SEQUESTRATION OF INGESTED \(^{14}C\)CISENECIONINE N-OXIDE IN THE EXOCRINE DEFENSIVE SECRETIONS OF CHRYSMELID BEETLES

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Abstract—Oreisca canaliculata (Chrysomelidae) sequesters in its elytra and pronotum defensive secretion the N-oxides of pyrrolizidine alkaloids (PA N-oxides) from its food plant Adenocaulon alliariae (Asteraceae). \(^{14}C\)Cisenecionine N-oxide was applied for detailed studies of PA N-oxide sequestration. An average of 11.4% of total radioactivity is taken up by individual beetles which had received \(^{14}C\)Cisenecionine N-oxide with their food leaves 8 days before. An average of 28.9% of the ingested radioactivity could be recovered from the defensive secretions collected twice, i.e., 3 and 8 days after tracer feeding. The tracer transfer into the secretion zones is a slow but progressive process as indicated by the high percentage of tracer still recovered from the secretion samples after 8 days. Chromatographic analysis revealed that \(^{14}C\)Cisenecionine N-oxide is the only labeled compound present in the defensive secretion. Bees that fed on tertiary \(^{14}C\)Cisenecionine sequestered only trace amounts of radioactivity (exclusively present as labeled N-oxide) in their secretions. O. speciosus, a species also adapted to PA containing food plants, was shown to sequester \(^{14}C\)Cisenecionine N-oxide with the same efficiency as O. canaliculata. O. bifrons, a specialist feed on Chrysobalanus icaco (Apocynaceae), injected PA treated leaf samples already at very low PA concentrations (10 microgram/piece). In both O. canaliculata and O. speciosus, \(^{14}C\)Cisenecionine N-oxide applied by injection into the hemolymph is rapidly transferred into the glands. O. bifrons, not adapted to

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Pyrrolizidine alkaloids (PAs) are a group of plant secondary compounds which are receiving much attention in the study of insect-plant relationships (see Boppel, 1986, 1990; Schneider, 1987). Although it is likely that they are produced by plants as protective agents against herbivory, some plant feeders have evolved to cope with these compounds or even use them for their own protection or as pheromone precursors. Well-known examples are the arctiid moths and the damself and lycaenine butterflies.

The leaf beetle *Oreina caccia* (Chrysomelidae) was recently shown to sequester plant-derived PAs in the form of their N-oxides (PA N-oxides) in its defensive glands (Pastoels et al., 1988a). This is the first time that PAs have been reported to be involved in exocrine chemical defense. Leaf beetles are specialist herbivores and chemically defended, but sequestration of plant chemicals for defense has been established unambiguously in only a few of them (Pastoels et al., 1988b), e.g., the sequestration-in hemolymph and eggs of cucurbitacin and conjugates by cucumber beetles (*Galerucinae*, *Luperina*; Ferguson and Metcalf, 1985).

In the subfamily Chrysomelinae, defensive compounds are typically biosynthesized de novo and stored in pronotal and elytral glands (Pastoels et al., 1988b). *Oreina caccia* was the first example of host plant influence on these sequestrations. It feeds on *Adenostyles alliariae*, *Senecio jacquinii*, and to a lesser extent on *Petasites paradoxa* (all members of the Asteraceae) but sequesters in its seeretions exclusively PA N-oxides derived from *A. alliariae* (Rowell-Rahier et al., 1991). *O. caccia* does not synthesize the cardenolides found in the secretions of all of the other species of the genus and of most members of the related genus *Chrysolina* (van Osdal et al., 1987, 1988; Rowell-Rahier et al., unpublished). Most interestingly, a second species, *O. speciosissima*, feeding on the same host plants as *O. caccia*, was also found able to sequester PA
N-oxides from *A. alliariae* but to still possess the capability for *de novo* cardiacide biosynthesis (Rowell-Bailie et al., 1991).

In this study, we investigated by means of 14C-labeled senecionine and its N-oxide the ability of *O. caucalis* to sequester PAs. Labeled PAs were offered either orally or administered by injection into the hemolymph in order to circumvent the gut barrier. In addition, the ability to sequester and N-oxide the tertiary PAs and to synthesize PAs *de novo* was tested. For comparison, *O. speciosissima* and the related *O. bifrons*, a species not adapted to PA-containing plants which feeds on *Chasaphyllum itrazutum* (Aplacaceae), were included in the experiments.

**MATERIAL AND METHODS**

**Insects**

The beetles were collected in May and June 1988/89 and kept at room temperature on their host plants or at 4°C for 1 to 3 weeks until use. 

