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Chemistry and biochemistry of *Solanum chacoense*, bitter steroidal alkaloids

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The Ohio State University, 1993
CHEMISTRY AND BIOCHEMISTRY OF SOLANUM CHACOENSE, BITTER STEROIDAL ALKALOIDS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy in the Graduate School of
The Ohio State University

By

David R. Lawson, B.A., M.S.

* * * * *

The Ohio State University
1993

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To Julie, Stephanie, and Jacob
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INTRODUCTION

*Solanum* steroidal glycoalkaloids are a large and important class of biologically-active secondary metabolites that have been isolated from more than 350 different plant species (Ripperger and Schreiber, 1981, Roddick, 1986). Despite the reference to *Solanum* in their name, steroidal glycoalkaloids and alkamines are not restricted to *Solanum* (Solanaceae), but have also been identified in *Lycopersicon* (Solanaceae), and *Veratrum* (Liliaceae) species.

This class of compounds has been associated with a number of biological activities across diverse taxonomic groups (Roddick, 1986). With regard to the steroidal alkaloids of the Solanaceae, these properties are both a concern and a benefit. Of concern is the potential toxicity to humans of certain steroidal glycoalkaloids that may commonly occur in food products, such as potato tubers (Jadhav et al., 1981; Morris and Lee, 1984). For this reason Roddick (1986) speculated that if potatoes were introduced as a new food product today, they probably would not be approved by Food Standards Committees. Of benefit, however, are the antifeedant activities of particular steroidal glycoalkaloids against economically-important insect pests, such as the Colorado potato beetle, *Leptinotarsa decemlineata*, Say, and the potential for incorporating this natural mechanism of insect resistance into potato cultivars. This approach of developing host-plant resistance has become increasingly important in the design of integrated pest management (IPM) strategies because of the rapid
development of insecticide-resistant Colorado potato beetle populations (Forgash, 1985) and the growing public concern over pesticide use.

All glycoalkaloids, however, are not equally effective deterrents to Colorado potato beetle feeding as demonstrated by the fact that the steroidal glycoalkaloids found in *S. tuberosum* (α-solanine and α-chaconine) are relatively ineffective as antifeedants to the beetle (Sinden, 1986a). The ineffectiveness of these compounds is due, in part, to the amazing ability of the Colorado potato beetle to develop populations resistant to toxins. In addition, efforts to improve the culinary quality of potatoes and reduce concentrations of potentially toxic and bitter-tasting steroidal glycoalkaloids in tubers has resulted in a reduction in glycoalkaloid content in commercial potato cultivars. As a consequence, potato improvement programs have sought to incorporate resistance traits into *S. tuberosum* cultivars from wild *Solanum* germplasm that contain effective glycoalkaloid antifeedants without elevating glycoalkaloid concentrations in the tubers above acceptable limits (Ross, 1966; Sinden et al., 1991).

One such species, *S. chacoense*, has demonstrated excellent natural resistance to Colorado potato beetle feeding, and resistance has been associated with leptine glycoalkaloids (Sturckow and Löff, 1961; Sinden et al., 1986a). *S. chacoense* has the added benefit of apparently limiting leptine biosynthesis to the foliage (Sinden et al., 1986b), and therefore reducing the risk of incorporating potentially toxic natural products into the tubers of hybrid progeny.

In spite of the fact that the natural resistance of leptine-producing accessions of *S. chacoense* has been known for more than 30 years, conventional breeding approaches to introducing this resistance has failed to produce a commercial potato cultivar. Therefore, other approaches may be needed to introduce leptine-mediated resistance to the Colorado potato beetle into commercial potatoes. With the present state of biotechnology, specific introduction of leptine biosynthesis from *S. chacoense*
into *S. tuberosum* would seem plausible, assuming proper knowledge of the biochemistry had been acquired. However, despite extensive research concerning the protection conferred by leptines against Colorado potato beetle defoliation, little research has investigated the biosynthesis of these ecologically-important antifeedants.

The structural relationship between solanidine (aglycone of α-solanine and α-chaconine), leptinidine (aglycone of the leptinines), and acetylleptinidine (aglycone of the leptines), implies that acetylleptinidine biosynthesis in *S. chacoense* may occur from solanidine with leptinidine as an intermediate. Specifically, two biosynthetic steps would be required, and would include the stereospecific hydroxylation of solanidine at C-23 to yield leptinidine (23-hydroxysolanidine), followed by acetylation of the C-23 hydroxyl of leptinidine to yield acetylleptinidine (23-O-acetylleptinidine). The first of these two steps has been demonstrated (Osman et al., 1987), but the conversion of leptinidine to acetylleptinidine has not. Since *S. tuberosum* already has the capacity to synthesize solanidine, acetylleptinidine biosynthesis might be transferred from *S. chacoense* to *S. tuberosum* using molecular techniques (e.g., *Agrobacterium*-mediated transformation, particle gun transformation), which may facilitate the specific introduction of genes coding for the enzymes of these two biosynthetic steps.

Therefore research was initiated to test the hypothesis that acetylleptinidine biosynthesis occurs from solanidine, via leptinidine, and experiments were conducted that addressed the following objectives:

1. Develop an analytical method for accurate quantitation of *S. chacoense* steroidal alkaloids.
2. Confirm the structural identities of solanidine, leptinidine, and acetylleptinidine isolated from *S. chacoense*.
3. Determine the effects of leaf physiological age on the concentrations and biosynthesis of solanidine, leptinidine, and acetylleptinidine.
4. Determine if leptinidine can be used as a precursor for acetylleptinidine biosynthesis by *S. chacoense* leaves.
REVIEW OF LITERATURE

Literature relating to steroidal glycoalkaloids and alkamines is reviewed. Specifically, attention is given to their chemistry, quantitation, biosynthesis, toxic effects on humans, and mode of biological action. In addition, their potential role in host-plant resistance to the Colorado potato beetle is briefly reviewed.

Chemistry

The organic chemistry of steroidal alkaloids has been reviewed regularly, including synthesis and reference to spectral characterization (see Ripperger and Schreiber, 1981 and references therein).

Structure

Steroidal alkaloids exist as glycosides in the plant, thus the name glycoalkaloid, and the glycosides are commonly referred to as 'Solanum' glycoalkaloids. The aglycones of steroidal glycoalkaloids are alkamines, and structurally are based upon a C_{27}-cholestane carbon skeleton. Five major structural types have been identified: (1) solanidane; (2) spirosolane; (3) 22,26-epiminocholestane; (4) \( \alpha \)-epiminocyclohemiketal; and (5) 3-aminopirostane (Roddick, 1986; Ripperger and Schreiber, 1981) (Fig. 1). Compounds belonging to the solanidane and the spirosolane structural classes have been the most intensely studied of the five.
Figure 1. Major structural classes of *Solanum* steroidal alkamines.
Steroidal glycoalkaloids may differ according to the structure of the aglycone, or the sugar moiety, or both (Fig. 2). For example, two common glycoalkaloids of potato, α-solanine and α-chaconine, share the same aglycone, solanidine, but differ only according to sugar composition. On the other hand, solasonine and α-solanine have identical sugar compositions but differ according to the structure of the aglycone. Aglycones often are closely related as well, differing simply by chirality, such as tomatidenol (22S, 25S) and solasodine (22R, 25R), or by a single degree of unsaturation, such as solanidine (solanid-5-en-3β-ol) and demissidine (solanidan-3β-ol) (Fig. 3).

Spectroscopy

Spectral characterization of alkamines has been extensive. Because of the structural similarity of this class of secondary metabolites among themselves and to other, closely-related natural products (e.g., sapogenins), published data can be used reliably to elucidate key structural features. Radeglia et al. (1977), for example, published $^{13}$C-NMR chemical shifts for eight important Solanum alkamines, which included solanidine, tomatidine, demissidine, and solasodine, but with some uncertainty in particular assignments. $^{13}$C-NMR data, frequently with some supporting $^1$H-NMR data, has also been reported for other spirosolane alkamines and their corresponding glycosides (Bird et al., 1979b; Weston et al., 1977; Yamashita et al., 1990), as well as for alkamines of the solanidane and epiminocholestane types (Bird et al., 1979a; Krishna Kumari et al., 1985; Murakami et al., 1985). Tables of $^1$H- and $^{13}$C-NMR data have also been compiled for other selected alkamines (Ripperger and Schreiber, 1981).
Figure 2. Aglycone and carbohydrate composition of selected steroidal glycoalkaloids.
Figure 3. Structures of selected *Solanum* steroidal alkamines.
In addition to NMR, MS has been a valuable tool in the identification of alkamines and glycoalkaloids. Ripperger and Schreiber (1981) compiled a table of diagnostic fragments of the spirosolanes, epiminocholestanes, solanidanes, 3-aminospirostanes, and epiminocyclohemiketals. Other reviews on the MS of steroidal alkaloids have been compiled by Budzikiewicz (1964, 1982, 1991). Fast atom bombardment MS has been used for the analysis of glycoalkaloids in crude extracts of potato peels and sprouts (Price et al., 1985). Several fragment ions were found to be diagnostic for individual glycoalkaloids, thus demonstrating the potential of this method for qualitative analysis. In addition, this method requires less rigorous sample preparation, thus minimizing the potential for generating hydrolytic artifacts that may be encountered using more conventional sample preparation procedures.

Quantitation

The amphipathic nature of steroidal glycoalkaloids challenges efficient extraction, since these compounds tend to readily partition into lipophilic cellular structures, and, despite their very polar sugar moieties, can be quite water insoluble often requiring rigorous and time-consuming acidic aqueous or acidic organic extraction procedures. Many sample preparation methods rely upon ammonia precipitation of glycoalkaloids from crude extracts before analysis (Gregory et al., 1981; Jellema et al., 1981; Morris and Lee, 1981; Sinden et al., 1986b). Although this method is suitable for glycoalkaloid analysis in S. tuberosum, ammonia precipitation does not quantitatively precipitate glycoalkaloids of some wild Solanum species, and may result in losses of up to 60% (Sinden et al., 1986b).

Methods of quantitation have been as simple as colorimetric assays to analyze total glycoalkaloid concentrations to as sophisticated as high-resolution gas chromatography (HR-GC) or high-performance liquid chromatography (HPLC) to
analyze individual alkamines and glycoalkaloids. Methods related to potato steroidal alkaloid analysis have been reviewed (Coxon, 1984; Jadhav et al., 1981).

**Colorimetric Assays**

Colorimetric assays (Bushway et al., 1980b; Coxon et al., 1979; Fitzpatrick and Osman, 1974; Sanford and Sinden, 1972) have proved useful in certain applications, particularly those for which only total glycoalkaloid concentrations are desired. However, since certain individual glycoalkaloids are now recognized as having ecological and toxicological significance, increasing emphasis is being placed on higher resolution chromatographic methods to resolve and quantitate glycoalkaloids of particular interest.

**High Performance Liquid Chromatography**

The high molecular weights of glycoalkaloids make them good candidates for HPLC analysis. However, the lack of a good UV-absorbing chromophore makes glycoalkaloid analysis by HPLC methods, which depend heavily upon UV detection, more challenging. Therefore, detection limits for underivatized steroidal alkaloids can be as high as 0.1 μg (Morris and Lee, 1981), and analysis is limited to unsaturated compounds. Although earlier HPLC methods (Hunter et al., 1976, 1980) used adsorption chromatography, solvent choices for normal phase separations are limited because of the high UV-cutoffs of conventional mobile phases. Crabbe and Fryer (1980) used carbohydrate columns and mobile phases consisting of methanol, isopropanol, and cyclohexane for HPLC of spirosolane alkamines and glycosides, permitting the separation to be monitored at 205 nm. Potato glycoalkaloids have also been resolved using carbohydrate columns but with tetrahydrofuran-water-acetonitrile mobile phases (Bushway et al., 1979, 1980a). Reverse-phase HPLC (RP-HPLC) of potato steroidal alkaloids has been reported by several (Bushway et al., 1979; Carmen
et al., 1986; Friedman and Dao, 1992; Hellenäs, 1986; Jonker et al., 1992; Morris and Lee, 1981), and permits quantitation at lower wavelengths (i.e., 200-205 nm). RP-HPLC was used to consistently quantitate seven glycoalkaloids present in *S. chacoense* foliage (Sinden et al., 1986b). Some HPLC methods are more suited for analyses of glycoalkaloids and their partial hydrolysis products than for aglycones since aglycones can be highly retained and may not elute (Morris and Lee, 1981). However, more recently reverse phase-ion pair chromatography (Friedman, 1992; Friedman and Dao, 1992) has succeeded in eluting solanidine from a mixture of acid hydrolysis products derived from α-solanine and α-chaconine.

Despite the absence of a good UV-absorbing chromophore in steroidal alkaloids, only one attempt seems to be reported in the literature to increase the extinction coefficients of these solutes through derivatization. Drewes et al. (1992) benzoylated solasodine for HPLC quantitation at 254 nm, but obtained a mixture of mono- and di-substituted derivatives. In addition, the method was only used for purchased standards.

**Gas Chromatography**

GC has also proved to be a powerful procedure for steroidal alkaloid analysis. Separations using GC have principally been limited to the lower molecular weight alkamines rather than their corresponding glycosides because the high operating temperatures necessary to achieve elution of the latter, although GC of permethylated glycoalkaloids has been reported (Herb et al., 1975). This limitation becomes a disadvantage when knowledge of the glycoalkaloid composition is desired and when one alkamine can be the aglycone of more than one glycoside. King (1980) conducted isothermal analysis of trifluoroacetate derivatives of solanidine and demissidine using a nitrogen phosphorous detector, and reported recoveries of 95% or more based upon
fortification of samples with glycoalkaloid standards. Van Gelder (1985) developed an HR-GC procedure that resolved structurally similar aglycone pairs such as solanidine and demissidine, and solasodine and tomatidine.

Thin-Layer Chromatography

Numerous TLC procedures have been developed for steroidal alkamines and glycoalkaloids (reviewed in Jadhav et al., 1981), but their application has been primarily limited to qualitative analysis. Popular detection reagents include iodine vapor, antimony trichloride, and Dragendorff's. Some TLC procedures have been developed for quantitative analysis (Jellema et al., 1980 and 1981) but have not gained widespread acceptance.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) have received relatively little attention but offer promise in terms of simplicity and sensitivity, and their specificity permits less rigorous sample workup. Assays have been developed that demonstrate specificity for structural features included in rings C through F (Morgan et al, 1983; Plhak and Sporns, 1992); however, because of limitations imposed by hapten linkage to the antigen, the assays cannot distinguish between saturated and unsaturated alkamine ring structures or glycoalkaloids having identical aglycones but different carbohydrate moieties.

