides which led to the smaller intake for each larva. The longer period over which the glucosides were fed in the second experiment may also have influenced the partitioning between sequestration and metabolism.

The isolated and purified glucosides of the two sets that had been dosed show a clear difference in their specific activity (Table 2); those larvae fed with labelled linamarin show 75 times higher label in isolated linamarin than in isolated lotaustralin; in those fed with labelled lotaustralin the label in the isolated lotaustralin is 170 times that of the isolated linamarin. Thus, the absorbed glucoside is not used to form the alternative glucoside, i.e. linamarin is not used to form lotaustralin and vice versa. On the other hand there is a large amount of glucoside consumed, *ca* 80% in the first experiment, *ca* 50–60% in the second, which is not stored intact and thus must have been metabolized. Further experiments will show whether the larvae are able to hydrolyse the consumed glucosides using the resulting HCN to supply nitrogen for metabolism via β-cyanoalanine, which has been detected in *Zygaena* spp. [8], and asparagine [6]. It cannot be ruled out from the present experiments that both cyanoglucosides are hydrolysed in the gut before absorption to the corresponding cyanohydrins, absorbed as such and then re-glucosylated by a larval glucosyltransferase. Such a pattern is discussed for sequestration of *N*-methylazoxymethanol glycosides by *Seirarctia echo* (Arctiidae, Lepidoptera) [9]. Further experiments to clarify this question will be done when new larvae are available.

The present results have been obtained with a special system in that the C-27 strain of *L. corniculatus* did not introduce cyanoglucoside hydrolysing β-glucosidase activity into the intestinal tract of the larvae. However, the gut content of larvae of *Z. trifolii*, which had been obtained by dissection, does not show any remarkable β-glucosidase activity [Ackermann, I. and Nahrstedt, A., unpublished results]. It is not known whether larvae of *Zygaena* spp. do feed in nature on acyanogenic *L. corniculatus*; in the laboratory the larvae of *Z. filipendulae* and *Z. lonicerae* showed no preference for either cyanogenic or acyanogenic *L. corniculatus* [10]. Nevertheless, these results clearly demonstrate that sequestration of linamarin and lotaustralin by the larvae is possible. It is, as far as we know, unique in the relationships between plants and insects that the herbivore is able to synthesize the same compounds as are stored in its foodplant and additionally acquires these compounds by feeding. The exceptionally high content of cyanoglucosides in *Zygaena* spp. feeding on cyanogenic *Lotus* spp. is probably influenced by this capacity; other cyanogenic lepidoptera, i.e. *Heliconius* spp. (Nymphalidae) which feed on plant resources lacking these same glucosides show far smaller amounts of linamarin and, if present, lotaustralin [7].

Table 2. Contents of linamarin and lotaustralin in three sets of larvae of *Zygaena trifolii*

Sample	Dry wt (mg)	per set (μmol)			per 1 g dry wt (μmol)		Sp. act. (dpm/μmol)	
		CN^-	lin	lot	lin	lot	lin	lot
Six larvae consumed [aglycone ^{14}C]linamarin 2.6×10^6 dpm (23 μmol)	154	52.9	35.4 (67%)	17.5 (33%)	230	114	0.22×10^5	0.003×10^5
Seven larvae consumed [aglycone ^{14}C]lotaustralin 4×10^6 dpm (13.2 μmol)	177	51.6	28.5 (55%)	23.2 (45%)	161	131	0.002×10^5	0.34×10^5
Five larvae consumed untreated leaves (control)	128	31.5	18.9 (60%)	12.6 (40%)	148	98	—	—

Two sets were fed with [aglycone ^{14}C]linamarin and lotaustralin on leaves of *Lotus corniculatus* (strain C-27); the third was given untreated leaves for control. The last column shows the specific radioactivity of the isolated glucosides.

EXPERIMENTAL

Plant material. Leaves of an acyanogenic strain of *Lotus corniculatus* (strain C-27, obtained from Prof. D. A. Jones, Hull) were produced in a greenhouse for use in feeding experiments. This strain contained less than 0.05 μmol/g dry wt of cyanoglucosides (cf. cyanogenic plants occurring in Braunschweig; *ca* 77 μmol/g dry wt) and a β-glucosidase (if present) hydrolysing less than 0.002 μmol linamarin/g fr. wt/min (plants in Braunschweig; 0.32 μmol linamarin/g fr. wt/min).