*O. caucalis* specimens originated from Wasserkiefern (Vosges, France) and Zastler (Schwarzwald, Germany), *O. speciosissima* from La Lécherette (Vaud, Switzerland) and Zastler (Schwarzwal, Germany), and *O. bifrons* from Höhwald (Vosges, France).

**Radioactive Tracers**

[14C]Senecionine (1.07 GBq/nmol) and its N-oxide were prepared biosynthetically from [1,4-14C]petasine (4.4 GBq/nmol) using root cultures of *Senecio vulgaris* according to Eltse et al. (1988). Labeled petasine was obtained from Amersham Buchler (Braunschweig, Germany).

**Tracer Feeding Experiments**

The beetles were placed individually in Petri dishes (5 cm diam.), kept at room temperature (20-22°C) and fed during 24 hr with a disk (1 cm diam.) or a piece of host plant leaf treated with 10 μl of a methanolic solution of [14C]senecionine N-oxide (8.3 kBq/μl) or [14C]petasine (8.7 kBq/μl). After 24 hr, the frass and leaf remains were collected, and the beetles were transferred to fresh untreated host plant leaves. This transfer was repeated every day until the end of the experiment. Frass and defensive secretions were collected at the time intervals given in the results section. At the end of the experiment, the beetles were deep frozen prior to chemical extraction.
Injection of Labeled Compounds

An ethanol solution (0.5 μl) of [14C]seneconine N-oxide (1.0 kBq/μl) or 1 μl of [14C]seneconine (0.8 kBq/μl), or 1 μl of [1,4-13C]putrescine (2.1 kBq/μl) was injected into the abdominal cavity of the beetles with a 1 μl microsyringe (Hamilton) or a individually calibrated micropipette. The beetles were subsequently treated as described above.

Collection of Defensive Secretions

The beetles were manipulated with fine forceps in order to provoke emission of the secretion which was collected on filter papers and immediately placed in MeOH until analysis.

Alkaloid Extraction

Individual deep-frozen beetles were ground with 2.0 ml acidic MeOH (1% HCl) and quartz sand in a mortar for 10 min. The homogenate was suspended in a total volume of 5.0 ml acidic MeOH and after centrifugation the supernatant was used directly for analysis of total radioactive by scintillation counting or TLC-analysis. Frass samples were extracted in the same way.

Thin-Layer Chromatography (TLC)

MeOH solutions of secretions and crude MeOH extracts of beetles and frass were analyzed by TLC and evaluated quantitatively by means of a TLC multichannel analyzer (Rin-32a, Raytest). Separation was obtained on Silica gel 60 Merck) using the solvent system: CH2Cl2:MeOH:NH4OH (25%) (82:15:3, by vol).

High Pressure Liquid Chromatography (HPLC)

Reverse-phase ion-pair HPLC (Wagner et al., 1986) was used to separate PA N-oxides according to Hartmann et al. (1988). [14C]-labeled PA N-oxides were detected with the HPLC radioactivity monitor LB-306D (Berthold) equipped with a 2 ml flow-cell and the split-mixer LB-5035.

RESULTS


In order to study the efficiency of PA uptake and storage, leaf-disks of A. alliariae treated with [14C]seneconine N-oxide and [14C]seneconine were offered individually to O. caucasus beetles. The beetles consumed the leaf-disks
usually within 24 hr and were subsequently allowed to feed on untreated food leaves. Specimens with pre-empted glands were employed. The defensive secretions were collected 5 days after the beginning of the experiment and in the N-oxide feeding experiment again 5 days later and were analyzed and compared with the MeOH-soluble radioactivity found in the frass and the bodies of the beetles (Table 1). In spite of great individual variation which is probably mainly due to differences in feeding behavior and activity, the data clearly show that a considerable proportion of the tracer is ingested by the beetles, i.e., an average of 11.4% (seneceonine N-oxide) and 4.8% (seneceonine). Only trace amounts of radioactivity could be recovered from the secretions of beetles that fed an tertiary [14C]seneceonine, whereas an average 28.9% of ingested radioactivity was found in the secretions of beetles that fed on [14C]seneceonine N-oxide. The tracer transfer into the secretion seems to be a slow and progressive process as indicated by the high percentage of tracer still recovered from the second secretion sampled after 8 days. Chromatographic analysis of the secretions revealed absolutely pure [14C]seneceonine N-oxide. Not even traces of labeled tertiary seneceonine or any other metabolite could be detected (Figure 1A). Reversed-phase ion-pair HPLC confirmed the purity; neither labeled senecephiline N-oxide nor any other potential derivative of seneceonine N-oxide could be detected. On the other hand in crude beetle extracts, [14C]seneceonine N-oxide was always found to be accompanied by at least trace amounts of labeled seneceonine and polar metabolites (Rf < 0.1; Figure 1B).