Biosynthesis

Alkamines

The biochemistry of steroidal alkaloids has received considerable attention, though lagging behind the organic chemistry and isolation of new compounds, and has been reviewed previously (Heftmann, 1983; Jadhav and Salunkhe, 1975; Jadhav et al.,
The chemical structures of *Solanum* glycoalkaloids suggest derivation from cholesterol, having origins from triterpene biosynthesis. Initial confirmation of these biosynthetic origins came from experiments that demonstrated incorporation of radiolabeled acetate into \( \alpha \)-tomatine in tomato (Heftmann, 1983). 4-\(^{14}\)C-Cholesterol administered to the leaves and flowers of *Lycopersicon pimpinellifolium* resulted in radioactive labeling of \( \alpha \)-tomatine (Heftmann et al., 1967), but the actual site of labeling in \( \alpha \)-tomatine was not determined. That \( \alpha \)-tomatine was the radiolabeled product was confirmed by TLC of the active fraction and TLC of oxidized products of the labeled compound. *Solanum* species were later demonstrated to utilize radiolabeled acetate (Guseva and Paseshnichenko, 1958), mevalonate (Guseva et al., 1961), cholesterol (Heftmann et al., 1967), cycloartenol (Ripperger et al., 1971), and lanosterol (Ripperger et al., 1971) for the biosynthesis of *Solanum* steroidal glycoalkaloids.

Following experimental confirmation of the obvious biosynthetic origins of this unique chemistry, research focussed on properly understanding the sequence of side chain oxidations, amination, and cyclization. Much of the biosynthesis has been elucidated using *Veratrum* species, and the biosynthetic events occurring in these species are generally perceived to mirror those occurring in *Solanum*. Kaneko et al. (1976, 1977) have proposed a hypothetical biogenetic scheme for solanidine biosynthesis in *Veratrum grandiflorum* (Fig. 4). Whereas etioline and teinemine have been isolated from *Veratrum* species, probably the first report of these compounds in *Solanum* was that of Lin et al. (1987).

The sequence of E and F ring closure remains unclear. Schreiber (1968) first hypothesized that F ring closure preceded E ring closure, and his hypothesis is supported by numerous occurrences of alkaloids having a closed F ring and an open E ring. Experimentally, this has been supported by Tschesche and Spindler (1978), who
Figure 4. Hypothetical pathway for solanidine biosynthesis in *Veratrum grandiflorum*. Adapted from Kaneko et al., 1976. I = Dormantinol; II = Dormantinone; III = Verazine; IV = Etioline; V = Etiolidine; VI = Teinemine; VII = Solanidine.
have demonstrated in *S. laciniatum* conversion of solacongestidine and dihydrosolacongestidine to soladulcidine. Experimental evidence also supports the alternative sequence, i.e., E ring closure prior to F ring closure: Both (25R) 26-aminocholesterol and (25R) 26-amino-16β-hydroxycholesterol (Tschesche and Brennecke, 1980) and 26-aminodihydrosoladulcidin (Tschesche and Piestert, 1975) have been utilized by *S. laciniatum* to synthesize solasodine.

In the biosynthesis of the spirosolane (configuration at C25 is S), soladulcidine, amination occurs immediately after C26-hydroxylation and before any further oxidation of the side chain (Tschesche and Brennecke, 1980). Tschesche et al. (1976) have demonstrated, using *S. lycopersicum* and *S. laciniatum*, that direct replacement of the C26 or C27 hydroxyl group occurs rather than transamination of a C26-oxo-intermediate. Their results also show that R or S configuration at C25 is determined by the C26-hydroxylating enzyme. The nitrogen in steroidal glycoalkaloid biosynthesis in *Veratrum* may originate from L-arginine (Kaneko et al., 1976), whereas the nitrogen appears to originate from glycine and alanine in *S. tuberosum* (Jadhav et al., 1973).

**Glycoalkaloids**

Liljegren (1971) hypothesized that glycosylation was the last step in solasonine and solamargine biosynthesis, and glycosylation via a glucosyltransferase of solanidine to solanine has been demonstrated in potato slices and enzyme extracts of potato sprouts incubated with UDP-U-14C-glucose (Jadhav and Salunkhe, 1973). More recently, a sequential addition of saccharide units to solanidine has been shown (Osman et al., 1980), which is in keeping with the formation of flavonoid glycosides (Miles and Hagen, 1968). Solanidine UDP-glucose glucosyltransferase (solanidine-GT; UDP-glucose: solanidine glucosyltransferase, EC 2.4.1) has been purified to near homogeneity from potato sprouts, having a native molecular weight of 36,000
(Stapleton et al., 1991), and having specificity for 3-β-hydroxy steroids containing a ring nitrogen. Solanidine-GT activity increases concomitantly with glycoalkaloid accumulation in wounded potato tubers (Bergenstråhle et al., 1992). Despite the polarity given to steroidal glycoalkaloids by the relatively large carbohydrate moiety, steroidal glycoalkaloid transport apparently does not occur in plant tissues (Eltayeb and Roddick, 1985; Roddick, 1982).

**Localization**

Glycoalkaloid concentrations are highest in regions of high metabolic activity, including meristematic regions, young leaves, growing sprouts, and flowers (Friedman and Dao, 1992; Lampitt et al., 1943; Wolf and Duggar, 1940), and are higher in leaves than in tubers (Deahl et al., 1973; Friedman and Dao, 1992). In tubers, glycoalkaloid concentrations are highest in the periderm and cortex, but concentrations are low in the pith (Lampitt et al., 1943). Mechanically damaged tubers accumulate glycoalkaloids, a process that has now been demonstrated to occur due to de novo synthesis (Bergenstråhle et al., 1992). That synthesis was responsible for higher glycoalkaloid concentrations in meristematic regions was supported when root tips were observed to synthesize α-tomatine more rapidly than other plant parts in tomato (Roddick, 1974).

In general, most steroidal glycoalkaloids are found in all plant parts; however, not all steroidal glycoalkaloids are synthesized constitutively. *S. chacoense* synthesizes leptines in the foliage but not in the tubers (Kuhn and Löw, 1961), and roots initiated from *S. chacoense* tubers synthesize commersonine and dehydrocommersonine while *S. chacoense* tubers do not (Zacharius and Osman, 1977). Steroidal glycoalkaloids may accumulate in the vacuole or in the soluble fraction of the cytoplasm or both, as α-
tomatine, leptines, and leptines have been found in mechanically expressed sap (Deahl and Sinden, 1987; Roddick, 1976).

At least part of the biosynthesis of steroidal glycoalkaloids appears to occur in membranes constituting microsomal fractions of tissue homogenates (Osman et al., 1987; Roddick, 1976). \( \alpha \)-Tomatine was almost exclusively isolated from 105,000 g supernatants of tomato homogenates (Roddick, 1976), and the hydroxylation of solanidine to both 23-hydroxysolanidine (leptinidine) and possibly another \( x \)-hydroxysolanidane was found to occur in microsomal fractions prepared from leaves of \( S. \ chacoense \) (Osman et al., 1987). The hydroxylation of solanidine required NADPH, suggesting the role of a cytochrome P-450.

In addition to evidence for the role of microsomal enzymes in the biosynthesis and metabolism of steroidal alkaloids, Ramaswamy et al. (1976) have reported the complete synthesis of solanidine in chloroplasts isolated from potato peel, which have been greened under low temperature and dim light conditions (Ramaswamy and Nair, 1974). These same authors (Ramaswamy and Nair, 1984) have provided evidence for a \( C_1 \)-pathway in which \( CO_2 \) is fixed into formic acid, which eventually becomes incorporated into triterpene biosynthesis -- a pathway for which there is support in isolated pea chloroplasts (Shah and Cossins, 1970). These data become more convincing in light of recent evidence for full autonomy of isoprenoid biosynthesis in chloroplasts (Cheniclet et al., 1992; Kuntz et al., 1992; Schulze-Siebert and Schultz, 1987; Soler et al., 1992), but the interdependence of chlorophyll biosynthesis with steroidal alkaloid biosynthesis is at variance with other work (reviewed in Jadhav and Salunkhe, 1975).
Metabolism

Catabolism of steroidal alkaloids has been observed in both *Solanum* and *Lycopersicon*. Solasodine content in fruits of *S. mammosum* increases in green fruit, but then declines during ripening (Telek et al., 1977). In tomato, degradation of $^{[14]C}$tomatine was observed in excised tomato fruits, with the extent of degradation dependent upon the developmental stage of the fruit and the length of incubation of radiolabel (Eltayeb and Roddick, 1985). Ripened fruit and longer incubation periods resulted in less recovery of radiolabel in $\alpha$-tomatine. In addition, radioactivity from metabolized $^{[14]C}$tomatine was found associated with chlorophylls, xanthophylls, and carotenes, results which the authors indicated may support the existence of an "isoprenoid-scavenging" pathway.

Synthesis in Tissue Culture

Obtaining steroidal glycoalkaloid levels in cultured cells comparable to that of fresh, whole plant tissue has largely been unsuccessful. Roddick and Butcher (1972) failed to positively influence levels of $\alpha$-tomatine in cultured excised tomato by additions of metabolic precursors (viz. acetate, mevalonic acid, and cholesterol) or ammonia supplements (viz. $(\text{NH}_4)_2\text{SO}_4$, urea, and casamino acids), providing support against the hypothesis that insufficient titers of key metabolites and metabolic affectors were the cause of low levels of $\alpha$-tomatine biosynthesis in cultured tomato tissues.

Other work seeking to increase steroidal glycoalkaloid titers in *S. laciniatun* has demonstrated that shoots cultured in photoautotrophic conditions provide higher solasodine levels on a mg/g dry weight basis than those cultured photoheterotrophically, heterotrophically (no light), or callus grown photoheterotrophically (Conner, 1987). Differences in solasodine levels of regenerated shoots grown photoautotrophically and those of field-grown plant were attributed to
poorer light quality given to in vitro-grown shoots (Conner, 1987). The data of this work also support a positive relationship between actively photosynthesizing chloroplasts and solasodine biosynthesis. The authors hypothesized that low solasodine yield in S. laciniatum cell cultures was due to differences in gene expression under culture conditions compared to gene expression under autotrophic conditions.

Hosoda and Yatzawa (1979) reported successful callus culture of S. laciniatum, which produced key sterols and a steroidal glycoalkaloid, on PN medium containing an optimum concentration of 0.5 ppm 2,4-D either in the presence or the absence of light. Kinetin supplements to the media were harmful to callus growth.

**Human Toxicity**

The toxicology of steroidal alkaloids has been reviewed exhaustively elsewhere (Jadhav et al., 1981; Keeler et al., 1990; Morris and Lee, 1984). Even though their toxic effects extend across diverse taxonomic groups (Roddick, 1986), the effects of steroidal alkaloids found in food frequently consumed by humans has understandably received considerable attention. Whereas glycoalkaloid concentrations are relatively low in cultivated potato tubers, physiological and mechanical stresses can cause their concentrations to exceed the 20 mg/100 g fresh weight threshold considered safe for consumption (Jadhav and Salunkhe, 1975; Jadhav et al, 1980 and 1981; Wu and Salunkhe, 1976).

The only recorded cases of glycoalkaloid ('solanine') poisoning of humans have involved glycoalkaloids of potato, with up to 30 deaths in over 2000 documented cases (Morris and Lee, 1984). Symptoms of glycoalkaloid poisoning usually occur 8-12 h after ingestion, and include vomiting, diarrhea, severe abdominal pain, drowsiness, apathy, confusion, weakness, depression, and sometimes unconsciousness (McMillan and Thompson, 1979; Morris and Lee, 1984). Vital signs associated with
glycoalkaloid poisoning include raised body temperature, rapid and weak pulse, low blood pressure, and rapid respiration.

Recently, the teratogenic effects associated with glycoalkaloids has been specifically addressed. Friedman et al. (1991) evaluated the teratogenicity of common steroidal alkaloids (solanidine, solasodine, demissidine) and glycoalkaloids (α-solanine, α-chaconine) in FETAX (frog embryo teratogenesis assay-Xenopus) assays. α-Chaconine was 3X more toxic than α-solanine and glycoalkaloids were more toxic than the alkamines, which emphasized the importance of the carbohydrate moiety (both its presence and structure) for toxicity. FETAX assays reportedly can predict mammalian developmental toxicity with 89% accuracy (Bantle et al., 1990). Keeler et al. (1990) reported induction of congenital craniofacial malformations in hamsters fed S. elaeagnifolium, S. dulcamara, the glycoalkaloid component of which contained predominantly solasodine glycosides, as well as those fed S. tuberosum sprouts, which contained both solanidine glycosides and free solanidine.

Natural Host-Plant Resistance to Colorado Potato Beetle

The Colorado potato beetle (CPB) is the most economically-important pest to potato (Jacques, 1988). To date, the most effective means for controlling this pest has been the use of synthetic insecticides. However, because of their extensive use, many of these insecticides are now ineffective in the control of many CPB populations. The intense selection pressure imposed on CPB populations by overuse of particular insecticides, along with the adaptability of the CPB to them, has rendered many of these products ineffective for control (Cutkomp et al., 1958; Forgash, 1985; Harris and Svec, 1976). Current research efforts strive to incorporate natural resistance to insects into economically-important solanaceous crops in an attempt to provide diversification in an integrated pest management (IPM) control scheme, including host-plant resistance
(Plaisted et al., 1992; Ross, 1966; Sinden et al., 1991; Torka, 1950), trap crops (Duchesne and Parent, 1991), and biological control (Anderson et al., 1988; Campbell et al., 1985; Pucci, 1992), to reduce the environmental and physical consequences of extensive synthetic pesticide use.

Glycoalkaloids as Antifeedants

*Solanum* steroidal glycoalkaloids have long been implicated in the natural defense of solanaceous crops to insects, and, because of their diverse biological effects and insect-deterrent activities, would appear to be good candidates to supplement existing natural defenses (e.g., trichomes). Whereas total glycoalkaloid content was often correlated with CPB resistance, it is now recognized that sufficiently high concentrations of specific glycoalkaloids are at least equally important (Sinden et al., 1986a; Tingey, 1984). Crystallized samples of α-solanine, α-chaconine, leptine I, leptine I, leptinine I, leptinine II, demissine, and α-tomatine discouraged food uptake by CPB (Sturckow and Löw, 1961). In addition, Bushway et al. (1987) identified anticholinesterase activity by several steroidal alkaloids (see below). Since many synthetic pesticides target cholinesterase inhibition as their primary mode of action, Bushway et al. (1987) proposed that the ability of CPB to adapt to extensive use of this class of insecticides may, at least in part, account for the adaptation of CPB to the natural deterrence formerly mediated by these secondary products. In addition, CPBs resistant to DDT were also more resistant to leptine, leptinines, and *S. chacoense* than "normal" CPBs.

Detoxification of some potato glycoalkaloids by CPB may also be due to rhamnosidase activity that has been isolated by Bergeron et al. (1988), which metabolizes α-chaconine to β2-chaconine in partially purified preparations, but does not metabolize α-solanine. Hydrolysis of the sugar moiety may affect binding of the
steroidal glycoalkaloids to membrane sterols, thereby suppressing their membrane lytic properties (see below).