Feeding. Final instar larvae (obtained from H. Seipel; Büttelborn) were kept individually in small (2.5 cm) petri dishes at room temp. (*ca* 20°). Before feeding they were starved for 24 hr. Fresh leaves of *L. corniculatus* were impregnated with a concd soln of the respective compound in MeOH, allowed to dry for several minutes and presented to the larvae which usually consumed 1–2 leaves per day. Residual plant material and faeces were collected in order to estimate their content of cyanogenics or radioactivity and hence to estimate cyanoglucoside consumption.

Preparation of labelled cyanoglucosides. To prepare [aglycone ^{14}C]linamarin and lotaustralin a set of 29 larvae of *Z. trifolii* was fed with L-[U^{14}C]valine and L-[U^{14}C]isoleucine (Amersham-Buchler, Braunschweig) on leaves of *L. corniculatus* for 2.5 days. The larvae were worked up as described previously [7] and both glucosides were separated and purified by HPLC resulting in 8 mg (30 μmol) linamarin with a specific activity of 1.1 × 10^5 dpm/μmol and 4 mg (16 μmol) lotaustralin with a specific activity of 2.5 × 10^5 dpm/μmol. Incidentally, these results show that the larvae of *Z. trifolii* can also synthesize both glucosides from the respective amino acids.

Extraction. After the feeding period the larvae were allowed to starve for 15 hr in order to allow elimination of as much plant material as possible from their intestine. They were then frozen in liquid N_2 and washed several times at −90° with MeOH in order to clean the surface of any adhering glucosides. The glucoside removed in this way were *ca* 0.5–1% in the first experiment (measured using the cyanide estimation) and 0.4–0.7% in the second one (counting the radioactivity). The larvae were then minced in liquid N_2 and freeze dried. The dry material was defatted with petrol (2 hr) by refluxing and extracted in the same way with MeOH (3 hr). The MeOH extract was used for further examinations.

Cyanide estimation. An aliquot of the MeOH extract was taken to dryness and incubated at 40° for 18 hr with phosphate buffer pH 6, 50 mg semicarbazide dihydrochloride and 10 μl of a highly purified β-glucosidase capable of hydrolysing linamarin and lotaustralin ('linamarase') from *Hevea brasiliensis* [11]. HCN was liberated by aeration [12] and trapped in 2–3 ml 0.1 N NaOH, from which it was estimated using the anthranilic acid method [13]. Total cyanide content represents the total cyanoglucoside content.

GC analysis. An aliquot of the MeOH extract was taken to dryness, dissolved in 0.05 ml dry pyridine and silylated with bistrimethylsilyltrifluoroacetamide (0.1 ml) and trimethylchlorosilane (0.05 ml). After 1 hr 1–3 μl were injected into the GLC system: OV-101 3% on gaschrom Q 100–120 mesh; 180 cm × 0.2 cm i.d., glass; N_2 20 ml/min; 155–230°, 2°/min; FID 230°, injector 200°; R_t linamarin *ca* 23 min, R_t lotaustralin *ca* 25.5 min. The percentage ratio of the glucosides was calculated from the area under each signal; the absolute amount of both glucosides was estimated from these values and the total cyanide (= glucoside) content.

HPLC separation. An aliquot of the MeOH extract was taken to dryness, suspended in H_2O and filtered through a 0.45 μm membrane filter. The filtrate was injected one or in parts into a HPLC system: RP-18, 1.6 × 25 cm; MeCN–H_2O, 1:19, 4.5 ml/min; RI-detector; R_t linamarin *ca* 20 min, R_t lotaustralin *ca* 47 min. The separate fractions were collected and lyophilized.

Estimation of radioactivity. As described in [7] the HPLC fractions were hydrolysed by the 'linamarase' from *Hevea brasiliensis* [11]. The HCN liberated was absorbed, estimated quantitatively [13] and its radioactivity measured in an Unisolve (Baker Chemicals) scintillator. These data allow calculations of the radioactivity in the nitrile group per μmol; multiplication by four gives the specific activity of linamarin, by five that of lotaustralin.

Calculations. The amount of glucoside consumed was calculated by subtraction of either the glucoside (estimated by HCN) or the radioactivity remaining on the glassware and unconsumed plant material, from the amount applied. In order to estimate the amount of glucoside apparently absorbed either the control value or the mean between the control value and value for the set fed the alternative glucoside, both based upon 1 g dry wt, were used. This gives the theoretical value that would have been obtained if the compound administered had not been absorbed as follows: theoretical value (μmol) = dry wt of set fed × glucoside content of control (μmol/g dry wt). Subtraction of this value from that measured gives the amount absorbed.

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