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The two faces of pyrrolizidine alkaloids: the role of the tertiary amine and its *N*-oxide in chemical defense of insects with acquired plant alkaloids

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Larvae of *Creatonotos transiens* (Lepidoptera, Arctiidae) and *Zonocerus variegatus* (Orthoptera, Pyrgomorphidae) ingest ^{14}C-labeled senecionine and its *N*-oxide with the same efficiency but sequester the two tracers exclusively as *N*-oxide. Larvae of the non-sequestering *Spodoptera littoralis* eliminate efficiently the ingested alkaloids. During feeding on the two alkaloidal forms transient levels of senecionine (but not of the *N*-oxide) are built up in the haemolymph of *S. littoralis* larvae. Based on these results, senecionine [^{18}O]*N*-oxide was fed to *C. transiens* larvae and *Z. variegatus* adults. The senecionine *N*-oxide recovered from the haemolymph of the two insects shows an almost complete loss of ^{18}O label, indicating reduction of the orally fed *N*-oxide in the guts, uptake of the tertiary alkaloid and its re-*N*-oxidation in the haemolymph. The enzyme responsible for *N*-oxidation is a soluble mixed function monooxygenase. It was isolated from the haemolymph of the sequestering arctiid *Tyria jacobaeae* and purified to electrophoretic homogeneity. The enzyme is a flavoprotein with a native M_r of 200000 and a subunit M_r of 51000. It shows a pH optimum at 7.0, has its maximal activity at a temperature of 40–45°C and an isoelectric point at pH 4.9. The reaction is strictly NADPH-dependent (K_m 1.3 μM). From 20 pyrrolizidine alkaloids so far tested as substrates, the enyzme *N*-oxidizes only alkaloids with structural elements which are essential for hepatotoxic and genotoxic pyrrolizidine alkaloids (i.e. 1,2-double bond, esterification of the allylic hydroxyl group, presence of a second free or esterified hydroxyl group at carbon 7). A great variety of related alkaloids and xenobiotics were tested as substrate, none was accepted. The K_m values of senecionine, monocrotaline and heliotrine, representing the three main types of pyrrolizidine alkaloids, are 1.3 μM, 12.5 μM and 290 μM, respectively. The novel enzyme was named senecionine *N*-oxygenase (SNO). The enzyme was partially purified from two other arctiids. The three SNOs show the same general substrate specificity but differ in their affinities towards the main structural types of pyrrolizidine alkaloids. The enzymes from the two generalists (*Creatonotos transiens* and *Arctia caja*) display a broader substrate affinity than the enzyme from the specialist (*Tyria jacobaeae*). The two molecular forms of pyrrolizidine alkaloids, the lipophilic protoxic tertiary amine and its hydrophilic nontoxic *N*-oxide are discussed in respect to their bioactivation and detoxification in mammals and their role as defensive chemicals in specialized insects. Pyrrolizidine-alkaloid-sequestering insects store the alkaloids as nontoxic *N*-oxides which are reduced in the guts of any potential insectivore. The lipophilic tertiary alkaloid is absorbed passively and then bioactivated by cytochrome *P*-450 oxidase.

Keywords: *Tyria jacobaeae* (Lepidoptera, Arctiidae); pyrrolizidine alkaloid; alkaloid uptake; senecionine *N*-oxygenase; chemical defense.

Pyrrolizidine alkaloids are unique among the some 20000 plant alkaloids in respect to their attractiveness for specialized insects. A number of species from diverse taxa have evolved adaptations to sequester and utilize plant pyrrolizidine alkaloids against insectivores (Hartmann, 1991; Hartmann and Witte, 1995). Larvae of the European cinnabar moth *Tyria jacobaeae* store pyrrolizidine alkaloids from their host plant *Senecio jacobaea* and retain the alkaloids during all stages of metamorphosis (Aplin et al., 1968; Aplin and Rothschild, 1972). In the arctiid moth *Utetheisa ornatrix*, which also sequesters pyrrolizidine alkaloids from its larval food plant, both parents provide pyrrolizidine alkaloids for egg protection. Females receive the alkaloids from males during copulation and transmit these pyrrolizidine alkaloids together with their own load to the eggs (Dussourd et al., 1988). The transmission process is governed by the pyrrolizidine-alkaloid-derived male courtship pheromone hydroxydanaidal which signals the male's systemic load of pyrrolizidine alkaloids to the female and thus stimulates its behavior (Dussourd et al., 1991). Similar biparental contributions to egg defense are known from the arctiid *Creantonotos transiens*

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Abbreviations. FMO, multisubstrate flavin monooxygenase; SNO, senecionine *N*-oxygenase; FAB-MS, fast-atom-bombardment mass spectrometry.

Enzymes. Dimethylaniline monooxygenase (*N*-oxide-forming), multisubstrate flavin monooxygenase (EC 1.14.13.8); unspecific monooxygenase, xenobiotic monooxygenase, microsomal *P*-450 (EC 1.14.14.1).

(Nickisch-Rosenegk et al., 1990), the danaid butterfly *Danaus gilippus* (Dussourd et al., 1989) and a number of Brazilian Ithomiinae butterflies (Brown, 1984a,b). Besides lepidopterans, the African grasshopper *Zonocerus variegatus* (Bernays et al., 1977; Biller et al., 1994) is known to sequester pyrrolizidine alkaloids from its host plants. More recently leaf beetles (Chrysomelinae) of the genus *Oreina* have been found to sequester pyrrolizidine alkaloids from their host plants and accumulate them in their exocrineous defensive secretions at levels well exceeding a 0.5 M concentration (Pasteels et al., 1988; Hartmann et al., 1997).

Pyrrolizidine alkaloids are strongly hepatotoxic and pneumotoxic to vertebrates (Mattocks, 1986; Cheeke, 1989) and genotoxic to insects (Frei et al., 1992). Since the pioneering discovery of Mattocks and his collaborators (Butler et al., 1970), who first suggested a pyrrolic metabolite as responsible for pyrrolizidine alkaloid toxicity, the mode of action of hepatotoxic pyrrolizidine alkaloids has been completely unraveled (Mattocks, 1986; Winter and Segall, 1989). In vertebrates microsomal cytochrome *P*-450 oxidases catalyze the conversion of pyrrolizidine alkaloids into unstable pyrroles which are highly reactive alkylating agents. The microsomal P-450 enzymes are part of xenobiotic-metabolism, mainly localized in liver and lung tissue, and participate in the transformation of lipophilic foreign compounds into excretable metabolites. In the case of the pyrrolizidine alkaloids, however, a *per se* nontoxic compound is converted into a toxic metabolite (bioactivation). Insects which have a similar xenobiotic metabolism with microsomal cytochrome *P*-450 enzymes (Hodgson, 1985; Brattsten, 1992) should be affected by potentially toxic pyrrolizidine alkaloids in the same way as vertebrates. Besides bioactivation, two other pathways of pyrrolizidine alkaloid metabolism have been established: hydrolysis of the ester bonds and, more important, *N*-oxidation. The two reactions are believed to be detoxification mechanisms (Cheeke, 1994). *N*-oxidation converts the potentially toxic tertiary alkaloid into a derivative which no longer can be transformed into a pyrrolic toxin. For example, guinea pigs possess a reactive microsomal multisubstrate flavin monooxygenase (FMO) which catalyzes efficiently the conversion of ingested pyrrolizidine alkaloids into their *N*-oxides. This explains the high resistance of guinea pigs to toxic effects of pyrrolizidine alkaloids. The *N*-oxygenation by far exceeds the cytochrome-*P*-450-dependent bioactivation (Miranda et al., 1991). On the contrary, rats which are highly susceptible to pyrrolizidine alkaloid intoxication show only very low *N*-oxidation activity (Williams et al., 1989).