**Sources of CPB Host-Plant Resistance**

Two *Solanum* species, *S. chacoense* and *S. berthaultii*, have shown consistently high resistance to CPB in the field (Carter, 1984). *S. chacoense* resistance has been attributed to leptine content, but loss of acetylation of leptines, i.e., to the formation leptinines, reduces their activity (Sturckow and Löw, 1961). Sinden et al. (1986a) determined that high-leptine (120-306 mg/100 g fresh weight) clones of *S. chacoense* had lower adult infestations in the field, resulting in less feeding damage than low-leptine (< 51 mg/100 g fresh weight) clones. Lower adult feeding rates and slower larval development were also observed on high-leptine clones. The authors assumed that observed differences between high- and low-leptine producing clones were likely due only to differences in foliar leptine concentrations, since *S. chacoense* clones containing leptines were significantly more resistant to CPB feeding than clones lacking leptines but having higher total foliar glycoalkaloid concentrations. However, possible additive or synergistic interactions of α-solanine and α-chaconine were not discussed. Although *S. chacoense* clones of high solanine plus chaconine concentrations (≥ 170 mg%) and no measureable leptine concentrations were generally less effective against CPB, some resistance was indicated, and synergism in biological activity of certain steroidal glycoalkaloids does occur (Roddick et al., 1988). Very high solanine plus chaconine concentrations (i.e., 704 mg% fresh weight) were as effective in some resistance parameters as a *S. chacoense* clone (320287-1) containing 120 mg% fresh weight leptines.
Leptine-producing clones of *S. chacoense* are excellent candidates for developing host-plant resistance in commercially-acceptable cultivars because genes for leptine biosynthesis are apparently rare (Sinden et al., 1986b) and thus selection pressure imposed on the CPB will have been minimal. In addition, important physiological regulation of leptine biosynthesis seems to already be in place, which appears to limit leptine biosynthesis to the foliage, thus reducing the potential for introducing toxic glycoalkaloids into the tubers of hybrid germplasm. (Kuhn and Löw, 1961, Sinden et al., 1986b).

Another wild *Solanum* species, *S. berthaultii*, has demonstrated resistance to aphids, leafhoppers, flea beetles, spider mites, and the CPB (Gibson and Turner, 1977). *S. berthaultii* mediates resistance to these insects via secretory trichomes, the exudate of which entraps small insects (Gibson, 1971; Gibson and Turner, 1977) and possibly CPB larvae (Gibson, 1976), although entrapment of CPB larvae has not been substantiated in the field (Dimock and Tingey, 1987). Effects of *S. berthaultii* on CPB included reduced oviposition (Wright et al., 1985), slower larval development, lower immigration rates, and higher larval mortality (Groden and Casagrande, 1986). However, after two generations of confinement on *S. berthaultii* in the field, beetles no longer demonstrated a reluctance to oviposit on the host (Groden and Casagrande, 1986). In addition, survival of larvae from third generation CPB reared on *S. berthaultii* was comparable to that of larvae raised on *S. tuberosum*. The resistance is heritable as reduced oviposition was observed in the F$_2$ of *S. tuberosum X S. berthaultii* in laboratory studies (Wright et al., 1985). A recent germplasm release, NYL 235-4, derived from *S. tuberosum X S. berthaultii* has demonstrated good CPB resistance (Plaisted et al., 1992).
Steroidal Glycoalkaloids and Sensory Perception

Generally, studies examining resistance of tomatoes and potatoes have not directly investigated effects of steroidal glycoalkaloids at the sensory level of the CPB. Harrison and Mitchell (1988) focused on the influence of selected steroidal glycoalkaloids (i.e., α-solanine and α-tomatine) on CPB sensory perception and feeding behavior. Specifically, these authors correlated reductions in feeding on alkaloid-treated plants with any changes that may have been observed in stereotyped, sampling behavior. Their comprehensive study, which included analyzing the behavior and feeding of beetles collected from three different geographic regions, and treatments that included both native plants and non-host adapted plants, failed to establish any sensory-based effects by α-solanine and α-tomatine.

Mode of action of Steroidal Glycoalkaloids

Anticholinesterase Activity

Symptoms of glycoalkaloid poisoning are consistent with their reported effects on cholinesterase. Orgell and Hibbs (1963) demonstrated anticholinesterase activity of crude preparations of α-solanine plus α-chaconine, leptine, α-tomatine, and demissidine. Subsequently, using purer materials, Bushway et al. (1987) tested seven glycoalkaloids (α-chaconine, β₂-chaconine, α-solanine, dehydrocommersonine, commersonine, demissine, and α-tomatine) and three alkamines (solanidine, tomatidine, and demissidine) for anticholinesterase activity. All of the glycoalkaloids, except α-tomatine, inhibited cholinesterase activity greater than 18%. Alkamines were slightly less effective, with solanidine and demissidine inhibiting cholinesterase activity between 10 and 15%. α-Tomatine and tomatidine each inhibited activity approximately 4%. Their data support the hypothesis that both the aglycone and the sugar moiety influence biological activity (Roddick and Rijnenberg, 1986). The nature of the sugar
moiety was also important to activity, as those glycosides having less than four sugar residues (e.g., α-solanine and α-chaconine) were more inhibitory than the one having four residues (i.e., α-tomatine) (Bushway et al., 1987).

Membrane Disruption

In addition to anticholinesterase activities, membrane lytic properties of these compounds has been well documented, and have been demonstrated to have biological significance (FETAX assay; Blankemeyer et al., 1992). While some have hypothesized that the biologically active moieties of steroidal glycoalkaloids are the aglycones (Segal et al., 1974; Segal and Milo-Goldzweig, 1975; Segal and Schlösser, 1975), others have provided strong evidence for the necessity of the entire glycoside (Roddick and Drysdale, 1984; Roddick and Rijnenberg, 1986). Evidence supports binding of certain steroidal glycoalkaloids to membrane sterols as a potential mode of action of steroidal glycoalkaloid biological effects (Roddick and Drysdale, 1984; Roddick and Rijnenberg, 1986). α-Tomatine, in particular, complexes with 3β-hydroxysterols (Steel and Drysdale, 1988). Sterol-containing liposomes were readily lysed by α-chaconine and α-tomatine, while sterol-free liposomes were not (Roddick and Drysdale, 1984; Roddick and Rijnenberg, 1986), and the extent of lysis by α-tomatine was directly correlated with the amount of membrane sterol present (Roddick and Drysdale, 1984); however, α-solanine lyses neither sterol-containing nor sterol-free liposomes (Roddick and Rijnenberg, 1986 and 1987). Even slight variations of the sugar moiety can adversely affect binding properties to membrane sterols (Roddick and Rijnenberg, 1986). For example, β2-chaconine, which lacks a single rhamnose, demonstrated no lytic properties (Roddick and Rijnenberg, 1986 and 1987), the results of which are consistent with the detoxification of α-chaconine to β2-chaconine by the CPB (see above). Also, partial hydrolysis of the sugar molecule of α-tomatine reduces its ability
to complex with 3β-hydroxysterols and reduces its *in vitro* fungitoxicity (Arneson and Durbin, 1968).

Further support for membrane interaction as a potential mode of action of some steroidal glycoalkaloids is that *Fusarium solani* mutants having reduced membrane sterol content are more resistant to α-tomatine than wild type fungi (Défago and Kern, 1983). Furthermore, *Phytophthora megasperma*, which can grow in the absence of membrane sterols (Steel and Drysdale, 1988), or will incorporate exogenously-supplied sterols into membrane structures, was more susceptible to α-tomatine treatments when cultured on 3β-hydroxysterol-containing media.

Evidence also exists for synergistic activity between steroidal glycoalkaloids. Although inactive alone, α-solanine does interact synergistically with α-chaconine in the lysis of sterol-containing liposomes (Roddick and Rijnenberg, 1987), erythrocytes, beet root cells, and *Penicillium notatum* protoplasts (Roddick et al., 1988). Other factors also affect the toxicity of steroidal glycoalkaloids. α-Tomatine is more toxic at high pH (deprotonated form) than at low pH (protonated form), and the deprotonated form complexes with cholesterol (a 3β-hydroxysterol), whereas the protonated form does not (Arneson and Durbin, 1968).
CHAPTER I
ANALYSIS OF SOLANUM ALKALOIDS USING INTERNAL STANDARDIZATION AND CAPILLARY GAS CHROMATOGRAPHY

ABSTRACT

A capillary gas chromatographic method has been developed for quantitation of principal steroidal alkaloids of Solanum chacoense and S. tuberosum. The method uses tomatine as an internal standard and requires less than 100 mg dry weight of plant tissue. Internal standard recoveries were approximately 80%-95%. Glycoalkaloids were concurrently extracted and hydrolyzed using 1 N HCl in methanol. The underivatized aglycones, solanidine, leptinidine, tomatidine, and acetylleptinidine, the peak areas of which constituted between 70% and 80% of the total peak area of the chromatogram, were resolved in less than 20 min using a nonpolar (Rt-x-1) megabore fused silica column, with retention times of 10.34, 14.47, 15.44 and 15.96 min, respectively. Retention times varied less than 0.2%. The procedure was applied to the quantitation of steroidal alkaloids from S. chacoense and S. tuberosum leaves and tubers, for which relative standard errors were typically less than 2% of the mean.
INTRODUCTION

The need to quantitate glycoalkaloids in potato cultivars has been emphasized repeatedly (Gregory, 1984; Osman, et al., 1978; Osman, 1980). While levels below 20 mg/100 g fresh weight of tuber pose no threat to consumers, excessive levels of specific glycoalkaloids impart bitterness (Sinden and Deahl, 1976) and may be toxic or teratogenic (Jadhav et al., 1981; Kuc, 1975; Maga, 1980; Morris and Lee, 1984; Roddick, 1986). Therefore, quantitation of individual glycoalkaloids is critical, especially in breeding programs that introduce germplasm from wild potato species for the development of new potato cultivars. Wild species, such as *Solanum chacoense* Bitter, commonly accumulate high levels of glycoalkaloids, and one *S. tuberosum* cultivar, Lenape, which had *S. chacoense* in its ancestry (Akeley et al., 1968) was withdrawn from production because of its high glycoalkaloid content (Zitnak and Johnston, 1970). Nonetheless, certain accessions of *S. chacoense*, which have high levels of leptine glycoalkaloids (Sinden et al., 1986b), have demonstrated excellent resistance to the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Carter, 1987; Sinden et al., 1986a; Sturckow and Löw, 1961) and have become important genetic resources for potato breeding programs (Deahl and Sinden, 1987; Ross, 1966; Sinden et al., 1986a; Sinden et al., 1986b).

The importance of quantitating potato glycoalkaloids and its challenge to potato researchers is reflected in the number of methods that have been developed. These methods vary in their approach and include colorimetric assays (Bushway et al., 1980; Coxon et al., 1979; Fitzpatrick and Osman, 1974; Sanford and Sinden; 1972), thin-layer chromatography (TLC) (Coxon and Jones, 1981; Deahl and Sinden, 1987; Hunter et al., 1976; Jellema et al., 1980, 1981; Shih and Kuc, 1974), high-performance liquid chromatography (HPLC) (Crabbe and Fryer, 1980; Morris and Lee, 1981), and gas chromatography (GC) (King, 1980; Van Gelder, 1985). However, these
chromatographic methods lack an accurate measure of glycoalkaloid recovery during sample preparation. In order to estimate recoveries, King (1980) fortified selected samples with glycoalkaloids already present in the tissue, but this technique fails to accurately account for sample-to-sample differences in recovery.

Further, substantial and variable losses in recovery can be incurred if glycoalkaloids are precipitated from crude extracts by adding concentrated ammonia (ammonia precipitation; Gregory et al., 1981; Jellema et al., 1981; Morris and Lee, 1981; Sinden et al., 1986b). Despite being used routinely in preparing extracts of *S. tuberosum* for glycoalkaloid analysis, ammonia precipitation does not quantitatively precipitate glycoalkaloids of some wild *Solanum* species, including *S. chacoense*. Losses greater than 60% have been reported (Sinden et al., 1986b).

In this chapter, the development of a procedure that combines the extraction and hydrolysis of glycoalkaloids into a single step, and uses capillary GC to quantitate principal steroidal glycoalkaloid (SGA) aglycones from *S. tuberosum* and *S. chacoense* leaves and tubers (Fig. 5) is reported. This procedure requires only a minimal amount of plant material (less than 100 mg dry weight), avoids ammonia precipitation during sample preparation, and uses a readily available internal standard for consistent, accurate quantitation.

**MATERIALS AND METHODS**

**Plant Material**

Tubers of *S. tuberosum* cv. Atlantic were field-grown using standard cultivation practices at the OARDC, Wooster, OH. Tubers of *S. chacoense*, PI 458310-1, were harvested from plants grown in the greenhouse. Foliar samples of *S. tuberosum* and *S. chacoense* were taken from plants (30-50 cm tall) grown in the greenhouse in a sand:soil:peat moss (1:1:1) potting mixture. Foliar samples also were taken from *S.
Figure 5. Structures of $C_{27}$-steroidal alkaloids.
chacoense plantlets cultured in vitro for 30 days (30 °C, 12-h photoperiod using fluorescent lighting) on Murashige and Skoog (1962) basal media containing 3% sucrose.

Alkaloid Standards

Demissidine, solanidine, solasodine, tomatidine and tomatine were purchased from Sigma Chemical Company (St. Louis, MO).

Purification of acetylleptinidine

Freeze-dried leaves (ca. 10 g dry weight) of S. chacoense, PI 458310-1, were homogenized 5 min in 350 ml of 2% acetic acid in methanol using a Waring blender. The homogenate was vacuum filtered through Whatman No. 1 filter paper, and the filtrate was evaporated to near dryness at 50 °C in vacuo. The residue was redissolved in 50 ml of 1 N HCl in methanol, capped under nitrogen, and hydrolyzed at 70 °C for 4 h in a shaking water bath. After cooling to room temperature, the hydrolysate was raised to pH 10 with concentrated ammonium hydroxide and centrifuged at 12000g for 6 min. The supernatant was partitioned twice (25 ml/each) against chloroform and the combined chloroform phases, which contained the aglycones, were evaporated to near dryness at 40 °C in vacuo.

The residue was redissolved in chloroform and subjected to two successive flash chromatography steps. The first step used a 1 X 4 cm silica gel (E.M. Merck, 40-60 μm) column, onto which approximately 100 mg (300 μl) of the preparation was applied. The column was eluted with 5 ml of hexane followed by 20 ml of ethyl acetate-hexane (2:8); 1 ml fractions were collected. Fractions containing acetylleptinidine according to TLC (see below) were combined, evaporated to dryness at 50 °C under a stream of nitrogen, and redissolved in chloroform. Twenty-five mg (50 μl) of the pooled acetylleptinidine-containing fractions were loaded onto a 0.8 X 25
33 cm silica gel (E.M. Merck, 40-60 μm) column, and acetylleptinidine was purified by first washing the column with 10 ml of ethyl acetate-hexane (1:9), and then eluting acetylleptinidine with ethyl acetate-hexane (2:8). Fractions of 1.2 ml were collected. The composition of each fraction was determined by TLC (see below). Fractions containing acetylleptinidine were combined, evaporated to dryness at 50 °C under a stream of nitrogen, and then recrystallized from methanol.