**Table 1. Distribution of MeOH-extractable Radioactivity (Mean ± SD) After Injection of Either [14C]Seneceonine N-Oxide or [14C]Seneceonine by O. cucullatus. Each Individual Beetle with Pre-Empted Glands was Fed with 8.3 kBq ([14C]Seneceonine N-Oxide) or 8.7 kBq ([14C]Seneceonine) Deposited on Leaf-Discs (A. alibaccia). After 24 hr, the Beetles were Allowed to Continue Feeding on Untreated Leaves**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of sampling (d)</th>
<th>[14C] Seneceonine N-Oxide (n = 9)</th>
<th>[14C] Seneceonine (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pass I</td>
<td>1</td>
<td>20.0 ± 6.6</td>
<td>18.5 ± 7.2</td>
</tr>
<tr>
<td>Pass II</td>
<td>2</td>
<td>3.9 ± 2.0</td>
<td>3.6 ± 2.5</td>
</tr>
<tr>
<td>Secrecion I</td>
<td>5</td>
<td>1.7 ± 1.1</td>
<td>Trace</td>
</tr>
<tr>
<td>Secrecion II</td>
<td>8</td>
<td>1.6 ± 1.3</td>
<td>Not collected</td>
</tr>
<tr>
<td>Secrecion = body</td>
<td>8</td>
<td>8.1 ± 4.2</td>
<td>4.8 ± 4.5</td>
</tr>
<tr>
<td>Percent in secretion§</td>
<td></td>
<td>11.4 ± 5.7</td>
<td>4.8 ± 4.5</td>
</tr>
<tr>
<td>Percent in secretion§</td>
<td></td>
<td>28.9 ± 11.4</td>
<td>11.4</td>
</tr>
</tbody>
</table>

*100% = Total radioactivity in the beetles (secretions + beetle extracts).
Fig. 1. Sequestration and elimination of $[^{14}C]$seneconine N-oxide fed orally to O. coarctator. TLC separation of crude MeOH extracts of A: defensive secretion, B: beetle extracts, C = frass (24 hr) extracts. Detection: TLC radioimaging.

The trace amount of radioactivity found in the secretions of $[^{14}C]$seneconine-fed beetles was exclusively in the N-oxide form, whereas extracts of these beetles were found to contain mainly tertiary $[^{14}C]$seneconine and only a small proportion of the N-oxide (ca. 20% of total alkaloid in the body).

The total amount of MeOH-soluble radioactivity eliminated by the beetles with the frass during 2 days was on average 23.9% (N-oxide fed) and 22.3% (seneconine fed), respectively. Most of the radioactivity was eliminated during the first 24 hr (Table 1, Figure 1). Within two days, the elimination of radioactivity decreased to a level of less than 1% per day (Figure 1). Chromatographic analysis of frass extracts showed that during gut passage considerable amounts of $[^{14}C]$seneconine N-oxide were reduced to tertiary seneconine and degraded to polar metabolites (Figure 1C). Only trace amounts of the N-oxide form were detected in frass extracts of beetles that fed on $[^{14}C]$seneconine.

The proportion of total radioactivity recovered as MeOH-soluble com-
pounds from secretions, beetles, and frass is on average only about 27% of radioactive given (Table 1). Apparently, considerable proportions of the tracers fed are transformed into MeOH insoluble material. The fate of insoluble radioactivity has not been followed in the course of the present experiments; however, in comparable feeding studies with Tyria jacobeae (Biller et al., unpublished), the loss of labeled seneconine N-oxide into insoluble material occurred most probably during the gut passage, and its endproducts were found to be associated with the frass.

In a second experiment, designed in the same way as that shown in Table 1, ten beetles that were “milked” prior to the tracer feeding were compared to ten individuals that were not “milked.” The results show that the incorporation into the secretion is significantly lower (ten times) in the beetles with full glands (1.6% ± 1.1) than in those with empty glands (13.9 ± 5.6) (data not shown).