In this context, it is notable that organisms adapted to deal with pyrrolizidine alkaloids generally keep these alkaloids in the *N*-oxide state. In plants such as *Senecio* species pyrrolizidine alkaloids are synthesized in the roots as *N*-oxides (Hartmann and Toppel, 1987). The *N*-oxides are specifically translocated via the phloem path into the shoots where they are stored in the cell vacuoles (Hartmann et al., 1989). A membrane carrier has been identified which selectively mediates the translocation of the polar *N*-oxides into the vacuole (Ehmke et al., 1988). Adapted insects store plant acquired pyrrolizidine alkaloids also preferentially as *N*-oxides (Mattocks, 1971). More than 80% of the pyrrolizidine alkaloids associated with *Utetheisa* eggs were found in the *N*-oxide state (Dussourd et al., 1988). Recent investigations revealed that in a number of alkaloid-sequestering species pyrrolizidine alkaloids are exclusively stored as *N*-oxides, e.g. Arctiidae (Ehmke et al., 1990; Hartmann et al., 1990; Trigo et al., 1993), Ithomiinae (Trigo et al., 1996), Orthoptera (Biller at al., 1994), chrysomelid leaf beetles (Pasteels et al., 1988, 1996; Hartmann et al., 1997). These findings indicate that storage of pyrrolizidine alkaloids as *N*-oxides must be advantageous to the sequestering species.

The present study was undertaken to understand the role of the *N*-oxide state of pyrrolizidine alkaloids in alkaloid sequestering insects. Particularly the mechanisms of resorption of orally ingested tertiary pyrrolizidine alkaloids or their *N*-oxides from the guts and their fate in the insect's body were investigated.

MATERIALS AND METHODS

Experimental insects. Larvae of *Creatonotos transiens* Walker (Lepidoptera, Arctiidae) were from a laboratory culture originated from females collected in Bali (Indonesia), larvae of *Arctia intercallaris* L. (Arctiidae) were obtained from a private breeder (Mr Kreuzer, Wackersdorf). The two species were maintained on artificial pyrrolizidine-alkaloid-free diet (Bergomaz and Boppré, 1986). *Arctia caja* L. larvae were obtained from a private breeder (Mr Sage, Niedergottsau) and reared on leaves of *Taraxacum officinale.* A total of $\approx$8000 late instar larvae of *Tyria jacobaeae* L. (Arctiidae) were collected in the field (Meijendal dunes, Leiden, The Netherlands) in July 1993 and July 1994), transported to the laboratory and kept in cages on their food plant *Senecio jacobaea* until haemolymph collection. *Spodoptera littoralis* Boisduval (Noctuidae) larvae were from a laboratory colony and reared on artificial diet (Bowling, 1967). *Zonocerus variegatus* L. (Orthoptera, Pyrgomorphidae) originated from a population collected in the Republic of Bénin (West Africa); specimens were reared from eggs on pyrrolizidine-alkaloid-free plants.

Preparation of [^{14}C]senecionine and [^{14}C]senecionine *N*-oxide. The two tracers were obtained biosynthetically by feeding of [1,4-^{14}C]putrescine (109 mCi · mmol^{-1}; Amersham Buchler) to root cultures of *Senecio vulgaris* as described by Hartmann (1994).

Feeding experiments with [^{14}C]senecionine and [^{14}C]senecionine *N*-oxide. Late instar larvae of *Spodoptera* or *Creatonotos* and adults of *Zonocerus* were fed individually with 1 mg senecionine or senecionine *N*-oxide containing 5×10^5 cpm of the respective tracer. The tracers were incorporated into small pieces of the respective artificial diet (*Spodoptera*, *Creatonotos*) or applied on glass-fiber discs (diameter 10 mm) (*Zonocerus*) and offered to the insects which had previously been starved for 12 h. After consumption of the labeled diet the insects were allowed to continue feeding on untreated diet or chinese cabbage (*Zonocerus*) for 48 h. If required (*Spodoptera*) the excrement was collected at intervals. After termination of the experiment the individuals were crushed in liquid nitrogen and extracted with 10 ml methanol/25% HCl, (100:1, by vol.) for 10 min. After centrifugation, the pellet was extracted twice in the same manner. Aliquots were taken to estimate total radioactivity by scintillation counting and for TLC separation of labeled senecionine and its *N*-oxide. The alkaloids were separated on silica gel 60 (Merck) with the solvent system methylene chloride/methanol/25% ammonium hydroxide (80:20:5, by vol.). Labeled senecionine (R_f = 0.87) and its *N*-oxide (R_f = 0.49) were localized by radiodetection using the TLC multichannel analyzer RITA (Raytest).

In injection experiments (*Spodoptera*) 2 µl Ringer's solution containing 3.5 µg alkaloid with 5×10^5 cpm of the respective tracer was injected into the haemolymph of late instar larvae with a 10-µl microsyringe (Hamilton). Exuding body fluid was removed with a piece of filter paper. Larvae were kept and further treated as described for oral feeding.

For analysis of the transient alkaloid concentration in the haemolymph of *Spodoptera*, larvae which were actively feeding on their diet containing the respective tracer were collected and the haemolymph was sampled immediately as described below

in the ^{18}O experiment and frozen in liquid nitrogen. After lyophilization, the dry haemolymph samples were dissolved in 50–200 µl methanol and analyzed for total radioactivity and ^{14}C-labeled senecionine and senecionine *N*-oxide as described above.

Preparation of [^{14}C]senecionine *N*-[^{18}O]oxide. A gas-tight all-glass apparatus was applied, consisting of a 100-ml round flask connected to the $^{18}O_2$ reservoir and a vacuum pump; it was sealed with a screw cap containing a teflon septum. [^{14}C]Senecionine (8.2 mg; 4.2×10^6 cpm) was added to the reaction flask and mixed with 25 ml of a solution of desalted crude senecionine *N*-oxygenase (SNO) in reaction buffer (10 mM potassium phosphate pH 7.0, 2 mM dithioerythritol). The apparatus was sealed and flashed/evacuated 10 times with >99.9% N_2. After one flash with $^{18}O_2$, the apparatus was filled with $^{18}O_2$ (98–99%; Linde) and the reaction was started by injecting 50 mg NADPH in 1 ml helium-flashed reaction buffer. The reaction was allowed to proceed for 24 h at 30°C and was then terminated by injection of 2.5 ml 25% HCl; precipitating protein was removed by centrifugation. The aqueous supernatant was dried under vacuum, the residue dissolved in 3 ml methanol and after centrifugation the solvent was evaporated. The product was purified by semipreparative HPLC to remove unreacted senecionine and particularly small amounts of contaminating seneciphylline *N*-[^{18}O]oxide which has the same M_r (i.e. 351) as unlabeled senecionine *N*-oxide. HPLC was performed using a RP-18 column (Nucleosil 7 µm, 250 mm long, 25 mm i.d.; Macherey & Nagel). The sample was redissolved in 800 µl water/trichloroacetic acid (1000:2, by vol.), centrifuged and applied onto the column via a Rheodyne rotary valve with a 500-µl loop. Separation was achieved isocratically using acetonitrile/water/trichloroacetic acid (150:850:2, by vol.) at a flow rate of 9 ml · min^{-1}, detection was by absorbance at 254 nm. The retention times (t_R) for the pyrrolizidine alkaloids are: seneciphylline, 27 min; seneciphylline *N*-oxide, 33 min; senecionine, 39 min; senecionine *N*-oxide, 47 min. The fractions containing senecionine *N*-oxide were recovered, the solvent removed by evaporation and the residue extracted with toluene at pH 11 (adjusted with NH_4OH) to remove any traces of non-*N*-oxidized senecionine. Three samples were prepared. The ^{18}O content was determined by fast-atom-bombardment mass spectrometry (FAB-MS); all measurements were corrected for natural abundance of ^{13}C. The specific radioactivity was determined by scintillation counting and quantitative GC analysis of the Zn/H$^+$-reduced sample. Sample A: 86% ^{18}O in *N*-oxide oxygen (specific radioactivity, 1.3×10^8 cpm/mmol); sample B: 97% ^{18}O in *N*-oxide oxygen (specific radioactivity, 1.3×10^8 cpm/mmol); sample C: 98% ^{18}O in *N*-oxide oxygen (specific radioactivity, 1.5×10^8 cpm/mmol).