TLC was performed using silica gel 60 (E.M. Merck, 0.25 μm) plates and ethyl acetate-hexane (1:1) for development. Spots were visualized with iodine vapor or Dragendorff’s reagent (Sigma Chemical Company, St. Louis, MO).

**Preparation of Leptinidine**

Leptinidine was prepared by hydrolyzing (70 °C for 30 min) recrystallized acetylleptinidine in 0.83 N sodium hydroxide in methanol-water. After cooling, the hydrolysate was partitioned twice against benzene; the combined benzene phases contained leptinidine, which was verified by TLC and GC (see below). Leptinidine was recrystallized from methanol.

**Sample Preparation**

Methodology is shown in Fig. 6. Freeze-dried foliar or tuber samples were ground to a fine powder using a mortar and pestle. Leaf (10-20 mg dry weight) or tuber (60-80 mg dry weight) tissue was placed into a 10 ml screw-cap vial, then 200 μg of tomatine (internal standard; dissolved in methanol) and 3 ml of 1 N HCl in methanol were added. Samples were capped under nitrogen and concurrently extracted and hydrolyzed in a shaking water bath for 4 h at 70 °C. After cooling to room temperature, extracts were made alkaline (pH≥10) with 2 ml of concentrated ammonium hydroxide and then centrifuged for 10 min at 1800g to remove insoluble materials. The supernatant was partitioned against 2 ml of benzene. Samples were
Freeze-Dried Tissue

Add Tomatine (Internal Standard)
Extract/Hydrolyze
(1 N HCl in Methanol)

Hydrolyzed Extract

Neutralize with Ammonium Hydroxide
Centrifuge

Supernatant

Partition vs. Benzene
Evaporate to Dryness
Redissolve in Chloroform

Aglycones

Apply to Silica-Gel Sep-Pak
Elute with Pyridine-Ethyl acetate-Hexane (2:5:5 v/v/v)
Evaporate to Dryness and
and Redissolve in Chloroform

GC Analysis using Rtx-1 Megabore
Capillary Column and FID

**Figure 6.** Flow diagram for extraction and quantitative analysis of *Solanum* alkaloids of *S. tuberosum* and *S. chacoense*. See Materials and Methods for details.
centrifuged at 1800g for 5 min to hasten phase separation. One ml of the benzene phase was removed and evaporated to dryness at 50 °C under a stream of nitrogen. The residue was redissolved in 0.5 ml of chloroform and either subjected to GC directly or applied to a silica-gel Sep-Pak cartridge (Waters Associates, Milford, MA). Aglycones were eluted from the Sep-Pak with 8 ml of pyridine-ethyl acetate-hexane (2:5:5, v/v/v), and the eluate was evaporated to dryness at 50 °C under a stream of nitrogen. The residue was redissolved in 0.5 ml of chloroform before GC analysis.

**GC Analysis**

One μl aliquots were injected onto a 15 m x 0.53 mm i.d. x 0.25 μm Rtx-1 fused silica column (Restek Corporation, Bellefonte, PA) fitted to an HP 5890A gas chromatograph equipped with a flame ionization detector (FID). The injector and detector temperatures were 270 and 280 °C, respectively. The column temperature was programmed from 210 to 260 °C at 2 °C/min. Helium was used as the carrier gas at a linear velocity of 45 cm/sec.

**RESULTS AND DISCUSSION**

An overview of the procedure (Fig. 6) reveals key elements that contributed to accurate SGA aglycone quantitation while minimizing technical input. These include (1) use of an internal standard (tomatine), (2) concurrent extraction and hydrolysis, and (3) megabore (530 μm i.d.) capillary GC of aglycones.

**Tomatine as an Internal Standard**

Coxon (1984) noted that there is no standard method for glycoalkaloid analysis. Ideally, any standard method should include an internal standard for reproducible, accurate quantitation. For *S. chacoense* and *S. tuberosum* cv. Atlantic, tomatine satisfied all of the criteria for an internal standard: (1) it was not a normal constituent,
(2) it was chemically and physically similar to the indigenous alkaloids, (3) its aglycone was resolved from the other analytes in the samples, (4) it eluted near the peaks of interest, and (5) it was readily available. Tomatine will not be a suitable internal standard for (glyco)alkaloid analysis of all *Solanum* species, specifically, those for which tomatidine cannot be resolved from indigenous components or tomatine is a normal constituent of the sample, but it can be used for (glyco)alkaloid analysis of several wild *Solanum* species and *S. tuberosum* cultivars. Tomatine was added to samples prior to extraction and hydrolysis (Fig. 6) to account for losses that may have occurred during preparation and analysis.

Even though several methods have been developed for *Solanum* (glyco)alkaloid quantitation using one or more of a number of colorimetric and chromatographic techniques, none of those routinely used accurately account for sample-to-sample variation due to sample workup. King (1980) obtained an average estimate of recovery by fortifying selected samples of *S. tuberosum* tubers and foliage with solanine, but an internal standard is the technique of choice, since its proper usage minimizes errors due to sample preparation, apparatus, and technique.

**Extraction and Hydrolysis**

Extraction and hydrolysis were combined into a single step (Fig. 6), which eliminated losses that could have occurred during grinding, filtration, and transfers. The parallel nature of sample preparation extends throughout the procedure, permitting several samples to be handled simultaneously, which reduces technical input, shortens sample preparation time, and permits "same-day" analysis. Any losses that were incurred during sample processing were accounted for by the internal standard, for which recoveries were (mean±SEM, n=9) 0.96±0.06 and 0.77±0.04 for leaves and tubers, respectively.
Traditional extraction and hydrolysis of glycoalkaloids in the absence of ammonia precipitation is usually a sequential process. Plant samples are extracted using a blender or homogenizer, filtered, and then concentrated before acid hydrolysis (Gregory et al., 1981; King, 1980). This three-step procedure requires constant technical input. In addition, samples may have to be processed serially in one or more of these steps, which increases sample preparation time.

Tissues were concurrently extracted and hydrolyzed for 4 h at 70 °C, after which time total alkaloid recoveries were maximized (Fig. 7). After 1 and 3 h, total alkaloid recoveries were only 75% and 90%, respectively, of those achieved at 4 h. Incubation for 5 h did not increase recoveries.

Accurate quantitation of aglycones can be affected by the method of hydrolysis. King (1980) and Van Gelder (1984) have found that conventional aqueous acid hydrolysis of glycoalkaloids results in degradation of some aglycones, including solanidine and tomatidine. To avoid this degradation, King (1980) hydrolyzed potato glycoalkaloids in ethanolic HCl and consistently isolated unmodified aglycones. Van Gelder (1984 and 1985) used a two-phase system to isolate unmodified aglycones, in which aglycones were trapped in carbon tetrachloride following hydrolysis in aqueous acid. I used methanolic HCl (Gregory et al., 1981; Sinden et al., 1986b), for simultaneous extraction and hydrolysis of glycoalkaloids. GC and TLC of glycoalkaloid standards hydrolyzed under conditions reported here revealed no evidence of aglycone degradation since no additional peaks (GC) nor additional spots (TLC) were detected as compared to GC and TLC of aglycone standards.

Since my procedure does not depend on ammonia precipitation during preparation of crude extracts, it is suitable for the analysis of both wild and cultivated Solanum species. Ammonia precipitation has been used reliably for precipitating solanine and chaconine from crude extracts of many S. tuberosum cultivars (Gregory et
Figure 7. Time course for concurrent extraction and hydrolysis of *S. chacoense*, PI 458310-1, alkaloids from freeze-dried leaf tissue. Each point represents one replicate. See Materials and Methods for details.
al., 1981; Jellema et al., 1981; Morris and Lee, 1981; Sinden et al., 1986b), but results in substantial and variable losses (more than 60%) in recovery when used in preparing extracts of some wild Solanum species (Gregory et al., 1981, Sinden et al., 1986b). Sinden et al. (1986b) reported for one S. chacoense clone that only 34% of the leptines, 37% of the leptinines, and 36% of solanine and chaconine were ammonia-precipitated. However, for another clone of S. chacoense, 78% of the leptines, 62% of the leptinines, and 91% of solanine and chaconine were recovered in the ammonia precipitate. Thus, ammonia precipitation not only fails to quantitatively precipitate glycoalkaloids unique to some wild species, but, in these species, fails to quantitatively precipitate glycoalkaloids that are normally ammonia insoluble (e.g., solanine and chaconine).

For the data reported here, and for routine analysis, glycoalkaloids were extracted and hydrolyzed from 10-20 mg or 60-80 mg dry weight of leaf or tuber tissue, respectively, in only 3 ml of solvent. However, this procedure has also been used to quantitate aglycones from as little as 0.3 mg dry weight of leaf tissue (Lawson, unpublished). These sample and solvent requirements are substantially lower than those reported previously. For example, glycoalkaloids have been extracted from 5 g fresh weight or more of leaf tissue in approximately 20 ml of solvent (Sinden et al., 1986b) and 10-300 g fresh weight of tuber tissue in 75-100 ml or more of solvent (Coxon et al., 1979; Herb et al., 1975; King, 1980; Van Gelder, 1984). It may be thought that larger sample sizes are needed for representative sampling. This possibility was tested by measuring the alkaloid contents of three subsamples of a single plant sample. Standard errors were approximately 1-2% of the mean. For instance, from a single leaf sample, alkaloid levels were (mean±SEM, n=3) 1762±21 µg solanidine/g dry weight, 1986±34 µg leptinidine/g dry weight, and 5413±18 µg acetylleptinidine/g dry weight, respectively.
Quantitation by GC of glycoalkaloid aglycones has proven to be an accurate means of determining glycoalkaloid content (King, 1980; Van Gelder, 1985). Response factors, retention times, resolution, and separation number were calculated for each alkaloid or alkaloid pair (Table 1). Since tomatine was the internal standard, its aglycone, tomatidine, was assigned a response factor of F=1. Chromatographic conditions were optimized based upon the resolution of tomatidine and acetylleptinidine, since these two alkaloids were the most difficult to separate. These conditions provided near-baseline resolution of tomatidine and acetylleptinidine (R=1.4; Table 1) with an analysis time of less than 20 min (Fig. 8); an R value of 1.5 constitutes baseline separation (Poole and Schuette, 1984). Thus, these conditions provided a good compromise between resolution and analysis time. Higher initial temperatures (i.e., 220 and 230 °C) shortened analysis time but reduced resolution. A lower initial temperature (i.e., 200 °C) provided baseline separation of tomatidine and acetylleptinidine (R=1.5), but increased analysis time by more than 6 min.

Van Gelder (1985) reported that column temperatures below 240 °C caused significant fronting (up to 52% peak asymmetry) in leading peaks and peak broadening, which he attributed to condensation of the sample in the column. None of these effects were observed at an initial column temperature of 210 °C (Fig. 8). On the other hand, Van Gelder (1985) provided evidence of aglycone decomposition at high temperatures (>280 °C). I avoided aglycone thermal decomposition by operating injector and column temperatures at or below 270 °C. GC of aglycone standards under optimized conditions revealed no evidence of degradation since no additional peaks were detected.
Table 1. Relative response factors (F), retention times (t_R), resolution (R), and separation number (SN) of *Solanum* glycoalkaloid aglycones.\(^a\)

<table>
<thead>
<tr>
<th>Aglycone</th>
<th>F</th>
<th>t_R</th>
<th>R</th>
<th>SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solanidine</td>
<td>1.52</td>
<td>10.339</td>
<td>15.2</td>
<td>12</td>
</tr>
<tr>
<td>Leptinidine</td>
<td>1.20</td>
<td>14.473</td>
<td>2.9</td>
<td>1</td>
</tr>
<tr>
<td>Tomatidine</td>
<td>1.00</td>
<td>15.444</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>Acetylleptinidine</td>
<td>1.30</td>
<td>15.960</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)See Materials and Methods for chromatographic details.
Figure 8. Chromatogram of *S. chacoense*, PI 458310-1, leaf extract. See Materials and Methods for chromatographic details. Peaks: 1, solanidine; 2, leptinidine; 3, tomatidine (internal standard); 4, acetylleptinidine.
The method was also tested for its effectiveness in resolving other steroidal alkaloids. Solasodine, an important starting material for the synthesis of pharmaceutical steroids (Roddick, 1986), was resolved from leptinidine (R=1.0) and tomatidine (R=2.0). However, demissidine, which has been implicated in plant resistance to insect feeding (Tingey, 1984), was not resolved from solanidine. When chromatographed individually, demissidine had a retention time 0.1 min longer than solanidine. Therefore, if the analyst required resolution of these two alkaloids, a 30 m column could be used to obtain R=0.6. Solasodine differs structurally from tomatidine in that solasodine is both $\Delta^5$ and (22R, 25R)- rather than (22S, 25S)-configuration, whereas demissidine differs structurally from solanidine only in that demissidine is not $\Delta^5$ (Fig. 5), which may account for the resolution of solasodine and tomatidine but not demissidine and solanidine. Van Gelder (1985) obtained resolution of solanidine and demissidine (R=1.9) using a 50 m x 0.22 mm i.d. x 0.12 $\mu$m CP-Sil 5 capillary column. King (1980) used 3-ß-trifluoroacetate derivatives to distinguish between these two alkaloids.

For total glycoalkaloid measurements, colorimetric assays (Bushway et al., 1980; Coxon et al., 1979; Fitzpatrick and Osman, 1974; Sanford and Sinden, 1972) have proved to be very useful. However, modern liquid or gas chromatography is necessary for quantitation of individual (glyco)alkaloids. Each technique has its limitations. For glycoalkaloid analysis, GC is unattractive principally because of the high operating temperatures (>300 °C) necessary to achieve elution, even if samples are derivatized (Herb et al., 1975). TLC has been limited primarily to qualitative or semi-quantitative analysis (Coxon and Jones, 1981; Deahl and Sinden, 1987; Hunter et al., 1976; Shih and Kúc, 1974), although a few exceptions exist (Jellema et al., 1980 and 1981). HPLC provides resolution of glycoalkaloids, but due to the absence of a good UV-absorbing chromophore, detection limits are 0.1 $\mu$g at best (Morris and Lee,
1981). The detection limit of underivatized alkaloids was approximately 3 ng using my megabore capillary GC method.