*Sequestration of Orally Fed [14C]Seneconine N-Oxide by O. speciosissima and O. bifrons*

With *O. speciosissima* beetles fed on labeled seneconine N-oxide, results were obtained which are comparable to those described above for *O. caucalis*. *O. bifrons*, a specialist feeding on *Chasenothrura hirsutum* (Apionaceae), that during its life history never gains access to pyridizidine alkaloids, behaves differently. At alkaloid N-oxide concentrations of 10 nmoles per leaf disk, the concentration at which the tracer was applied, nine out of ten beetles injected their alkaloid-treated host plant, whereas control beetles fed well on untreated leaves. No radioactivity was found in the secretions collected from the one individual that was willing to feed on a treated leaf.

*Sequestration of Injected [14C]Seneconine N-Oxide by O. caucalis, O. speciosissima, and O. bifrons*

To test the ability of the three species to sequester and excrete seneconine N-oxide, [14C]-labeled N-oxide was directly injected into the hemolymph of the beetles. The distribution of radioactivity in frass, secretion, and beetle was analyzed (Table 2). The results are given individually for nine specimens in each series, to demonstrate the variability between replicates. To some extent, this variability is due to the difficulty of reproducibly injecting 0.5 µl into the abdominal cavity of the beetle. Nevertheless, the results clearly show that 5 days after injection, considerable amounts of radioactivity are in the defensive secretions and bodies of *O. caucalis* and *O. speciosissima*. Another part of the radioactivity is rapidly eliminated with the frass. This elimination seems to be more rapid in *O. caucalis*. Here, only trace amounts of radioactivity were detected in the frass collected on the second day, whereas in *O. speciosissima*, substantial amounts of radioactivity were measured in the frass of the second
<table>
<thead>
<tr>
<th>Time of sampling (d)</th>
<th>O. confluent</th>
<th>O. speciosus</th>
<th>O. bifrons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.8</td>
<td>14.2</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>22.7</td>
<td>4.7</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>8.3</td>
<td>0.9</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>5.8</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>6.4</td>
<td>nd</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>4.2</td>
<td>nd</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>5.1</td>
<td>nd</td>
<td>tr</td>
</tr>
<tr>
<td>9</td>
<td>19.2°</td>
<td>13.5</td>
<td>21.1</td>
</tr>
<tr>
<td>Mean value (±SD)</td>
<td>7.4°</td>
<td>12.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Sampling after 4 d; nd = not determined, tr = traces, — = not detectable.
day. *O. bifrons*, not adapted to pyrrolizidine-containing plants, is, as expected, unable to sequester [14C]senecionine N-oxide either in the body or in the secretion. It is, however, as efficient as the other two species in eliminating injected alkaloid from its body. Most of the radioactivity is voided with the frass within the first 24 hr (Figure 2).

Analysis of the different fractions again revealed [14C]senecionine N-oxide as the only labeled compound in the defensive secretions, whereas in beetle extracts, a considerable amount of unknown labeled polar metabolites was detected in addition to the N-oxide.

Only trace amounts of [14C]senecionine N-oxide were found in the secretions of *O. calicai* if labeled seneconine was injected instead of its N-oxide.

**Injection of [14C]Putrescine into O. calicai**

To test the ability of the beetle to synthesize de novo pyrrolizidine alkaloids, ten beetles were injected with labeled putrescine, a known alkaloid precursor. In none of the beetles was activity found in the secretions, nor could any traces of alkaloid be detected.

**DISCUSSION**

[14C]senecionine N-oxide is efficiently taken up by *O. calicai* and transported via the exocrine glands into the defensive secretions. On its way from the gut into the secretion, it has to pass at least two sets of membrane barriers,
the gut epithelium and the gland cell membranes. The rapid uptake of the PA
N-oxide from the gut and its accumulation in the beetle seems to be followed
by a slow and continuous transfer into the defensive glands (see Table 1). This
transport is highly specific in two respects: (1) [14C]Seneconine N-oxide is
recovered from the defensive secretion in a virtually pure state. No labeled
transformation or degradation products are detectable. This is in accordance
with the observation that PA N-oxides from food plants such as Adonis amurensis
and Senecio fuchsii are taken up rather unspecifically by the beetle,
but only the PA N-oxides acquired from A. alliariae are transferred into the
glands (Rowell-Rahier et al., 1991). (2) Only the N-oxide form is translocated
into the secretions, not even traces of the tertiary form being detectable (see
Figure 1A). This is in good agreement with the identification exclusively of PA
N-oxides in the secretions of O. cucullae collected in nature (Pustelko et al.,
1988a; Rowell-Rahier et al., 1991). In this respect, O. cucullae behaves like
other specialized PA sequestering insects, e.g., Tyria jacobaeae (Ehmske et al.,
1990) and Creatonotos transiens (von Nickisch-Rosenegk et al., 1990; Hart-
mann et al., 1990). In contrast to these insects, however, which easily transform
tertiary PAs into the respective N-oxides and thus are able to sequester the
tertiary form with the same efficiency as the N-oxide, O. cucullae does not
possess this ability. If labeled seneconine is offered orally or administered by
injection into the hemelymph, only traces of radioactivity (exclusively
[14C]seneconine N-oxide) are detected in the secretions.