Oral feeding of [^{14}C]senecionine *N*-[^{18}O]oxide. *Creatonotos* final instar larvae and *Zonocerus* adults were fed individually with 1.3 mg tracer each applied on glass-fiber discs (diameter 5–10 mm). *Arctia* larvae were treated in the same way but the tracer was offered with artificial diet. All insects had been starved for 12 h. After consumption of the entire sample, the arctiid larvae were allowed to feed on untreated diet and *Zonocerus* on chinese cabbage for 48 h.

Injection of [^{14}C]senecionine *N*-[^{18}O]oxide. The tracer (0.7 mg/3 µl Ringer's solution) was injected into the haemolymph of *C. transiens* last instar larvae between the 3rd and 4th segment with a 10-µl microsyringe (Hamilton). Exuding body fluid was removed with a piece of filter paper. With *Z. variegatus* adults the injection was directly into the abdominal cavity between the 2nd and 3rd abdominal segment. After injection the insects were kept on their normal diets for 72 h.

Preparation of haemolymph and alkaloid analysis. Haemolymph of cooled (5 min at −20°C) individuals was collected with a calibrated glass capillary after cutting off a pseudopod (arctiid larvae) or leg (*Z. variegatus*). After recording the total volume the haemolymph of each individual was immediately added to 0.6 ml methanol/25% HCl (6:1, by vol.). The precipitated protein was removed by centrifugation and the supernatant evaporated under an air stream. Labeled senecionine *N*-oxide was purified by HPLC and further analyzed as given for preparation of [^{14}C]senecionine *N*-[^{18}O]oxide.

Capillary gas chromatography. Sample preparation, separation and quantitative analysis of pyrrolizidine alkaloids as tertiary alkaloids were performed according to the standard methods described by Witte et al. (1993).

Enzymatic assay of senecionine *N*-oxygenation (tracer assays). Enzyme activity was assayed by determining the formation of radioactively labeled senecionine *N*-oxide from [^{14}C]-senecionine either by radioactive thin-layer chromatography (TLC method) or by determination of the remaining substrate senecionine due to its selective solubility in toluene (toluene method). The reaction mixture contains in a total volume of 300 µl: 10 mM potassium phosphate pH 7.0 plus 2 mM dithioerythritol (standard buffer), 0.2 mM [^{14}C]senecionine ($2-3\times10^4$ cpm/assay), 1.2 mM NADPH and enzyme in standard buffer. The reaction was started by addition of NADPH and generally allowed to proceed 30 min at 37°C. The reaction was terminated by addition of 100 µl 2 M HCl.

TLC method. The reaction mixture was evaporated under air flow, redissolved in 20 µl methanol and subjected to radioactive TLC analysis. Enzyme activity was calculated from the substrate (senecionine)/product (senecionine *N*-oxide) ratio.

Toluene method. The enzyme assay as described above was performed in a scintillation vial (4 ml). The reaction was terminated by addition of 100 µl 2 M HCl, then 3 ml of a mixture of toluene/Lipo-Luma (Baker) (1:1, by vol.) was added. After addition of 100 µl 5 M NaOH and thorough shaking, the mixture was centrifuged and, without separation, was directly analyzed in a scintillation counter. The labeled polar alkaloid *N*-oxide remains quantitatively in the aqueous layer and does not interfere with scintillation counting. Generally an addition of 20000 cpm labeled substrate/assay was sufficient to give reliable results. Controls without addition of NADPH were treated in the same way.

Enzymatic assay of senecionine *N*-oxygenation (photometric assay). The enzyme assay was as given above. The reaction was started by the addition of the substrate and followed continuously by the decrease of the absorption (NADPH) at 334 nm (Eppendorf photometer) or 340 nm (Pye Unicam UV/VIS) at 37°C.

Enzyme purification. The haemolymph was collected from *Tyria jacobaeae* late instar larvae and kept on ice until a total volume of ≈8 ml had been obtained (≈80 individuals). Then 40 ml cooled saturated ammonium sulfate in 0.1 M potassium phosphate pH 7.0 plus 2 mM dithioerythritol (standard buffer) was slowly added. The suspension was stirred for 60 min, the precipitate recovered by centrifugation (20 min at 20000 · *g*) and stored at −20°C. In this way crude enzyme preparations could be preserved without significant loss of activity for more than one year.

Step 1 (Phosphocellulose, Sigma). Phosphocellulose medium mesh (Sigma) was cycled following the manufacturer's instructions. The preserved ammonium sulfate precipitate of the haemolymph from 80 individuals was dissolved in 20 ml standard buffer and desalted via Sephadex G-25 (PD-10 columns, Pharmacia). The desalted solution was applied to a phosphocellulose column (bed volume 40 ml; 75 mm length, 26 mm i.d.) previously equilibrated with 150 ml standard buffer. The column was washed with 80 ml buffer at a flow rate of 0.5 ml · min^{-1}.

Protein was fractionated by linear gradient elution of 0–1 M potassium phosphate pH 7.0 (100 ml). Fractions of 2.5 ml were collected, enzyme activity eluted in fractions 14 –20.

Step 2 (Hydroxyapatite Bio-Gel HT, Bio-Rad). The desalted fractions with enzyme activity from step 1 were applied to the a hydroxyapatite column (bed volume 4 ml; 50 mm length, 10 mm i.d.) previously equilibrated with 40 ml standard buffer. The column was eluted with 20 ml buffer followed by a 70-ml linear gradient of 0–0.5 M potassium phosphate pH 7.0. The flow rate was 0.5 ml · min^{-1}; 1.5-ml fractions with enyzme activity were eluted between 125–250 mM potassium phosphate.

Step 3 (Mono Q 5/5 HR, Pharmacia). Active protein obtained from step 2 was applied to an FPLC-Mono Q column, which had previously been equilibrated with 20 ml standard buffer. Protein was eluted with a 10-ml 0–0.1 M KCl gradient followed by a 60-ml 0.1–0.3 M KCl gradient. Fractions of 0.5 ml were collected; enzyme activity was found in fractions 23–30.

Determination of M_r. For the determination of the native M_r, the purified enzyme was applied to a Superdex 200 HR (10/30) column (Pharmacia) and was eluted with standard buffer at a flow rate of 0.5 ml · min^{-1}. As reference proteins, the kit MS II (Serva) was used. The M_r of the denatured enzyme was obtained by SDS/PAGE (Laemmli, 1970). A protein ladder of 12-kDa steps between 10–120 kDa (Life Technologies) was used as molecular marker. The purified samples (Mono Q eluate) were desalted on PD 10 columns and concentrated on Centricon-30 concentrators (Amicon) if necessary.

Determination of pI. The pI was determined on an Ampholine® PAGplate pH 3.5–9.5; length 110 mm (Pharmacia) using the horizontal electrophoresis system Multiphor II (Pharmacia). The protein reference mixtures V (Merck) (pI 4.75–10.6) and Serva (pI 3.50–10.65) were used for calibration. The enzyme was localized by silver staining (Heukeshoven and Dernick, 1988). In addition, one gel was cut into 10-mm slices and protein was eluted with standard buffer. Subsequently SNO activity was localized by applying the TLC method.