**Analysis of S. tuberosum and S. chacoense Alkaloids.**

The alkaloid contents of *S. tuberosum* and *S. chacoense* leaves and tubers were quantitated using this GC procedure (Fig. 8, Table 2). The purity of all alkaloid peaks was verified by GC-MS (see Chapter II). Leaves contained higher levels of alkaloids than tubers in both *S. chacoense* and *S. tuberosum*, which is consistent with previous reports (reviewed in Gregory, 1984 and Maga, 1980). Greenhouse-grown *S. chacoense* leaves contained higher (2.5X) total alkaloid levels than *S. tuberosum* leaves, and *S. chacoense* contained leptinidine and acetylleptinidine, which were not detected in *S. tuberosum*. Acetylleptinidine was the predominant alkaloid of *S. chacoense* leaves (54%) followed by leptinidine (36%) and solanidine (10%), which is similar to the proportions calculated from data reported by Sinden et al. (1986b). However, the trend was different in *S. chacoense* plantlets grown in vitro, the leaves of which contained predominantly solanidine (66%), followed by acetylleptinidine (20%) and leptinidine (14%). These data suggest that *S. chacoense* alkaloid levels are quantitatively influenced by the environment (Sinden et al., 1984)

Acetylleptinidine was not detected in *S. chacoense* tubers, suggesting that the biosynthesis of this alkaloid is under developmental regulation, since it was detected in *S. chacoense* leaves. The absence of acetylleptinidine in PI 458310-1 tubers is consistent with a previous report (Sinden et al., 1986b).

In this chapter, the use of tomatine as an internal standard, which was added prior to extraction, for the quantitation of *Solanum* alkaloids is reported. Alkaloids were quantitated underivatized using megabore capillary GC. The applicability of this procedure for the analysis of *S. chacoense* and an *S. tuberosum* cultivar has been
Table 2. Levels of principal SGA aglycones from *S. tuberosum* cv. Atlantic and *S. chacoense*, 458310-1, leaves and tubers grown in the greenhouse (GH), field (F) or tissue culture (TC).

<table>
<thead>
<tr>
<th>Source</th>
<th>Solanidine</th>
<th>Leptinidine</th>
<th>Acetylleptinidine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. tuberosum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubers (F)</td>
<td>590±38</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>leaves (GH)</td>
<td>6288±139</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>S. chacoense</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubers (GH)</td>
<td>2877±43</td>
<td>38±0</td>
<td>ND</td>
</tr>
<tr>
<td>leaves (GH)</td>
<td>1731±13</td>
<td>5990±114</td>
<td>8909±153</td>
</tr>
<tr>
<td>leaves (TC)</td>
<td>6807±83</td>
<td>1419±25</td>
<td>2102±36</td>
</tr>
</tbody>
</table>

*a* Values are the mean±SEM of three replications.

*b* Not detected.
demonstrated, but (1) the concurrent extraction and hydrolysis, (2) the parallel nature of sample preparation, and (3) the analytical capability of capillary GC has application for (glyco)alkaloid analyses of other wild and cultivated Solanum species. This procedure was used to investigate the developmental regulation and the biochemistry of Solanum alkaloid biosynthesis in S. chacoense.
LITERATURE CITED


Sinden SL, Sanford LL, Cantelo WW, Deahl KL (1986a) Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in Solanum chacoense. Environ Entomol 15: 1057-1062


CHAPTER II
NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MASS SPECTROMETRY OF SOLANIDINE, LEPTINIDINE, AND ACETYLEPTINIDINE. STEROIDAL ALKALOIDS FROM SOLANUM CHACOENSE, BITTER.

ABSTRACT
Solanidine, leptinidine, and acetylleptinidine from Solanum chacoense, Bitter were structurally characterized by high-resolution MS and high-field $^1$H-NMR and $^{13}$C-NMR. $^1$H- and $^{13}$C-NMR chemical shifts were assigned using 1D- and 2D-NMR techniques, and by comparison with literature values for related compounds. The methyl assignments reported here for solanidine differ from those of some previous reports, but were corroborated by 2D-NMR CH-correlation data.
INTRODUCTION

*Solanum chacoense*, Bitter contains leptines, leptinines (Kuhn and Löw, 1957; 1961), α-solanine, and α-chaconine (Fig. 9). The leptines are natural antifeedants to the Colorado potato beetle (*Leptinotarsa decemlineata*, Say; Carter, 1987; Sinden et al., 1986; Sturckow and Löw, 1961). Since the glycoalkaloid component of insect resistance is considered to be so significant, it has been suggested that selection and breeding for insect resistance be based on specific deterrent glycoalkaloid content (Sinden et al., 1986; Tingey, 1984). Further, it has even been suggested that it may be possible to screen for deterrent glycoalkaloid content in lieu of resistance testing (Deahl and Sinden, 1987). Because of the potential ecological and toxicological significance of certain steroidal glycoalkaloids (McMillan and Thompson, 1979; Friedman, 1992), several methods have been developed to assay (glyco)alkaloid content in leaves and tubers of *Solanum* species (Coxon, 1984; Coxon and Jones, 1981; Crabbe and Fryer, 1980; Deahl and Sinden, 1987; Jellema et al., 1980,1981; King, 1980; Lawson et al., 1992; Morris and Lee, 1981; Van Gelder, 1985).

Unfortunately, neither the leptines, the leptinines, nor their aglycones, acetylleptinidine and leptinidine, respectively, can be purchased commercially. Since analytical/chromatographic standards of these alkaloids must be purified by the analyst, spectral information should be readily available in order to confirm the structural identity of isolates. Although spectral data has been published for many steroidal alkaloids, a thorough search of the chemical literature (CA Search, 1967 to 1991; Chemical Abstracts, 1957 to 1966 and 1992) failed to retrieve specific publications regarding mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy of either acetylleptinidine or leptinidine. Also, whereas $^{13}$C-NMR spectra
Figure 9. *Solanum chacoense* C27-steroidal alkaloids.
have been reported for solanidine (Radeglia et al., 1977), the aglycone of α-solanine and α-chaconine, neither high-field $^1$H-NMR nor high-resolution MS (HR-MS) data were found to be reported for this alkaloid. Fast atom bombardment MS of solanidine has been performed (Price et al., 1985), but only the [M+H]$^+$ and $m/z$ 204 species was reported. In this chapter, HR-MS and high-field $^1$H- and $^{13}$C-NMR data for acetylleptinidine, leptinidine, and solanidine are reported.

**MATERIALS AND METHODS**

**Acetylleptinidine**

Acetylleptinidine was isolated and purified from *S. chacoense*, PI 458310-1, as previously described (Lawson et al., 1992). Briefly, freeze-dried leaves were homogenized in 2% acetic acid in methanol using a Waring blender. The homogenate was filtered and the filtrate was evaporated to near dryness at 50 °C *in vacuo*. The residue was redissolved in 1 N HCl in methanol, capped under nitrogen and hydrolyzed at 70 °C for 4 hours. After cooling, the hydrolysate was raised to pH 10 with concentrated ammonium hydroxide and centrifuged at 12000g for 6 min. The supernatant was partitioned against chloroform and the chloroform phase was evaporated to near dryness at 40 °C *in vacuo*. The residue was redissolved in chloroform and acetylleptinidine was purified by flash chromatography using silica gel columns and mobile phases of hexane and ethyl acetate-hexane. Thin-layer chromatography (TLC; silica gel, chloroform:methanol 7:3 v/v) was used to determine the composition of collected fractions. Acetylleptinidine was recrystallized from methanol.
Leptinidine

Leptinidine (21 mg) was prepared by hydrolyzing (70 °C for 30 min) recrystallized acetylleptinidine (22 mg) in 0.83 N sodium hydroxide in methanol-water. After cooling, the hydrolysate was partitioned twice against benzene; the benzene phase contained leptinidine, which was recrystallized from methanol.

Diacetylleptinidine

Acetylleptinidine (10 mg) was dissolved in a minimum amount of pyridine and combined with 2 ml of acetic anhydride. The solution was then incubated at 90 °C for 90 min. After the reaction mixture cooled to room temperature, 5 ml of water was added to destroy excess acetic anhydride. The solution was again cooled before partitioning three times against 1 ml portions of dichloromethane. The reaction was quantitative according to TLC. White needles, mp 196 - 210 °C; 1H-NMR (500 MHz) 3-H (δ 4.80, p), 6-H (δ 5.38, d, J=4.6), 18-H3 (δ 0.98, s), 19-H3 (δ 0.99, s), 21-H3 (δ 0.82, d, J=6.7), 26-H2 (δ 2.94, dd, J=10.3 and 3.1), 27-H3 (δ 1.04, d, J=6.6); EI-MS (70 eV) m/z (rel. int.) 497 [C31H47NO3] (11), 454 [C29H44NO3] (8), 437 [C29H43NO2] (100), 395 [C24H43O4] (1), 262 [C16H24NO2] (11), 208 [C12H18NO2] (6), 148 [C10H14N] (13).

GC and GC-MS

GC-flame ionization detector (FID) analysis of S. chacoense leaf extracts was performed as described previously (Lawson et al., 1992). GC-MS was performed under the same conditions as for GC-FID analysis of leaf extracts, but using an HP 5890 GC interfaced with an HP 5970 Quadrapole Mass Selective Detector (70 eV).
High-Resolution Mass Spectrometry

A VG 70-250S high-resolution mass spectrometer was used to obtain electron impact mass spectra. The mass spectrometer ionization was set at 70 eV, and the source temperature was 180 °C.

Nuclear Magnetic Resonance Spectroscopy

$^1$H- and $^{13}$C-NMR spectra were acquired using a Bruker WM-500 spectrometer at 500.13 MHz and 125.76 MHz, respectively. Samples were dissolved in either CDCl$_3$ or C$_5$D$_5$N with 0.3% SiMe$_4$ as the internal standard.

RESULTS AND DISCUSSION

Solanidine

The presence of solanidine in *S. chacoense* leaf extracts was tentatively identified by TLC. The solute had an Rf of 0.64 in chloroform:methanol (7:3 v/v), co-migrated with purchased solanidine, and reacted positively with Dragendorff's reagent (Krebs et al., 1969).

GC-MS of leaf extracts revealed a parent ion at $m/z$ 397 for a peak having the same retention time as authentic solanidine. Also, the fragmentation patterns from both GC-MS and HR-MS (Table 3) were identical to that of authentic solanidine and included fragments at $m/z$ 204 (C$_{14}$H$_{22}$N; Fig. 10) and $m/z$ 150 (C$_{10}$H$_{16}$N; Fig. 10), which are diagnostic of solanidane-based alkaloids (Budzikiewicz, 1964). HR-MS (Table 3) provided a parent peak at 397.334653680, supporting the molecular formula (C$_{27}$H$_{43}$NO requires a molecular weight of 397.6428) for solanidine.

$^1$H-NMR exhibited a pentet at $\delta$ 3.51 corresponding to the 3-H alpha to the 3-$\beta$-hydroxyl (Ripperger and Porzel, 1992). Other recognizable signals included 6-H ($\delta$ 5.34, s), 16-H ($\delta$ 3.37, d, $J$=8.4 Hz), 18-H$_3$ ($\delta$ 1.21, s), 19-H$_3$ ($\delta$ 0.94, s), 21-H$_3$ ($\delta$
Table 3. Principal EI-MS fragment ions of *S. chacoense* steroidal alkaloids.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Fragment ions, m/z (relative abundance, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solanidine</td>
<td>397 (19.18), 382 (11.12), 368 (1.63), 354 (0.96), 341 (1.02), 326 (0.86), 272 (0.69), 259 (0.58), 204 (25.87), 178 (4.26), 150 (100.00), 136 (7.13), 124 (4.50), 98 (7.84), 79 (3.79), 67 (3.62), 55 (6.34)</td>
</tr>
<tr>
<td>Leptinidine</td>
<td>413 (15.65), 398 (5.29), 369 (3.38), 354 (2.62), 342 (3.45), 326 (1.57), 312 (0.86), 294 (1.32), 267 (1.15), 236 (0.87), 220 (25.97), 207 (3.06), 194 (4.22), 181 (4.15), 166 (100.00), 148 (5.28), 137 (9.59), 122 (4.87), 105 (7.44), 91 (9.78), 82 (8.59), 67 (8.45), 55 (12.95)</td>
</tr>
<tr>
<td>Acetylleptinidine</td>
<td>455 (6.23), 440 (5.48), 412 (13.48), 395 (100.00), 380 (6.54), 368 (2.65), 354 (1.72), 340 (1.73), 326 (1.32), 262 (21.97), 249 (1.82), 236 (3.42), 223 (2.51), 208 (11.68), 162 (11.64), 148 (47.90), 134 (8.54), 119 (5.23), 105 (8.64), 91 (11.20), 79 (9.31), 67 (9.62), 55 (13.42), 43 (22.33)</td>
</tr>
</tbody>
</table>
Figure 10. Diagnostic fragment ions from EI-MS of solanidine, leptinidine and acetylleptinidine.
1.03, d, \( J=6.5 \) Hz), 25-H (\( \delta 2.84, m \)), 26-H\(_2\) (\( \delta 3.57, dd, J=11.2 \) and 3.5 Hz), and 27-H\(_3\) (\( \delta 0.93, d, J=6.6 \) Hz). The methyl assignments differ from those reported previously for other solanidane steroidal alkaloids (Krishna Kumari et al., 1985; Murakami et al., 1985; Table 4), and these differences may be due to the increased resolution in the methyl region of the proton spectrum afforded by high-field NMR (500 MHz). In addition, even though methyl assignments could be made from spectra of samples dissolved in CDCl\(_3\), C\(_5\)D\(_5\)N appeared to provide better resolution in the methyl region than did CDCl\(_3\) (data not shown; Raymond Doskotch, personal communication). \(^1\)H-NMR assignments presented in this chapter were also aided by 2D NMR CH-correlation, which clearly indicated methyl proton couplings to their respectively assigned carbons.

\(^{13}\)C-NMR chemical shift assignments (Table 5) were based primarily upon multiplicities from the DEPT experiment and literature values for solanidine (Radeglia et al., 1977). However, methyl chemical shifts were reassigned from literature values (Radeglia et al., 1977) according to 2D NMR CH-correlation data.