This once again emphasizes the physiological importance of the N-oxide
form of the pterylidine alkaloids in plants as well as in adapted insects. Plants
are known to synthesize PAs as N-oxides (Hartmann and Toppel, 1987) and to
use the polar, salt-like N-oxide as the molecular form for specific transport and
cellular accumulation (Hartmann et al., 1989). Carrier-mediated membrane
transport has been demonstrated for PA-containing plants (Senecio vulgaris)
(Ehmske et al., 1988) as well as for PA-sequestering insects (Creatonotos tran-
siens; Wink and Schneider, 1988). Characterization of the two different carrier
systems present in O. cucullae and O. speciosissima, i.e., of the gut epithelia
cell membranes and the gland cell membranes, will be challenging tasks.

It is surprising that O. speciosissima sequesters orally fed or injected
[14C]seneconine N-oxide with same efficiency as O. cucullae, but it confirms
previous findings (Rowell-Rahier et al., 1991). In nature, O. speciosissima feeds
preferentially on Pseudaletia variolosa, the leaves of which contain no PAs or at
least amounts undetectable with our analytical methods (Rowell-Rahier et al.,
1991). Specimens collected in nature are reported to sequester either no PA N-
oxides (Pustelko et al., 1988a) or only very small amounts, which represent the
PA pattern found in A. alliariae (Rowell-Rahier et al., 1991). On the other
hand, in contrast to O. cucullae, O. speciosissima still synthesizes cardenolides
(Rowell-Rahier et al., 1991). Why does not O. speciosissima use in nature its
ability to sequester PAs, and why has it evolved if it is not used? These are
intriguing questions which must await a better understanding of the biological significance of this sequestration.

Sequestration in the glands does not seem to have evolved as a detoxification mechanism, since the absolute amount sequestered is negligible compared to the amount ingested and excreted. Furthermore, the mechanisms which eliminate the polar N-oxides from the body via the gut seem to be as efficiently developed in unspecialized beetles, e.g., O. bifrons, as demonstrated by the injection experiment (see Table 2).

The role of PA N-oxide sequestration in defense is suggested by the facts that it occurs in secrections which are known to be defensive in related chrysomelids, and that in O. coccineus PAs have completely replaced the production of other toxins, i.e., cardenolides. PA N-oxide concentrations as high as 0.3 mol/l reported for the secretion of O. coccineus (Rowell-Rahier et al., 1991) should ensure an efficient protection, judging by the concentrations found in other sequestering insects. In caterpillars of Tyria jacobaeae the PA N-oxide concentrations may reach concentrations of 10 nmol/kg fresh weight (Elmke et al., 1990). The tissue concentrations are somewhat higher if we consider the integument as the major site of PA N-oxides storage. PAs are said to be strong repellents for a wide range of animals (Boettger, 1986). A convincing example is the giant tropical orb spider Nephila clavipes, an important potential predator of butterflies. Nephila cuts out field-caught lithomniids unharmful from its web, but readily eats freshly emerged adults, which are still free of PAs (Brown, 1984). A Nephila bioassay is now used, because the spider rejects sensitively PA-protected butterflies, but eats most other "unpalatable" aposematic insects. O. bifrons used in the experiments described here is a second example. Amounts of seneconine N-oxides as low as 10 nmol offered with a piece of food leaf prevented the beetles from feeding. For comparison, no feeding detergency was observed when seneconine or monacrotaline and their N-oxides were offered in amounts of 40 μg of per leaf disk to the PA-adapted O. coccineus and O. spectabilis (Rowell-Rahier et al., 1991).

The PA N-oxides found in the defensive secretions are acquired defensive chemicals which are exclusively derived from the food plant. There is no indication of de novo synthesis of PA N-oxides by the beetles. Furthermore, there is no evidence for chemical modification of acquired PA N-oxides, as documented recently for insect-specific PA N-oxides. The callimorphines in Tyria jacobaeae (Elmke et al., 1990) and the creatonoxines of Creatonoxus transiens (Hartmann et al., 1990) are synthesized by the insects from a necine base acquired from the plant, esterified with a necine acid of insect origin.

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