Pyrrolizidine alkaloids applied as enzyme substrates. Seneciphylline and retrorsine were obtained from Roth, monocrotaline from Aldrich, heliotrine from Corkwood Enterprises. The other pyrrolizidine alkaloids were isolated and purified from respective plant sources: senecionine, senecivernine and senkirkine from *Senecio vernalis* inflorecences according to Hartmann and Zimmer (1986), axillaridine and axillarine from seeds of *Crotalaria scasselatii* according to Wiedenfeld et al. (1985); triangularine and sarracine were isolated from *S. sylvaticus*, indicine was isolated from flowers and lycopsamine from seeds of *Heliotropium indicum*, rinderine from flowers of *Eupatorium laevigatum* and phalaenopsine from flowers of *Phalaenopsis* hybrids. Purity and identity of the structures was verified by GC/MS and, if necessary, NMR analysis. All pyrrolizidine alkaloids were purified as tertiary alkaloids. The respective *N*-oxides were prepared according to Craig and Purushothaman (1970). Retronecine and heliotridine were prepared by alkaline hydrolysis of monocrotaline and heliotrine, respectively (Hartmann et al., 1990). ^{14}C-Labeled pyrrolizidine alkaloids were isolated from the respective root cultures after feeding of labeled putrescine as biosynthetic precursor (Hartmann, 1994).

RESULTS

Sequestration of pyrrolizidine alkaloids by *Creatonotos* and *Zonocerus*. [^{14}C]Senecionine and [^{14}C]senecionine *N*-oxide were fed to late instar larvae of *Creatonotos* and to adults of *Zonocerus*. The results (Table 1) give two main answers: (a) exclusively [^{14}C]senecionine *N*-oxide could be detected in extracts of insects which had fed on either of the two alkaloidal forms; (b) there is no significant difference in the amount of sequestered alkaloid *N*-oxide between insects having fed on senecionine and senecionine *N*-oxide.

Table 1. Sequestration of pyrrolizidine alkaloids by *Creatonotos* larvae and adults of *Zonocerus* after feeding on [^{14}C]senecionine and [^{14}C]senecionine *N*-oxide. Alkaloid (1 mg = 100%) was fed per individual; after consumption of the labeled alkaloid, individuals were kept on untreated diet for 48 h prior to analysis. Mean ± SEM is given; $n = 5$ (*Creatonotos*); $n = 4$ (*Zonocerus*).

Species	Senecionine *N*-oxide sequestered	
	senecionine fed	senecionine *N*-oxide fed
	%	
Creatonotos transiens	7.7 ± 2.3	7.5 ± 5.6
Zonocerus variegatus	28.9 ± 6.9	36.3 ± 10.2

Uptake and elimination of pyrrolizidine alkaloids by the noctuid *Spodoptera*. Larvae of *Spodoptera* easily feed on pyrrolizidine-alkaloid-containing plants but do not sequester the alkaloids (Aplin and Rothschild, 1972). Feeding experiments with labeled senecionine and its *N*-oxide show that with both tracers the radioactivity is almost completely eliminated with the excrement within the first 24 h; less than 2% of the radioactivity offered with the alkaloids is retained in larval bodies after 48 h (Fig. 1A, B). The tracers are even more rapidly eliminated if they are internally applied, i.e. injected into the haemolymph of larvae (Fig. 1C, D). Considerable amounts (40–80%) of [^{14}C]senecionine could be recovered from methanolic excrement extracts after oral and internal application of the two tracers, whereas [^{14}C]senecionine *N*-oxide was detectable in traces only (<2%) in excrement extracts after oral application of the two alkaloidal tracers. In the injection experiments, labeled *N*-oxide was not detectable in excrement extracts. Thus, *Spodoptera* efficiently eliminate orally or internally administered senecionine and its *N*-oxide. The alkaloids are preferentially excreted in the reduced state (i.e. as tertiary alkaloid).

A more detailed experiment revealed that, during oral uptake of the two labeled alkaloids, considerable amounts of senecionine were transiently detectable in the haemolymph of larvae (Table 2). [^{14}C]Senecionine was the only labeled metabolite detectable in the haemolymph, not even traces of the *N*-oxide were found. [^{14}C]Senecionine *N*-oxide injected into the haemolymph is efficiently excreted but not even traces of tertiary alkaloid could transiently be detected in the haemolymph. In conclusion, these results strongly favor the following fate of orally ingested pyrrolizidine alkaloids by *Spodoptera*: (a) the alkaloid *N*-oxide is easily reduced in the guts; (b) the tertiary alkaloid is passively absorbed into the haemolymph and again eliminated by efficient excretion.

Mechanism of pyrrolizidine alkaloid absorption in alkaloid sequestering arctiids and *Zonocerus*. For pyrrolizidine alkaloid absorption in alkaloid-storing insects, two possible mechanisms have to be taken into consideration: (a) the existence of a specific carrier which particularly favors the uptake of the salt-like polar alkaloid *N*-oxides as suggested by Wink and Schneider (1988); (b) reduction of pyrrolizidine alkaloid *N*-oxide in the guts, uptake of the lipophilic tertiary alkaloids in their unprotonated form by simple diffusion followed by rapid re-*N*-oxygena-

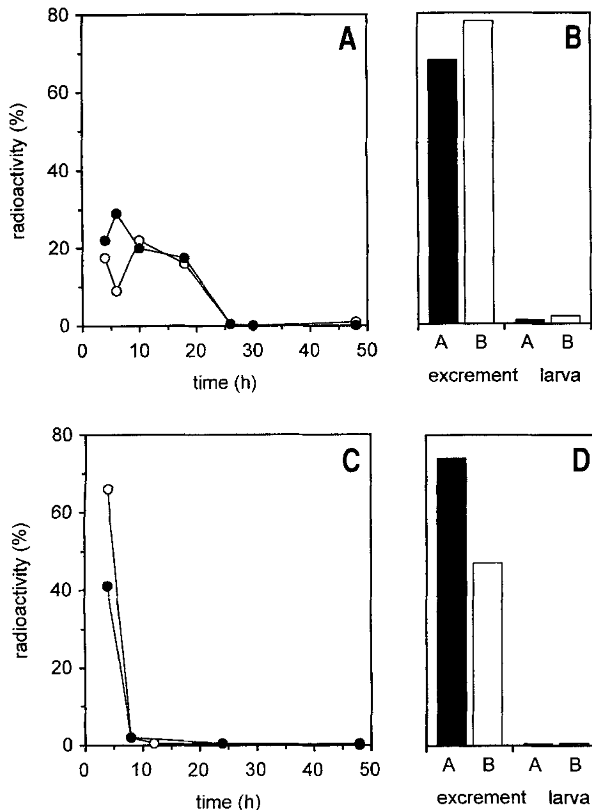

Fig. 1. Time course of elimination of radioactivity by *Spodoptera littoralis* following adminstration of [^{14}C]senecionine *N*-oxide (●——●) and [^{14}C]senecionine (○——○). (A, B) Tracers were fed orally; (C, D) tracers were applied by injection into the haemolymph. Columns: total radioactivity in total excrement extracts (excrement) and larval extracts (larva) after 48 h; (A) after feeding of [^{14}C]senecionine *N*-oxide, (B) after feeding of [^{14}C]senecionine.

Table 2. Transient concentration of [^{14}C]senecionine in the haemolymph of late instar larvae of *Spodoptera littoralis* feeding on labeled senecionine and its *N*-oxide. Larvae (n = 4, senecionine; n = 8, senecionine *N*-oxide) were fed on artificial diet containing the labeled alkaloids in the concentration indicated; actively feeding larvae were removed and the haemolymph was immediately collected. Mean ± SEM is given; n.d. = not detectable.