**Leptinidine**

The presence of leptinidine in leaf extracts was initially indicated by GC-MS, which revealed a peak having a parent ion at \( m/z \) 413 and was thus consistent with the molecular formula (C\(_{27}\)H\(_{43}\)NO\(_2\) requires 413.6422) for leptinidine. Purified sample (white needles from methanol) had a mp of 238 - 241°C, which was similar to that reported for leptinidine by Kuhn and Löw (239 - 240 °C; 1957, 1961), and had an Rf of 0.55 chloroform:methanol (7:3 v/v). Spots corresponding to leptinidine following TLC of leaf extracts co-migrated with leptinidine derived from base hydrolysis of purified acetylleptinidine (see below), and reacted positively with Dragendorff's reagent (Krebs et al., 1969).
## Table 4. $^1$H chemical shifts for solanidine steroidal alkaloid methyl carbons.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Solanidine</th>
<th>Literature Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>18</td>
<td>1.21</td>
<td>0.85</td>
</tr>
<tr>
<td>19</td>
<td>0.94</td>
<td>0.89</td>
</tr>
<tr>
<td>21</td>
<td>1.03</td>
<td>0.98</td>
</tr>
<tr>
<td>27</td>
<td>0.93</td>
<td>1.20</td>
</tr>
</tbody>
</table>

* $^\delta$ values in ppm downfield from SiMe$_4$

b in CDCl$_3$ + C$_5$D$_5$N

A = Krishna Kumari et al. (1985) for 3β-amino-5α,22αH,25βH-solanidane-23β-ol in CDCl$_3$

B = Murakami et al. (1985) for dihydroleptidinedine in CDCl$_3$
Table 5. Carbon chemical shifts* for solanidine, leptinidine, and acetylleptinidine.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Solanidine\textsuperscript{b}</th>
<th>Leptinidine\textsuperscript{c}</th>
<th>Acetylleptinidine\textsuperscript{c}</th>
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<td>CO\textsubscript{2}CH\textsubscript{3}</td>
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<td>21.1</td>
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</tbody>
</table>

\textsuperscript{*} δ values in ppm downfield from SiMe\textsubscript{4}\n\textsuperscript{b} in CDCl\textsubscript{3} + C\textsubscript{5}D\textsubscript{5}N\n\textsuperscript{c} in C\textsubscript{5}D\textsubscript{5}N
GC-MS and HR-MS exhibited diagnostic fragments at \( m/z \) 166 and \( m/z \) 220 (Table 3, Fig. 10), which were 16 amu greater than the corresponding fragments (\( m/z \) 150 and \( m/z \) 204) from solanidine. These data indicated the presence of a hydroxyl group in ring E or F. GC-MS and HR-MS (Table 3) of the base hydrolysate of purified acetylleptinidine (see below) had a fragmentation pattern identical to that of leptinidine found in \textit{S. chacoense} leaf extracts and included the major fragments reported previously (Osman et al., 1987). HR-MS (Table 3) of the base hydrolysate of acetylleptinidine produced a parent peak at \( m/z \) 413.329380080.

\(^{1}\text{H}-\text{NMR} \) also supported the presence of a hydroxyl at C-23, by exhibiting a signal at \( \delta \) 3.78 (d, \( J = 7.5 \) Hz), which, according to 2D NMR CH-correlation was coupled to C-23. This assignment is consistent with that previously given to the 23\( \alpha \)-H of dihydroleptinidine (Krishna Kumari et al., 1985). \(^{1}\text{H}-\text{NMR} \) further revealed signals that were assigned to 3-H (\( \delta \) 3.51, p), 6-H (\( \delta \) 5.35, d, \( J = 5.0 \) Hz), 18-H\(_3\) (\( \delta \) 1.02, s), 19-H\(_3\) (\( \delta \) 0.87, s), 21-H\(_3\) (\( \delta \) 0.97, d, \( J = 6.8 \) Hz), 26-H\(_2\) (\( \delta \) 2.87, d, \( J = 10.6 \) Hz) and 27-H\(_3\) (\( \delta \) 0.86, d, \( J = 8.8 \)).

In addition to \(^{1}\text{H}-\text{NMR} \) data, evidence for an axial (\( \beta \)) orientation for the C-23 hydroxyl came from expected substituent effects of axial hydroxyl groups, which result in downfield shifts of \( \alpha \)-, and \( \beta \)- carbons and the upfield shift of \( \Gamma \)- carbons (Eggert et al., 1976). A comparison of the \(^{13}\text{C}-\text{NMR} \) chemical shifts of leptinidine with solanidine revealed downfield shifts of +33.58 for C-23, +2.11 for C-22, and +7.79 for C-24, and upfield shifts of -2.24 for C-25 and -4.46 for C-20 (Table 5). These chemical shifts agree with those reported by Krishna Kumari et al. (1985) and Bird et al (1979).
Acetylleptinidine

GC-MS of leaf extracts from *S. chacoense* also revealed a peak having a parent ion at *m/z* 455, which is consistent with the molecular formula (C_{29}H_{45}NO_{3} requires 455.6794) for acetylleptinidine. Purified sample (white needles from methanol) had an Rf of 0.78 chloroform:methanol (7:3 v/v) and reacted positively with Dragendorff's reagent (Krebs et al., 1969). The mp (194 - 199 °C) was slightly higher than the 191 - 196 °C reported by Kuhn and Löw (1961) for x-acetylleptinidine.

GC-MS and HR-MS (*m/z* [M]+ 455.3385315; Table 3) of the purified sample produced fragmentation patterns identical to that produced by the *m/z* [M]+ 455 peak identified by GC-MS of leaf extracts. Fragments were also observed at *m/z* 208 (C_{12}H_{18}NO_{2}) and *m/z* 262 (C_{16}H_{24}NO_{2}) (Fig. 10), which corresponded to the acetoxyl analogs of the *m/z* 150 and *m/z* 204 fragments, respectively, of solanidine, and indicated substitution of the acetoxyl in ring E or F. MS also revealed a base peak at *m/z* 395 (C_{27}H_{41}NO), which is consistent with the loss of acetate [M-CH$_3$CO$_2$H]+.

The presence of an acetoxyl was also supported by $^1$H-NMR, which revealed a singlet at δ 2.16 corresponding to the methyl alpha to the carbonyl, and $^{13}$C-NMR (Table 5), which exhibited both a singlet at δ 170.87 and a quartet at δ 21.15, which were assigned to the carbonyl carbon and the methyl carbon alpha to the carbonyl, respectively. Substitution of the acetoxyl at C-23 was further indicated by a proton chemical shift at δ 5.30, which is similar to that reported for the 23α-H for dihydroleptinidine diacetate (Murakami et al., 1985; Schreiber and Ripperger, 1967), and was confirmed by 2D NMR CH-correlation. A β-orientation for the acetoxyl was deduced since base hydrolysis of acetylleptinidine yielded leptinidine.

The position of the acetoxyl group was also verified by direct comparison of the $^{13}$C-NMR chemical shifts of diacetylleptinidine with those of 3-acetylisosoladine and solasodine (Bird et al., 1979). Since rings A, B, C, and D of these compounds have
the same structure, such a comparison would indicate whether the acetoxyl was substituted at C-3. The data (Table 6) show that the chemical shifts for C-1 through C-6 of acetylleptinidine do not agree with those of 3-acetylsolasodine, but are nearly identical to those reported for solasodine. However, acetylation of acetylleptinidine brought the chemical shifts of these carbons into excellent agreement with those reported for 3-acetylsolasodine, and thus indicated that the acetoxyl group of acetylleptinidine was not at C-3.

$^1$H-NMR also exhibited resonances assignable to 3-H (δ 3.86, p), 6-H (δ 5.43, d, J=5.1 Hz), 18-H$_3$ (δ 1.00, s), 19-H$_3$ (δ 1.06, s), 21-H$_3$ (δ 1.04, d, J=6.7 Hz), 26-H$_2$ (δ 2.95, dd, J=10.5 and 2.9 Hz) and 27-H$_3$ (δ 0.82, d, J=6.7 Hz). $^{13}$C-NMR chemical shifts (Table 5) were assigned by direct comparison with solanidine and 2D NMR CH-correlation.
Table 6. $^{13}$C-NMR chemical shifts* of solasodine, 3-acetylsolasodine, acetylleptinidine, and diacetylleptinidine.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
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<tr>
<td>solasodine b</td>
<td>31.6</td>
<td>71.7</td>
<td>42.3</td>
<td>141.0</td>
<td>121.5</td>
</tr>
<tr>
<td>3-acetylsolasodine b</td>
<td>27.8</td>
<td>73.9</td>
<td>38.1</td>
<td>139.8</td>
<td>122.4</td>
</tr>
<tr>
<td>acetylleptinidine c</td>
<td>31.8</td>
<td>71.4</td>
<td>43.6</td>
<td>142.1</td>
<td>121.3</td>
</tr>
<tr>
<td>3,23-diacetylleptinidine c</td>
<td>28.1</td>
<td>74.0</td>
<td>38.6</td>
<td>140.0</td>
<td>122.8</td>
</tr>
</tbody>
</table>

*δ values in ppm downfield from SiMe$_4$

b in CDC$_3$
c in C$_5$D$_5$N
LITERATURE CITED


Sinden SL, Sanford, LL, Cantelo, WW, Deahl, KL (1986) Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in Solanum chacoense. Environ Entomol 15: 1057-1062


CHAPTER III
LEAF PHYSIOLOGICAL AGE AFFECTS SOLANUM CHACOENSE, BITTER
STEROIDAL ALKALOID CONCENTRATIONS AND BIOSYNTHESIS

ABSTRACT

The effects of leaf physiological age on concentrations and biosynthesis of solanidine, leptinidine, and acetylleptinidine in Solanum chacoense, Bitter were investigated. Steroidal alkaloid concentrations were measured for individual leaves harvested at several physiological ages as determined by plastochronic development. In addition, alkaloid concentrations were measured for leaves at all plastochronic intervals of developing plants. Individual and total steroidal alkaloid concentrations were highest when leaves were young and declined (up to 70%) as leaves matured. Solanidine concentrations in young leaves exceeded acetylleptinidine and leptinidine concentrations by as much as 4X. Relative steroidal alkaloid concentrations changed from solanidine > acetylleptinidine ≥ leptinidine when leaves were young to acetylleptinidine > leptinidine > solanidine when leaves were mature. Incorporation of [14C]mevalonate into solanidine, leptinidine, and acetylleptinidine of young and mature leaves corroborated relative alkaloid concentrations determined by static measurements and supported the hypothesis that the variations in individual alkaloid concentrations were due to differences in relative rates of biosynthesis.
INTRODUCTION

Steroidal glycoalkaloids are a class of plant secondary metabolites found principally among Solanum, Veratrum, and Lycopersicon species. Many of these compounds have broad-spectrum biological activities, with some even having pharmacological significance. For example, some Solanum alkaloids (e.g., solasodine) have become important starting materials for the industrial synthesis of steroidal hormones because they can be chemically transformed into key, synthetic intermediates (Sato et al., 1951). In addition, the toxicological aspects of steroidal glycoalkaloids (McMillan and Thompson, 1979; Friedman, 1992) have also brought considerable attention to this class of natural products, and have been the subject of several reviews (reviewed in Jadhav et al., 1981; Keeler et al., 1990; Morris and Lee, 1984; Roddick, 1986).

Some steroidal glycoalkaloids also have potential ecological significance. The leptines of S. chacoense, Bitter (Fig. 11) are potent antifeedants to the Colorado potato beetle, Leptinotarsa decemlineata, Say, (Sinden et al., 1986a; Sturckow and Löw, 1961). As a result, accessions of S. chacoense containing high concentrations of leptines have become important resources for potato breeding programs (Carter, 1987; Deahl and Sinden, 1987; Gregory et al., 1981; Ross, 1966; Sinden et al., 1986a,b).

Whereas the antifeedant properties of S. chacoense glycoalkaloids have received much attention, comparatively little research has focused on the developmental regulation and biosynthesis of these alkaloids. Sinden, et al. (1986b) and Lawson et al. (1992) have identified leptines and acetylleptinidine, respectively, in the shoots of leptine-producing clones of S. chacoense, but not in the tubers, indicating that acetylleptinidine/leptine biosynthesis may be developmentally regulated. In addition, glycoalkaloid content varies qualitatively between S. chacoense accessions. For
Figure 11. Structures of *S. chacoense*, Bitter steroidal alkaloids.
example, leptines and leptinines have not been detected in some accessions, but only solanine and chaconine (Sinden et al., 1986b). These qualitative differences may be due either to repression of gene expression or to an absence of the genes necessary for leptinine and leptine biosynthesis (Sinden et al., 1986b).

Compared to wild potato species, much more is known about the distribution of glycoalkaloids in *S. tuberosum*. The toxicity of certain steroidal glycoalkaloids to humans and their potential ecological significance to the cultivated potato have prompted investigations in which these alkaloids were measured in tuber or foliar samples of commercial potato cultivars and insect-resistant wild potato species. Glycoalkaloid concentrations are high in actively growing sprouts, meristematic regions (Lampitt et al., 1943; Wolf and Duggar, 1940) and flowers (Lampitt et al., 1943), and are higher in leaves than in tubers (Deahl, et al, 1973; Friedman and Dao, 1992; Lawson et al., 1992). In the tuber, glycoalkaloid concentrations are highest in the periderm and cortex and decrease toward the center, with low glycoalkaloid concentrations in the pith (Lampitt et al., 1943).

Although these studies report the general distribution of glycoalkaloids in *Solanum* plants, they do not provide detailed information on the effects of physiological development on steroidal alkaloid (SA) concentrations. Nonetheless, these reported differences in the distribution of glycoalkaloids within the plant suggest that *Solanum* SA biosynthesis is under physiological control. Analytical methods developed previously (Lawson et al., 1992) were used to investigate the effects of *S. chacoense* leaf physiological development on the concentrations and biosynthesis of solanidine, leptinidine, and acetylleptinidine.
MATERIALS AND METHODS

Plant Culture

Apical cuttings of *S. chacoense*, Bitter, PI 458310-1, were rooted in sand and then transplanted to sand:soil:peat moss (1:1:1, v/v/v; 8 in. pots) in a greenhouse (14-h light/10-h dark photoperiod with supplemental lighting). One month following transplanting, plants were topped to initiate axillary bud growth. Physiological ages of developing axillary shoots were defined according to plastochronic development. Leaves were numbered sequentially from youngest to oldest.

Steroidal Alkaloid Concentrations of Individual Developing Leaves

To investigate the effects of leaf physiological development on SA concentrations of individual leaves, newly emerging leaves of plants at different stages of development were tagged, and then harvested at other, specified stages. SAs were quantitated as described below.

Steroidal Alkaloid Concentration of Leaves at Different Leaf Positions of Developing Plants

The effects of leaf position on foliar SA concentrations were determined by quantitating SA concentrations of leaves at all plastochronic intervals of individual plants, which were harvested at specified stages of plant development.

Steroidal Alkaloid Analysis

Harvested leaves were transported on ice from the greenhouse to the laboratory where they were washed in deionized water, blotted dry, and their fresh weights recorded. Leaves were freeze-dried and SAs were quantitated using capillary GC and tomatine (Sigma Chemical Company, St. Louis, MO) as an internal standard (Lawson
et al., 1992). Peak identities and aglycone structures were confirmed using GC-MS (Chapter II).