Alkaloid fed	Concentration fed	Feeding time	Concentration in haemolymph of	
			[^{14}C]senecionine	[^{14}C]senecionine *N*-oxide
	μM	min	nM	
[^{14}C]Senecionine	6.27	22–30	82 ± 26	n.d.
[^{14}C]Senecionine *N*-oxide	6.27	20–33	157 ± 161	n.d.

tion in the haemolymph. This second mechanism is supported by the results described in the preceding sections. There is one crucial experiment to unequivocally demonstrate which of the two hypotheses is correct: feeding of a senecionine *N*-oxide with an ^{18}O-labeled *N*-oxide oxygen. If the first mechanism (carrier hypothesis) is correct, the ^{18}O should be retained in the senecionine *N*-oxide recovered from the haemolymph. In contrast,

Table 3. Administration of [^{14}C]senecionine *N*-[^{18}O]oxide to three pyrralizidine-alkaloid sequestering insect species and analysis of the ^{16}O/^{18}O ratio and the specific radioactivity of the senecionine *N*-oxide recovered from the haemolymph.

Experiment	Ratio ^{16}O/^{18}O	10^{-8} × specific radioactivity
		cpm/μmol
Creatonotos transiens		
N-Oxide administered (control)	3:97	1.3
N-Oxide in haemolymph (oral feeding)	86:14	1.1
N-Oxide in haemolymph (injection)	12:88	1.2
Arctia intercallaris		
N-Oxide administered (control)	2:98	1.5
N-Oxide in haemolymph (oral feeding)	99: 1	1.3
Zonocerus variegatus		
N-Oxide administered (control)	14:86	1.3
N-Oxide in haemolymph (oral feeding)	88:12	0.8
N-Oxide administered (control)	2:98	1.5
N-Oxide in haemolymph (injection)	3:97	1.4

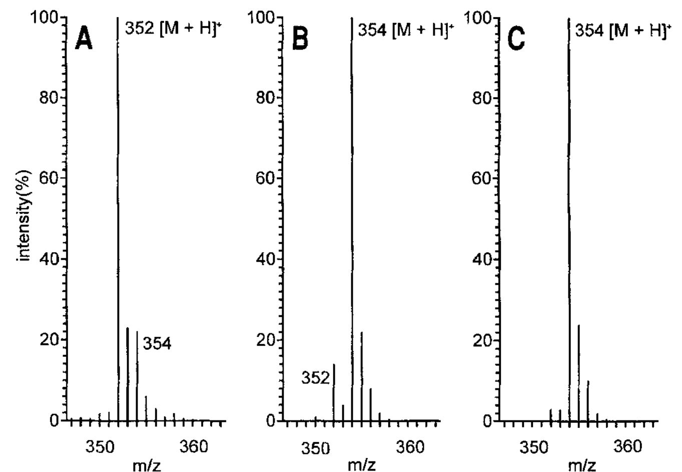

Fig. 2. FAB-MS of senecionine *N*-oxide isolated from the haemolymph of *Creatonatos transiens* larvae after (A) oral feeding of [^{14}C]senecionine *N*-[^{18}O]oxide, (B) injection of [^{14}C]senecionine *N*-[^{18}O]oxide into the haemolymph, and (C) analysis of synthetic [^{14}C]senecionine *N*-[^{18}O]oxide administered to the larvae (control). For FAB-MS analysis (Finnigan Mat 8430) 2–10 μg purified senecionine *N*-oxide were applied; glycerol was used as matrix.

with the second mechanism the ^{18}O should be completely replaced by ^{16}O.

Double-labeled [^{14}C]senecionine [^{18}O]*N*-oxide was prepared and fed orally to larvae of the two arctiids *Creatonotos* and *Arctia* and to adults of the grasshopper *Zonocerus*. To prevent any complications with background levels of pyrrolizidine-alkaloids the insects had been reared on pyrrolizidine alkaloid-free diets. After sequestration of the labeled alkaloid, the haemolymph was collected, the senecionine *N*-oxide extracted and purified by HPLC. Identity and purity of the isolated compound was verified: (a) by comparison of its specific radioactivity with that of the tracer applied (Table 3); (b) after reduction by GC/MS. The purified *N*-oxide was analyzed for its ^{18}O content by means of FAB-MS. The result of one measurement is illustrated in Fig. 2. It shows that, in comparison to the control, the ^{18}O label in the senecionine *N*-oxide recovered from the haemolymph is almost completely exchanged for ^{16}O. With the three insects 86–99%

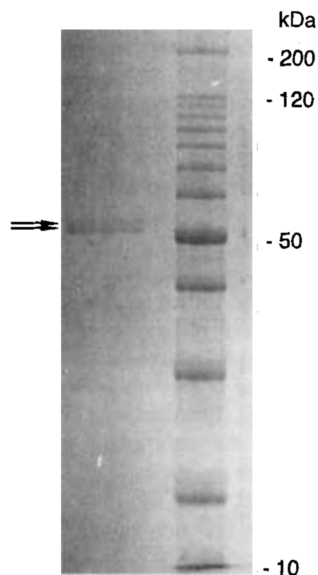

Fig. 3. SDS/PAGE of purified SNO from *Tyria jacobaeae*. The two bands of SNO (left) migrate slightly above the 50-kDa protein marker. The molecular mass markers (right) represent a protein ladder of 12-kDa steps between 10 kDa (bottom) and 120 kDa. The top band represents a 200-kDa fragment. Proteins were stained with Coomassie brilliant blue.

of the ^{18}O label was lost during sequestration of orally fed ^{18}O-labeled senecionine *N*-oxide (Table 3). If the ^{18}O-labeled *N*-oxide is directly injected into the haemolymph, the label is retained completely stable for at least 72 h. This excludes any significant turnover of the *N*-oxide oxygen in the haemolymph and proves that the reduction of the orally ingested alkaloid *N*-oxide must have been taken place in the guts. The significant loss of 12% of ^{18}O label in the experiment with *Creatonotos*, which was not observed with *Zonocerus*, can be explained by the larvaes' licking their wounds. After injection small droplets of haemolymph were observed to ooze out the wound. These droplets were ingested by licking and any labeled *N*-oxide contained in the wound droplets would loose its label during the gut passage.

Detection of senecionine *N*-oxygenating enzyme activity in pyrrolizidine-alkaloid-storing insects. The tracer experiments with the ^{18}O-labeled senecionine *N*-oxide indicate the presence of efficient *N*-oxidizing enzyme activity in the insects' bodies. In preliminary studies, isolated haemolymph of a number of pyrrolizidine-alkaloid-sequestering species was tested for the ability to *N*-oxidize [^{14}C]senecionine. Significant *in vitro* enzyme activity could be detected in the following species and metamorphic stages: family Arctiidae, *Tyria jacobaeae* (larvae, pupae); *Callimorpha dominula* (larvae); *Creatonotos transiens* (larvae); *Arctia caja, A. intercallaris* (larvae); *Amerila phaedra* (adults) and family Danainae, *Idea leuconoe* (pupae). In the grasshopper *Zonocerus* the *N*-oxygenating enzyme activity is associated with the fat body and not with the haemolymph. As expected, no enzyme activity was detected in *Spodoptera* which does not sequester pyrrolizidine alkaloids.

Purification of senecionine *N*-oxygenase (SNO) from haemolymph of *T. jacobaeae*. Due to their easy availability larvae of the European cinnabar moth, *Tyria jacobaeae*, were chosen as enzyme source. A soluble NADPH-dependent mixed-function monooxygenase was detected in crude haemolymph preparations. In ammonium sulfate precipitates of freshly collected haemolymph the enzyme activity could be preserved for months without significant loss of activity. Purification to electrophoretic purity (Fig. 3) was achieved in a three-step procedure by

Table 4. Purification of senecionine *N*-oxygenase (SNO) from haemolymph of *Tyria jacobaeae* larvae. Crude extract: desalted ammonium sulfate precipitate of ≈8 ml haemolymph (corresponding to 80 late instar larvae).

Purification step	Protein	Total activity	Specific activity	Yield	Purification
	mg	nkat	nkat/mg	%	-fold
Crude extract	228	24.2	0.11	100	1
Phosphocellulose	4.5	8.2	1.81	34	17
Hydroxyapatite	0.619	5.6	8.98	23	85
Mono Q	0.045	3.5	77.56	14	731

combination of phosphocellulose chromatography, hydroxyapatite chromatography and anion-exchange chromatography (Mono Q) (Table 4). The purification via phosphocellulose was not cation-exchange but phosphate-affinity chromatography since elution of enzyme activity was only possible with potassium phosphate but not potassium chloride. The matrix-fixed phosphate binds to proteins known to react with phosphate esters (Sternbach, 1991).

General and molecular properties of SNO. The purified enzyme from *Tyria* haemolymph shows a pH optimum of 7.0 in standard buffer. It has its maximal activity at a temperature of 40–45°C; the activation energy (E_a) of senecionine *N*-oxygenation was determined over a temperature range of 10–35°C and was found to be 53.1 kJ/mol. The reaction is strictly dependent on molecular oxygen. The reaction is not dependent on cytochrome *P*-450, as indicated by the following lines of evidence: (a) it is a soluble enzyme; (b) it is not carbon-monoxide-sensitive and the carbon monoxide difference spectrum of the dithionite-reduced enzyme did not show the typical carboxy ferrocytochrome absorption maximum at 450 nm (Hodgson, 1985); (b) typical cytochrome *P*-450 inhibitors (e.g. metyrapone, SKF 525A, *n*-octylamine and guanethidine) did not significantly reduce enzyme activity. Rather, the oxygenase is a flavoprotein. An absolute spectrum of purified SNO in the presence of oxygen

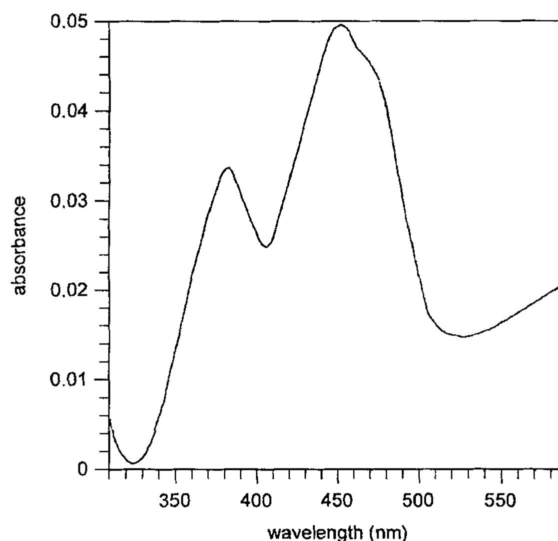

Fig. 4. Ultraviolet/visible spectrum of purified SNO from *Tyria jacobaeae*. The spectrum was recorded with 131 µg protein in the oxidized state of the enzyme, i.e. in presence of substrate (senecionine) and O_2 and absence of NADPH.

Table 5. Substrate specificity of senecionine *N*-oxygenase from the haemolymph of three arctiids. Supinine, supinidine and isoretronecanol were available only as ^{14}C-labeled substrates and were assayed in comparison to [^{14}C]senecionine with the TLC method. All other compounds were assayed photometrically under standard assay conditions at 170 μM. Senecionine, triangularine, sarracine, phalaenopsine and retronecine were assayed photometrically and as labeled substrates with the TLC method. See Fig. 5 for the chemical structures. Activities are given as percentages of that with senecionine as substrate; n.d., not detected.

Substrates	Activity with SNO from		
	Tyria	*Creatonotos*	*Arctia*
	%		
Senecionine type			
Senecionine	100	100	100
Seneciphylline	95	119	117
Retrorsine	97	93	100
Senecivernine	81	113	107
Senkirkine	0	0	0
Triangularine type			
Triangularine	60	n.d.	n.d.
Sarracine	<1	n.d.	<1
Monocrotaline type			
Monocrotaline	92	110	103
Axillarine	74	38	55
Axillaridine	76	93	79
Lycopsamine type			
Lycopsamine	20	75	93
Rinderine	23	81	79
Indicine	35	78	90
Heliotrine	25	115	86
Supinine	0	0	0
Phalaenopsine type			
Phalaenopsine	0	0	0
Necines bases			
Retronecine	0	0	0
Heliotridine	0	0	0
Supinidine	0	0	0
Isoretronecanol	0	0	0

and its substrate senecionine and in the absence of NADPH is shown in Fig. 4. The spectrum exhibits the typical absorption pattern for an oxidized flavoprotein, i.e. absorption maxima at 375 and 456 nm with a ratio A_{456}/A_{375} of 1.5 (Dawson et al., 1986). Basing on a subunit M_r of 51000 of SNO and a molar absorption coefficient for FAD of $11300 \cdot M^{-1} \cdot cm^{-1}$, a molar apoenzyme/FAD ratio of 0.4 was estimated. Assuming that one mole of each apoenzyme subunit binds one mole of FAD, the ratio should be 1. Due the small quantity of pure enzyme available and the low absorption values, a considerable error of calculations has to be taken into account. However the estimated ratio is well within the expected order of magnitude. This strongly supports the assumption that SNO is a flavoprotein.

The isoelectric point of the homogeneous enzyme is at pH 4.9 ± 0.1. The native M_r of the enzyme determined by gel permeation chromatography with a Superdex 200 HR column was found to be 200000 ± 10000. SDS/PAGE always shows two sharp bands of almost identical migration and intensity (Fig. 3) with an M_r of 51000 ± 1000, which indicates that the active enzyme is a tetramer composed of subunits of nearly identical size.

Substrate specificity and substrate kinetics of SNO from three arctiids. The purified enzyme from *Tyria* and partially purified enzyme preparations obtained from the haemolymph of *Creatonotos* and *Arctia caja* larvae were chosen for comparative studies on substrate specificity and substrate kinetics of larval SNO. In Table 5 all available pyrrolizidine alkaloids are listed which were tested as substrates for SNO. They comprise examples from the main structural types of pyrrolizidine alkaloids according to the classification given by Hartmann and Witte (1995) (Fig. 5). The three enzymes show the same substrate specificity but differ in the relative efficiency for individual substrates. Not all pyrrolizidine alkaloids are *N*-oxidized. Ester alkaloids lacking an hydroxyl group at carbon seven (e.g. supinine and phalaenopsine), 1,2-dihydro-alkaloids such as sarracine and phalaenopsine and unesterified necine bases are not accepted as substrates. Senkirkine the otonecine analogue of senecionine, is also not *N*-oxidized. This, however, is not surprising as the otonecine derivatives are the only plant pyrrolizidine alkaloids which are never found as *N*-oxides.

In addition to pyrrolizidine alkaloids a large number of related structures and potential substrates of multisubstrate FMOs were tested as substrates of SNO. None of these compounds, which include tropane alkaloids, quinolizidine alkaloids, nicotine, indole alkaloids, caffeine, isoquinolines and synthetic heterocyclic tertiary amines, were accepted as substrate by SNO from *Tyria*.

The SNO-catalyzed reaction is strictly NADPH-dependent; NADH cannot replace NADPH as cosubstrate. The K_m values of NADPH for the enzymes from *Tyria* and *Creatonotos* are 1.3 μM and 2.2 μM, respectively.

The substrate kinetics were established for three selected substrates representing the major classes of pyrrolizidine alkaloids (Table 6). With an apparent K'_m value of 1.4 mM the *Tyria* enzyme has a high affinity for its substrate senecionine which represents the type of pyrrolizidine alkaloids of its exclusive host plant *Senecio jacobaea*. It displays a considerably lower affinity toward other structural types such as the monocrotaline (10-fold lower) and particularly the monoester heliotrine (200-fold lower), which are not present in *S. jacobaea*. These differences are less expressed with the enzymes from the two other arctiids which, in contrast to *T. jacobaeae*, are generalists feeding on a variety of alkaloidal food plants with different pyrrolizidine alkaloid patterns.