Incorporation of $[^{14}C]$Mevalonic Acid into Steroidal Alkaloids of Immature and Mature Leaves

R-[2-$^{14}$C]Mevalonic acid was generated from R-[2-$^{14}$C]mevalonic acid lactone (Amersham Corp., 53.4 mCi/mmol, 50 μCi/ml) by pipeting an appropriate aliquot of the lactone into a microcentrifuge tube and evaporating the solution to dryness. The residue was then redissolved in deionized, distilled water to the appropriate volume, yielding the corresponding acid in an aqueous solution. Shoot tips (upper 4 cm of the shoot) and mid-leaves (leaf no. 6) were removed from plants at the 12-leaf stage of development. Samples were transported to the laboratory on ice and washed with deionized water. For shoot tip cuttings, all leaves except the first leaf (terminal leaf) were removed and the stem was cut to a final length of 2.5 cm. For mid-leaves, all leaflets except the terminal leaflet were removed from the petioles. Stems of shoot tips were placed in $1 \mu$Ci (20 μl) each of $[^{14}$C]mevalonate (2 shoot tips/replicate); petioles of mid-leaves were placed in $2 \mu$Ci (40 μl) each of $[^{14}$C]mevalonate (1 mid-leaf terminal leaflet/replicate). After radiolabel was taken up, it was chased with 40 μl of deionized water, and then shoot tips and leaflets of mid-leaves were incubated for 48 h in deionized water (14-h light/10-h dark photoperiod, 23 °C). Shoot tip leaf and mid-leaf terminal leaflet controls were fed 0.02 μmole/20 μl and 0.04 μmole/40 μl of unlabeled R-mevalonic acid (Sigma Chemical Company, St. Louis, MO) in water, respectively.

Following incubation, terminal leaves and terminal leaflets were removed from the stems and petioles, respectively, and prepared for SA analysis as described previously (Lawson et al., 1992). Final SA fractions were spotted on silica gel TLC
plates (Whatman LK6D) and sequentially developed to 16 cm in (1) chloroform:methanol (95:5, v/v) and (2) chloroform:methanol (7:3, v/v). Lanes to which SA fractions from shoot tips were applied were spiked with unlabeled cholesterol (Sigma Chemical Company, St. Louis, MO), solanidine (Sigma Chemical Company, St. Louis, MO), leptinidine, and acetylleptinidine, and lanes to which SA fractions from terminal leaflets of mid-leaves were applied were spiked with cholesterol. Leptinidine and acetylleptinidine were obtained using procedures reported previously (Lawson et al., 1992). Bands were visualized using iodine vapor. Silica gel at the Rfs corresponding to solanidine, leptinidine, and acetylleptinidine was scraped from the plate and radioactivity determined by liquid scintillation counting using a Beckman LS 1801 liquid scintillation counter and Hionic Fluor (Packard) liquid scintillation cocktail.

Purity of Rf positions corresponding to solanidine, leptinidine, and acetylleptinidine was determined by scraping silica gel from TLC plates, eluting with chloroform:methanol (1:1, v/v), and analyzing the eluates using GC (Lawson et al., 1992). From GC analysis, bands with Rfs corresponding to solanidine, leptinidine, and acetylleptinidine contained their respective alkaloids at 99+%, 98%, and 92% purity.

RESULTS

Steroidal Alkaloid Concentrations of Individual Developing Leaves

For this experiment, newly-emerging leaves of plants at the specified stages of development were tagged and SA concentrations were measured for tagged leaves throughout their development.
Total Steroidal Alkaloid Concentrations

Total SA concentrations of individual, developing leaves generally declined during leaf maturation (Fig. 12). First and second emerging leaves underwent declines in SA concentrations of 69% and 71%, respectively, during maturation until plants reached the 6-leaf stage of development, after which time declines in total SA concentrations in these leaves were less pronounced (Fig. 12 A, B). Newly-emerging leaves of older plants (e.g., leaf no. 1 of plants at the 6-leaf stage of development), however, underwent more gradual (41%) declines in mean total SA concentration during maturation (Fig. 12C).

Individual Steroidal Alkaloid Concentrations

Initially, mean solanidine concentrations were higher in immature leaves and exceeded acetylleptinidine concentrations (Fig. 12). However, in young plants (i.e., plants at the 1- to 2-leaf stage) mean solanidine concentrations declined (29%) and mean acetylleptinidine concentrations increased (34%) as first emerging leaves matured (Fig. 12A, B). Thus, relative mean SA concentrations changed during maturation from solanidine > acetylleptinidine ≥ leptinidine to acetylleptinidine > leptinidine > solanidine. Marked changes in individual SA concentrations also occurred in newly-emerging leaves of more mature plants. In plants at the 6-leaf stage, mean solanidine concentrations in newly-emerging leaves (i.e., leaves at the first leaf position) exceeded acetylleptinidine concentrations by as much as 3.4X. As these leaves matured to become the leaves at the fifth leaf position of plants at the 10-leaf stage, mean solanidine concentrations declined 62% while acetylleptinidine concentrations increased 116% (Fig. 12C).
Figure 12. Individual and total (insets) alkaloid concentrations throughout the development of newly-emerging leaves of developing plants. Each point represents the mean of three replicates ± SE. Numbers in parentheses indicate the plastochronic age of plants (i.e., number of leaves/plant) when first- (A), second- (B), and sixth- (C) emerging leaves occupied the leaf positions specified on the abscissa. • = solanidine, ♦ = leptinidine, ■ = acetylleptinidine.
A

B

C

Leaf Position (Youngest to Oldest)
Steroidal Alkaloid Concentrations of Leaves at Different Leaf Positions of Developing Plants

For this experiment, SA concentrations were measured of leaves at all plastochnic intervals of plants at the specified stages of development.

Total Steroidal Alkaloid Concentrations

For plants at the 10- to 14-leaf stages of development, total SA concentrations declined linearly (p<0.05) with leaf age (i.e., higher leaf number; Fig. 13B,C,D). However, total SA concentrations were consistently greater in leaf no. 4 than in leaf no. 3.

For plants at the 6-leaf stage, mean total SA concentration in the youngest leaf was 1.8X more than mean total SA concentration in the oldest leaf (2048 µg/g leaf fresh weight in leaf no. 1 to 1124 µg/g leaf fresh weight in leaf no. 6), but the effect of leaf position on total SA concentration was not significantly correlated (Fig. 13A).

Individual Steroidal Alkaloid Concentrations

Individual SA concentrations in leaves of plants at the 10- to 14-leaf stages of development were negatively correlated (linear, p≤0.05) with leaf age, with solanidine concentrations declining sharply over the first two to three leaf positions (Fig. 13B,C,D). At the 6-leaf stage of development, solanidine concentrations again followed a declining trend with leaf age, but leptinidine and acetylleptinidine concentrations remained relatively unaffected by leaf age (Fig 13A).

Mean solanidine concentrations were highest and exceeded mean concentrations of acetylleptinidine and leptinidine in leaves at the first leaf position of plants at the 6- to 12-leaf stages (Fig. 13A,B,C). However, the solanidine to acetylleptinidine ratio in these leaves steadily declined from 3.4 in plants at the 6-leaf stage of development to 1.6, 1.3, and 1.1 in plants at the 10-, 12-, and 14-leaf stages of development,
Figure 13. Individual and total (insets) alkaloid concentrations of leaves at different leaf positions of plants at the 6- (A), 10- (B), 12- (C), and 14- (D) leaf stages of development. Each point represents the mean of three replicates ± SE. • = solanidine, ♦ = leptinidine, ■ = acetylleptinidine.
respectively. As with total SA concentrations, acetylleptinidine concentrations were consistently higher in leaf no. 4 than in leaf no. 3 of plants at the 10- to 14-leaf stages (Fig. 13A,B,C). Relative SA concentrations of acetylleptinidine > leptinidine > solanidine in mid- and more mature leaves were principally due to the strong negative correlation between solanidine concentrations and leaf age over the first three leaf positions (Fig. 13B,C,D). This trend was different for leptinidine and acetylleptinidine, the concentrations of which varied less or were slightly higher over the first three to four leaf positions.

Incorporation of [14C]Mevalonate into Steroidal Alkaloids

Similar trends were observed for [14C]mevalonate incorporation on either a total DPM or DPM/g fresh weight basis as were observed from static SA measurements. In immature leaves, a larger proportion of the radiolabel that had become incorporated into the total SA pool accumulated in solanidine (53%), as compared to leptinidine (21%) and acetylleptinidine (26%) (Table 7). However, this trend of incorporation reversed in mid-leaves, in which 56% of the radiolabel incorporated into the total SA pool accumulated in acetylleptinidine, as compared to leptinidine (29%) and solanidine (15%).

Mid-leaves incorporated more radiolabel into individual and total SA pools than immature leaves. Incorporation of [14C]mevalonate into total SAs averaged 0.4% in immature leaves and 4% in terminal leaflets of mid-leaves.

DISCUSSION

The effects of leaf physiological age on individual and total SA concentrations were observed by monitoring SA concentrations throughout the development of newly-emerging leaves (Fig. 12) and in leaves at different leaf positions of individual plants (Fig. 13). Generally, leaf and plant physiological development significantly affected
Table 7. Incorporation of 2-[\textsuperscript{14}C]-mevalonate into steroidal alkaloids of \textit{S. chacoense}, PI 458310-1, immature leaves and mid-leaves.

Results are the means of duplicates from a representative experiment.

<table>
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<tr>
<th>Leaf</th>
<th>Alkaloid</th>
<th>Total DPM</th>
<th>DPM/g FW (X $10^{-3}$)</th>
</tr>
</thead>
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<tr>
<td>Immature</td>
<td>SD</td>
<td>8820</td>
<td>5405</td>
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<td></td>
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<td>4270</td>
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<tr>
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<td>SD</td>
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<tr>
<td></td>
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<td>55275</td>
<td>1262</td>
</tr>
<tr>
<td></td>
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<td>108660</td>
<td>2494</td>
</tr>
</tbody>
</table>
individual and total SA concentrations. SA concentrations were highest when
individual, developing leaves were young and in leaves of shoot tips (Figs. 12 and 13),
which is in agreement with previous reports that glycoalkaloid concentrations in \textit{S. tuberosum} are highest in regions of high metabolic activity, including meristematic
regions and young leaves (Lampitt et al., 1943; Wolf and Duggar, 1940). However,
total and individual SA concentrations generally declined as individual leaves matured,
and were lower in successively older leaves (i.e., higher leaf number) of individual
plants (Figs. 12 and 13). The initial decline in total SA concentrations was greatest as
newly-emerging leaves of very young plants matured (1- and 2- leaf stage; Fig.
12A,B). Similar trends were also observed over the first two to three leaf positions of
individual plants (Fig. 13). Lower total SA concentrations were coincident with lower
solanidine concentrations in these leaves.

Lower total and individual SA concentrations in mature leaves, as compared to
immature and mid-leaves, would imply that mature leaves may be more susceptible to
Colorado potato beetle feeding damage. However, even though total glycoalkaloid
levels had long been implicated in the insect resistance of some \textit{Solanum} species,
sufficiently high concentrations of specific glycoalkaloids are now recognized as having
at least equal importance (Sinden et al, 1986a; Tingey, 1984). Sinden et al. (1986a)
determined that \textit{S. chacoense} clones containing leptines were significantly more
resistant to Colorado potato beetle feeding than clones lacking these specific
glycoalkaloids but having higher total foliar glycoalkaloid concentrations. Thus, that
mature leaves had lower total SA concentrations than young and mid-leaves (Fig. 13) is
not as ecologically significant as the observation that acetylleptinidine concentrations in
mature leaves generally corresponded to leptine concentrations near or above 1 mg/g
leaf fresh weight, which reportedly is sufficient to deter Colorado potato beetle feeding
(Sturckow and L\text{"o}w, 1961). Young leaves, despite having higher concentrations of
solanidine than acetylleptinidine, also generally maintained these relatively high acetylleptinidine concentrations (Figs. 12 and 13).

These concentrations (> 1 mg/g fresh weight) of acetylleptinidine/leptine in leaves would be a highly desirable trait in commercial potato cultivars. However, levels of glycoalkaloid expression in S. chacoense hybrids can depend upon the non-S. chacoense parent, whose genetic regulation could reduce acetylleptinidine/leptine concentrations below efficacious levels. For example, Veilleux et al. (1992) have found that in hybrids generated from crosses between S. chacoense and S. phureja, total SA concentrations varied depending on the S. phureja parent. S. phureja parents producing relatively high concentrations of solanidine glycoalkaloids produced hybrids containing higher concentrations of leaf SAs (solanidine, leptinidine, and acetylleptinidine) than hybrids generated from crosses involving an S. phureja parent producing relatively low concentrations of solanidine glycoalkaloids. Thus, potential genetic regulation of SA biosynthesis become important considerations when selecting parents for S. chacoense hybridization, and individual SA concentrations of hybrids must be monitored closely.

The effects of leaf physiological development on individual and total SA concentrations prompted experiments to test the hypothesis that the observed variations in individual SA concentrations were due to differences in relative rates of biosynthesis. Plants at the 12-leaf stage were used for the comparative biochemistry between immature and mid-leaves because distinct differences in individual SA concentrations existed at both leaf positions in these plants (Fig. 13C).

SAs are derived from triterpene biosynthesis (reviewed in Heftmann, 1983), thus originating from acetate (Guseva and Pasechnichenko, 1958; Kaneko et al., 1972) and mevalonate (Guseva et al., 1961), with cycloartenol (Ripperger et al., 1971), lanosterol (Ripperger et al., 1971), and cholesterol (Heftmann et al., 1967; Tschesche
et al. 1976) as key intermediates. Since more immediate radiolabeled precursors to SA biosynthesis were not commercially available, radiolabeled intermediates of triterpene biosynthesis were considered for biochemical experiments. For the comparative biochemistry, radiolabeled mevalonate was chosen because higher levels of incorporation of radiolabel into SAs are obtained with mevalonate as compared to acetate (Guseva et al., 1961), and because the poor solubility of cholesterol along with the technical difficulties associated with the topical application of cholesterol (Bennett and Heftmann, 1965) can hinder efficient incorporation.

The incorporation of radiolabeled mevalonate into solanidine, leptinidine, and acetylleptinidine (Table 7) agrees with previous reports that SAs are products of triterpene biosynthesis (Guseva and Pasechnichenko, 1958; Guseva et al., 1961; Heftmann et al., 1967; Ripperger et al., 1971). In the youngest leaf (leaf no. 1) of a plants at the 12-leaf stage of development, relative SA concentrations (± SE) were solanidine (1343 ± 210 µg/g fresh weight) > acetylleptinidine (1029 ± 152 µg/g fresh weight) > leptinidine (740 ± 19 µg/g fresh weight). Likewise, the pattern of relative net incorporation of [14C]mevalonate into individual SA pools agreed with this relative ranking of SA concentrations (Table 7). Similarly, leaf no. 6 of plants of the same age contained SA concentrations (± SE) of 297 ± 28 µg solanidine/g fresh weight, 484 ± 32 µg leptinidine/g fresh weight, and 708 ± 15 µg acetylleptinidine/g fresh weight, and the pattern of net [14C]mevalonate incorporation into the individual SA pools again corroborated the relative ranking of SA concentrations determined by static measurements. These data (Table 7) support the hypothesis that relative differences in solanidine, leptinidine, and acetylleptinidine of leaves of different maturities are due to differences in relative rates of net biosynthesis of these alkaloids, and not merely due to dilution. In addition, differences caused solely by dilution would be expected to cause proportional differences in SA concentrations, and not the relative differences in
concentrations of individual SAs that were observed between immature and mid-leaves (Fig. 13C).