DISCUSSION

Uptake behavior of the two forms of pyrrolizidine alkaloids (i.e. tertiary alkaloid and its *N*-oxide) was studied with *Spodoptera littoralis*, a species which tolerates but does not sequester pyrrolizidine alkaloids and several pyrrolizidine-alkaloid-sequestering species. For both non-sequestering and sequestering species, only the tertiary was taken up into the body, irrespectively of whether the tertiary alkaloid or its *N*-oxide had been fed. The ingested alkaloid *N*-oxide has to be reduced in the gut prior to absorption. This was directly proved in the case of *Spodoptera* by analysis of transient levels of tertiary alkaloid in the haemolymph. With the sequestering species, which in their bodies store only the *N*-oxide form, feeding of [^{18}O]-*N*-oxide-labeled alkaloid revealed that the alkaloid *N*-oxide recovered from the haemolymph had lost all of its ^{18}O label. Since [^{18}O]-*N*-oxide directly injected into the haemolymph completely retained its ^{18}O label, the reduction must have occurred in the guts. It has long been known that in mammals any pyrrolizidine alkaloid *N*-oxide ingested with food is easily reduced in the gut and the lipophilic tertiary alkaloid is passively absorbed (Mattocks, 1986). The same happens in insects. In *Spodoptera* alkaloid diffuses passively into the haemolymph creating a transient

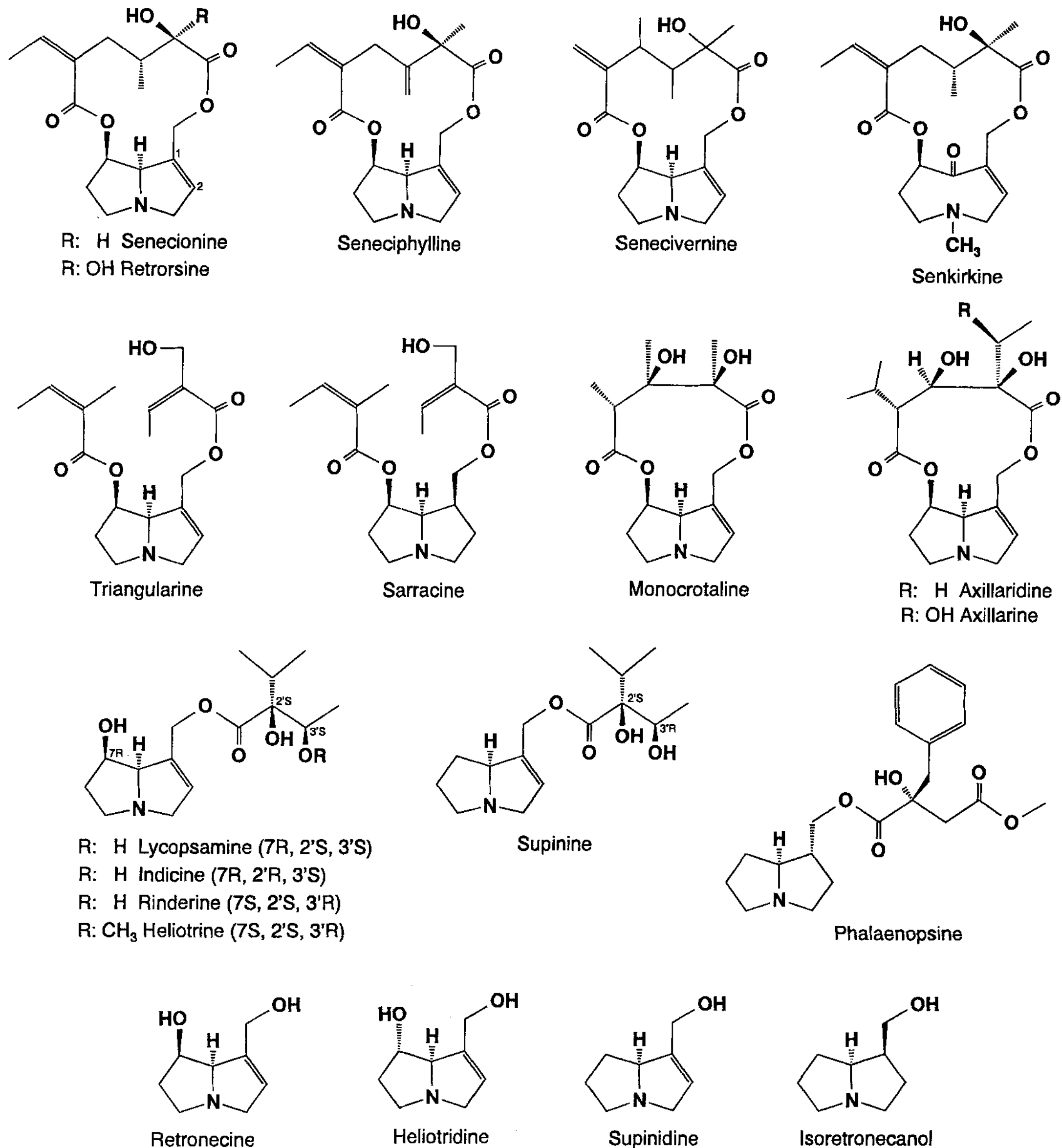

Fig. 5. The structures of pyrrolizidine alkaloids applied as potential substrates of SNO.

level of tertiary alkaloid. *Spodoptera* larvae which are adapted to feed on plants containing pyrrolizidine alkaloids obviously prevent deleterious poisoning by rapid and efficient excretion of the absorbed tertiary alkaloid. Even the alkaloid *N*-oxide, which never enters the haemolymph via the guts, is rapidly excreted if administered internally. As demonstrated with the ^{18}O-labeled senecionine *N*-oxide, pyrrolizidine alkaloid uptake in alkaloid-sequestering lepidopterans and the taxonomically unrelated grasshopper *Zonocerus* proceeds exactly in the same way as in *Spodoptera*, i.e. reduction in the guts followed by uptake of the tertiary amine into the haemolymph. Two mechanistic features facilitate alkaloid accumulation in pyrrolizidine-alkaloid-sequestering species: efficient re-*N*-oxidation and lack of *N*-oxide excretion (metabolic trapping). The ^{18}O experiment clearly disproves a specific carrier-mediated uptake of senecionine *N*-oxide as recently claimed for *Creatonotos transiens* (Wink and Schneider, 1988). We cannot exclude a carrier-mediated uptake for tertiary alkaloids in pyrrolizidine-alkaloid-sequestering insects. However, uptake of tertiary pyrrolizidine alkaloids by passive

Table 6. Substrate kinetics (apparent K_m values) for SNO from three arctiids. One representative substrate from each of the three major pyrrolizidine alkaloid types was applied.

Substrate	K'_m	V	V/K'_m
	µM	pkat · mg^{-1}	%
Tyria jacobaeae			
Senecionine	1.4	202	100
Monocrotaline	12.5	155	9
Heliotrine	284.0	124	<1
Creatonotos transiens			
Senecionine	3.1	554	100
Monocrotaline	2.9	344	67
Heliotrine	22.9	628	15
Artia caja			
Senecionine	4.8	198	100
Monocrotaline	5.1	168	81
Heliotrine	18.6	222	29

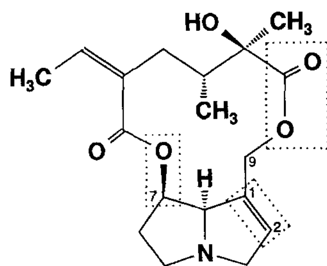

Fig. 6. Essential structural features of the alkaloidal substrates of SNO. These features are: esterification of the allylic hydroxyl group at C9; 1-2 double bond and a free or esterified hydroxyl group at C7.

diffusion as demonstrated for the non-sequestering species *Spodoptera* and well known from vertebrates (Mattocks, 1986) makes the existence of such a carrier unlikely.

In the pyrrolizidine-alkaloid-sequestering arctiids the *N*-oxidation of the passively absorbed tertiary alkaloid is catalyzed by a specific monooxygenase (SNO). Purified SNO shares its flavin dependency and ability to *N*-oxidize pyrrolizidine alkaloids with microsomal multisubstrate flavine monooxygenase (FMO) but is completely different with respect to its substrate specificity. FMO has been purified from mammalian liver, lung and kidney (Ziegler and Mitchell, 1972; Tynes and Hodgson, 1985; Gasser et al., 1990). The enzyme shows an extraordinarily broad substrate specificity and oxidizes a variety of nucleophilic organic nitrogen (i.e. lipophilic alicyclic and cyclic aliphatic amines) and sulfur compounds (Ziegler, 1993). Multisubstrate FMO is not known in insects (Brattsten, 1992). Aside from FMO and two functionally related enzymes from bacteria (Ryerson et al., 1982) and *Trypanosoma* (Agosin and Ankley, 1987), SNO is the only characterized non-cytochrome-*P*-450 enzyme which converts an amine into its *N*-oxide. In contrast to FMO, it is not microsomal but soluble and specifically *N*-oxidizes only pyrrolizidine alkaloids which combine the following structural features: (a) presence of an 1,2-double bond (1,2-dehydropyrrolizidines); (b) an allylic esterified hydroxyl group (at C9); (c) a free or esterified second hydroxyl group at C7 (see Fig. 6). These are precisely the structural features of pyrrolizidine alkaloids found to be hepatotoxic in vertebrates (Winter and Segall, 1989) and genotoxic in the *Drosophila* wing spot test (Frei et al., 1992). The nontoxic free necine bases and 1,2-saturated alkaloids such as sarracine and phalaenopsine (see Fig. 5) are not *N*-oxidized by SNO. No other substrates outside pyrrolizidine alkaloids have been found to be accepted by the enzyme so far.

N-Oxidation seems to be a prerequisite for alkaloid sequestration in arctiids and *Zonocerus* and it is reasonable to assume that this mechanism has been selected to keep the alkaloid in the insect's body in a nontoxic and metabolically inert state. This view is substantially supported by the substrate specificity of SNO. It suggests that SNO must have been selected and optimized during the coevolutionary adaptation of sequestering insects to plants containing pyrrolizidine alkaloid. From the mechanistic point of view, this idea is further supported by a comparison of the substrate specificities of the enzymes from the three arctiids. *Tyria jacobaeae* is a specialist, feeding almost exclusively on *Senecio jacobaea* which contains only pyrrolizidine alkaloids of the senecionine type (Hartmann and Witte, 1995). Thus, not unexpectedly, senecionine is the best substrate of *Tyria* SNO, whereas monocrotaline and heliotrine as representatives of the monocrotaline type and lycopsamine type, respectively, which are not found in *Senecio*, are less efficient substrates with 10-fold and 200-fold higher K'_m values, respectively (see Table 6). On the contrary, *Creatonotos* and *Arctia* are generalists with larvae feeding on a variety of plants containing pyrrolizidine alkaloid, which may possess quite different alkaloid patterns. Consequently the differences in the affinity of their SNOs towards the three substrates are less pronounced (Table 6). Further studies must show to what extent the relative substrate specificity of SNO is related to the alkaloidal type of the insect's preferential pyrrolizidine alkaloid sources. Preliminary experiments with Brazilian Ithomiinae butterflies which are adapted to sequester pyrrolizidine alkaloids of the lycopsamine type revealed that *N*-oxidation is more efficient with these monoesters than with macrocyclic senecionine (Trigo, J. R. and Hartmann, T., unpublished results). Sequence data of SNO should help us to answer mechanistic questions of structure/activity relationships with respect to substrate specificity as well as questions concerning the evolutionary origin of the enzyme.

Why are pyrrolizidine alkaloids so unique among alkaloids with regard to their role in insects? From the mechanistic point of view, our knowledge of the chemical and biological attributes of pyrrolizidine alkaloids is now substantial enough to address this question conclusively. The essential feature of pyrrolizidine alkaloids is that they can exist in two interchangeable forms:

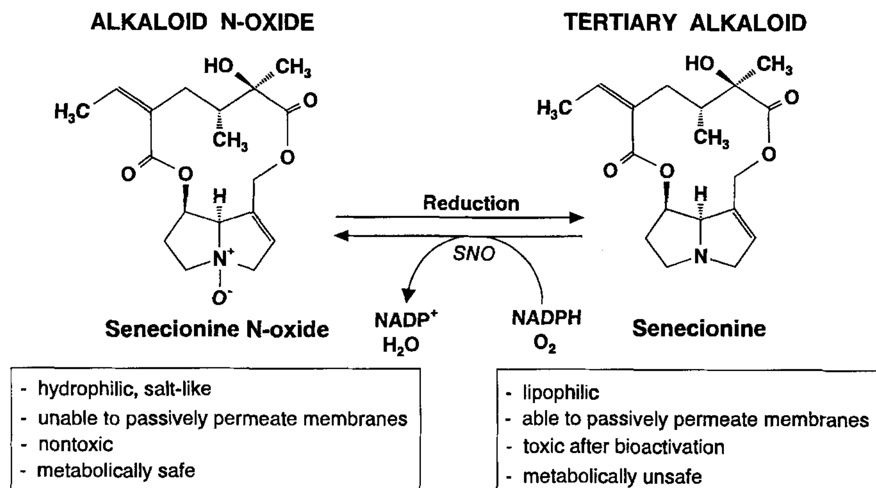

Fig. 7. The two faces of pyrrolizidine alkaloids. The nontoxic alkaloid *N*-oxide is easily converted into its toxic tertiary state; lepidopterans which sequester pyrrolizidine alkaloids for their own defense possess a specific monooxygenase (SNO) which keep the alkaloid in the metabolically safe state of its *N*-oxide.

the alkaloid *N*-oxide and the respective tertiary alkaloid (Fig. 7). Plants synthesize, translocate and store pyrrolizidine alkaloids as hydrophilic *N*-oxides (Hartmann et al., 1989). During the ingestion of food loaded with *N*-oxides by a herbivore, the *N*-oxides are reduced in the guts and absorbed passively as tertiary alkaloids. The tertiary alkaloid is deleterious for organisms or cells possessing a microsomal multisubstrate cytochrome *P*-450 and this accounts for its toxicity to perhaps any herbivore. Some organisms have evolved adaptations to overcome this potential toxicity by efficient *N*-oxidation (e.g. guinea pigs) or rapid excretion [e.g. *Spodoptera* as shown in this study or *Melanoplus* (Ehmke et al., 1989)]. Pyrrolizidine-alkaloid-sequestering insects are safe as long as they are able to keep the alkaloid in the *N*-oxide state. In lepidopterans and *Zonocerus*, SNO guarantees this safety. Leaf beetles of the genus *Oreina* (Chrysomelidae) sequester pyrrolizidine alkaloids in their defensive glands also exclusively as *N*-oxides, but they are not able to *N*-oxidize tertiary pyrrolizidine alkaloids. Contrary to lepidopteran species, they are able to take up directly the plant-derived pyrrolizidine alkaloid *N*-oxides and eliminate tertiary pyrrolizidine alkaloids (Ehmke et al., 1991; Rowell-Rahier et al., 1991).

In addition to aposematically colored lepidopterans, leaf beetles provide the strongest arguments favoring the role of pyrrolizidine alkaloids as powerful defensive compounds. One species, *O. cacaliae*, has even lost the ability to autogenously produce cardenolides for defense but instead accumulates pyrrolizidine alkaloid *N*-oxides acquired from its food plant in its defensive secretion (Pasteels et al., 1996). These beetles were found to be better protected against predation by birds than a closely related species which secretes autogenous cardenolides (Rowell-Rahier et al., 1995).

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