Even though mid-leaves incorporated more total dpm into SAs, immature leaves incorporated more radiolabel into SAs on a per g fresh weight basis. These data again agree with previous reports (Lampitt et al., 1943; Roddick, 1974; Wolf and Duggar, 1940) that SA concentration/biosynthesis is highest in young, actively-growing tissues. Comparisons concerning total incorporation between immature and mature leaves cannot be made because uptake kinetics may have been different between stems (immature) and petioles (mature leaflet) even though such a comparison reflects the same trend of higher total SA concentrations in immature leaves observed from static measurements.

In summary, leaf physiological age affected total and individual SA concentrations, which were generally higher in young leaves and declined with leaf maturation. Relative steroidal alkaloid concentrations were also affected by leaf age, with solanidine being the predominant SA in young leaves, and acetylleptinidine being the predominant SA in mature leaves. Incorporation of [14C]mevalonate into individual alkaloid pools supported the hypothesis that the variations in individual alkaloid concentrations between young and mature leaves were due to differences in relative rates of biosynthesis.
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CHAPTER IV

METABOLISM OF LEPTINIDINE TO ACETYLLEPTINIDINE BY LEAVES OF
SOLANUM CHACOENSE, BITTER

ABSTRACT

[14C]leptinidine was isolated to chemical and radiochemical purity following incubation of detached leaflets with R-[2-14C]mevalonic acid. The hydrochloride salt of [14C]leptinidine was fed to leaflets from detached mid-leaves, which were incubated for 48 or 96 h. Radiochromatograms generated from PTLC indicated metabolism into acetylleptinidine and other, non-steroidal alkaloid metabolites, which may have included plant pigments. Generally, leptinidine metabolism seemed to more extensive (6 - 10 X) in leaflets during the longer incubation period. Incorporation into acetylleptinidine ranged between 0.1 (585 dpm) and 0.4% (1886 dpm) of the total radiolabel administered. These data indicate that leptinidine can be used as a precursor for acetylleptinidine biosynthesis by S. chacoense leaves, and support the hypothesis that acetylleptinidine biosynthesis proceeds from leptinidine.
INTRODUCTION

The leptine glycoalkaloids (Kuhn and Löw, 1957, 1961) of *Solanum chacoense*, Bitter have potential ecological significance as natural antifeedants to the economically-important insect pest, the Colorado potato beetle, *Leptinotarsa decemlineata*, Say (Sturckow and Löw, 1961, Sinden et al., 1986). The steroidal glycoalkaloids α-solanine, α-chaconine, and leptinines are also found in *S. chacoense*, but are relatively ineffective as antifeedants to the Colorado potato beetle (Sturckow and Löw, 1961, Sinden et al., 1986).

The aglycones of *S. chacoense* glycoalkaloids, solanidine (α-solanine and α-chaconine), leptinidine (leptinines), and acetylleptinidine (leptines) are products of triterpene biosynthesis (Chapter III), as are all other steroidal alkaloids and steroidal sapogenins (Heftmann, 1983). A hypothetical biosynthetic pathway has been proposed for solanidine from alkaloid research involving *Veratrum grandiflorum* (Kaneko et al., 1976), but information is lacking regarding the enzymology of proposed biosynthetic steps. Osman et al. (1987) identified metabolism of uniformly labeled [14C]solanidine to leptinidine by microsomal fractions prepared from *S. chacoense* leaves, but also reported the apparent metabolism of solanidine to what may have been another x-hydroxysolanidine.

The structural relationship between solanidine, leptinidine, and acetylleptinidine, in addition to the evidence for conversion of solanidine to leptinidine (Osman et al., 1987), imply that acetylleptinidine biosynthesis in *S. chacoense* proceeds from solanidine, via leptinidine, in a two-step reaction sequence (Fig. 14). In this chapter, experiments are reported that test the hypothesis that leptinidine is a biosynthetic precursor to acetylleptinidine.
Figure 14. Hypothetical pathway for acetylleptinidine biosynthesis in *Solanum chacoense*, Bitter.
MATERIALS AND METHODS

Plant Culture

Apical cuttings of *S. chacoense*, Bitter, PI 458310-1, were rooted in sand and then transplanted to sand:soil:peat moss (1:1:1, v/v/v; 8 in. pots) in a greenhouse (14-h light/10-h dark photoperiod with supplemental lighting). One month after transplanting, plants were topped to initiate axillary bud growth. Physiological ages of developing axillary shoots were defined according to plastochronic development.

Steroidal Alkaloid Standards

Solanidine was purchased from Sigma Chemical Company (St. Louis, MO). Acetylleptinidine and leptinidine were purified using procedures reported previously (Lawson et al., 1992) and their identities confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy (Chapter II).

[^14C]Leptinidine and[^14C]Leptinidine·HCl

*Administration of R-[2-^14C]mevalonic acid lactone to leaves*

R-[2-^14C]Mevalonic acid was generated from R-[2-^14C]mevalonic acid lactone (Amersham Corp., 53.4 mCi/mmol, 50 µCi/ml) by pipeting an appropriate aliquot of the lactone into a microcentrifuge tube and evaporating the solution to dryness. The residue was then redissolved in deionized, distilled water to the appropriate volume, yielding the corresponding acid in an aqueous solution. Two mid-leaves (leaf no. 6) were removed from plants at the 12-leaf stage of development. Samples were transported to the laboratory on ice and washed with deionized water. All leaflets except the terminal leaflet (ca. 130 to 150 mg fresh weight) were removed from the petioles, and petioles were placed in 5 µCi (40 µl) each of ^14C]mevalonate. After
radiolabel was taken up, it was chased with 40 µl of deionized water, and then leaflets were incubated for 48 h in deionized water (14-h light/10-h dark photoperiod, 23 °C).

Purification of $[^{14}C]$acetylleptinidine

Following incubation, leaflets were freeze-dried and prepared for steroidal alkaloid analysis as described previously (Lawson et al., 1992). The crude steroidal alkaloid fraction was evaporated to dryness (50 °C, N$_2$), redissolved in chloroform, and then subjected to two sequential solid phase extraction (SPE) steps using 1 X 4 cm silica gel (E.M. Merck, 40-60 µm) columns. For the first SPE step, the column was equilibrated with dichloromethane, and then, following sample application, the column was first washed with dichloromethane (5 ml) and then $[^{14}C]$acetylleptinidine was eluted with dichloromethane:methanol (97:3, v/v; 10 ml). The latter fraction, which contained $[^{14}C]$acetylleptinidine according to TLC, was evaporated to dryness (50 °C, N$_2$), redissolved in a minimum volume of chloroform, and loaded onto a second SPE column, which had been equilibrated with hexane. After sample application, the column was washed with hexane and $[^{14}C]$acetylleptinidine was eluted with ethyl acetate:hexane (2:8, v/v; 10 ml). The latter fraction, which contained $[^{14}C]$acetylleptinidine according to TLC, was evaporated to dryness (50 °C, N$_2$), redissolved in a minimum volume of chloroform, and subjected to flash liquid chromatography on a 0.7 X 7.0 cm silica gel column, which had been equilibrated with hexane. Chromatography was effected using ethyl acetate-hexane (15:85, v/v); fractions of 100 drops were collected. $[^{14}C]$Acetylleptinidine eluted in fractions 10 to 15 according to TLC.

Preparation of $[^{14}C]$leptinidine

$[^{14}C]$Acetylleptinidine was then base hydrolyzed in 0.83 N sodium hydroxide in methanol-water (70 °C for 30 min) to yield $[^{14}C]$leptinidine (Lawson et al., 1992).
[\textsuperscript{14}C]Leptinidine from base hydrolysis was subjected to flash liquid chromatography using a 0.7 X 7.0 cm silica gel column equilibrated with dichloromethane. Chromatography was effected with dichloromethane:methanol (97:3); fractions of 100 drops were collected. The composition of individual fractions was determined by TLC.

Column liquid chromatographic separations were monitored by TLC using silica gel 60 (E.M. Merck, 0.25 \textmu m) plates and dichloromethane:methanol (8:2, v/v) for development. Spots were visualized with iodine vapor.

Purified [\textsuperscript{14}C]leptinidine (1.0 \mu Ci, 2.2 \mu Ci/umole) was verified to be radiochemically pure by TLC (Merck silica gel, 0.25 \mu m) using dichloromethane:methanol (8:2, v/v) developed to 10 cm. Chromatographic purity was determined using GC (Lawson et al., 1992). [\textsuperscript{14}C]Leptinidine·HCl (1.0 \mu Ci) was generated using 10% HCl-diethyl ether (103% experimental yield; Matuszak and Matuszak, 1967).

Administration of [\textsuperscript{14}C]Leptinidine·HCl

Mid-leaves (leaf no. 6) of plants at the 12-leaf stage of development were harvested and terminal leaflets of leaves were prepared as for R-[2-\textsuperscript{14}C]mevalonic acid administration (above). Petioles were immersed in 40 \mu l (451,557 dpm)/each of [\textsuperscript{14}C]leptinidine·HCl in water in 1.5 ml microcentrifuge tubes. After radiolabel was taken up, it was chased with 40 \mu l deionized water, and then leaflets were incubated for 48 or 96 h (2 leaflets/incubation period). Controls were treated in the same way but using unlabeled leptinidine·HCl.

Sample Preparation

Following incubation, leaflets and petioles were prepared for steroidal alkaloid analysis as described previously (Lawson et al., 1992). Final steroidal alkaloid fractions (100 \mu l) were spotted for preparative TLC (PTLC; E.M. Merck silica gel 60, 0.25 \mu m). Plates were developed to 10 cm in dichloromethane:methanol (8:2, v/v) and
border lanes visualized with iodine vapor. Silica gel at Rfs corresponding to solanidine, leptinidine, and acetylleptinidine according to the migration of authentic standards was scraped from the plate and eluted with ethyl acetate:hexane (1:1, v/v). Eluates were concentrated to 500 μl and their chemical purities evaluated by GC (Lawson et al., 1992). Eluates from silica gel at Rfs corresponding to solanidine and acetylleptinidine Rfs were subjected to further PTLC in dichloromethane:methanol (8:2, v/v) and ethyl acetate:hexane (1:1, v/v), respectively, and evaluated for chemical purity by GC.

**Liquid Scintillation Counting**

Aliquots of eluates were dispensed into liquid scintillation vials, mixed with 3.0 ml of Hionic Fluor and radioactivity was measured using a Beckman LS 1801 liquid scintillation counter. Data for radiochromatograms of PTLC were generated by scraping silica gel from 30% of the PTLC chromatogram at indicated Rfs into liquid scintillation vials, adding 300 μl methanol to elute solutes, and measuring radioactivity as described above.

**RESULTS AND DISCUSSION**

The metabolism of leptinidine by *S. chacoense* leaves was investigated by administering [14C]leptinidine-HCl in aqueous solution via petiolar absorption to terminal leaflets of mid-leaves. Plants at the 12-leaf stage of development were chosen because previous work (Chapter III) has found that mid-leaves of *S. chacoense* plants at this age have a high capacity for the synthesis of steroidal alkaloids, and because distinct differences in concentrations of individual steroidal alkaloids exist in these leaves. The efficient uptake of radiolabel (typically 99%) was attributed to the solubility afforded by the hydrochloride salt. This method of incorporation has distinct advantages over topical application, after which incorporations of radiolabel can be
impeded by physical barriers and degradation of the radiolabeled precursor (Bennett and Heftmann, 1965). Terminal leaflets were used rather than entire leaves or entire plants to minimize dilution, which would reduce the specific activities of both the [14C]leptinidine that was administered and radiolabeled products.

PTLC of final steroidal alkaloid extracts following standard sample workup (Lawson et al., 1992) revealed metabolism of leptinidine to products of higher and lower polarity according to TLC (Figs. 15A and 16A). Specifically, approximately 1% of the total radioactivity administered to 48-h leaflets was recovered at Rfs more polar than leptinidine, while 1.5% of the total radioactivity was recovered at Rfs less polar than leptinidine (Fig. 15A). For 96-h incubations, radioactivity found at lower and higher Rfs was 6% and 9%, respectively, of the total administered (Fig. 16A). In general, metabolism of leptinidine seemed to be more extensive (6 to 10 X) in leaves during the longer incubation period. The pattern of metabolism in both treatments was similar according to TLC except near the Rf of acetylleptinidine (hRf = 80) where relative levels of radioactivity were higher in the 48-h treatment (Figs. 15A and 16A).

Radioactivity associated with individual steroidal alkaloids was determined using a combination of PTLC and GC. [14C]Acetylleptinidine recovered following PTLC in dichloromethane:methanol (8:2, v/v) was isolated to chemical purity (according to GC) by PTLC using an ethyl acetate:hexane (1:1, v/v) mobile phase. Radiochromatograms indicated a peak of radioactivity at the Rf of acetylleptinidine that was baseline resolved from other regions of radioactivity (Figs. 15B and 16B). These data support the hypothesis that leptinidine can be used as a precursor for acetylleptinidine biosynthesis in S. chacoense leaves. Previously, Osman et al. (1987) demonstrated the conversion of solanidine to leptinidine in microsomal fractions prepared from S. chacoense leaf tissues. Therefore, in the context of this previous work, data reported here support the
Figure 15. PTLC radiochromatograms of (A) crude steroidal alkaloid fraction, and (B) acetylleptinidine Rf from (A) of 48-h incubation. Brackets indicate leading and trailing edges of standards. hRfs: (A) leptinidine (LD) = 47; solanidine (SD) = 56, acetylleptinidine (ALD) = 77; and (B) acetylleptinidine (ALD) = 29. See Materials and Methods for additional experimental details.
Figure 16. PTLC radiochromatograms of (A) crude steroidal alkaloid fraction, and (B) acetylleptinidine Rf from (A) of 96-h incubation. Brackets indicate leading and trailing edges of standards. hRfs: (A) leptinidine (LD) = 58; solanidine (SD) = 62, acetylleptinidine (ALD) = 80; and (B) acetylleptinidine (ALD) = 42. See Materials and Methods for additional experimental details.
hypothesis that acetylleptinidine is synthesized from solandine, via leptinidine, by a two-step reaction sequence (Fig. 14).

The specific activity of [14C]acetylleptinidine isolated from rep 2 of leaflets incubated with [14C]leptinidine-HCl for 48 h was 2.0 to 2.7 X higher than that of acetylleptinidine isolated from leaflets incubated for 96 h (Table 8), and indicate that metabolism of [14C]acetylleptinidine may also have been occurring. These data are consistent with results from [14C]mevalonate labeling of steroidal alkaloids in detached leaflets of S. chacoense. Maximum incorporation of [14C]mevalonate into steroidal alkaloid pools occurred following a 24- to 48-h incubation period, after which time (e.g., 96 h) radioactivity in alkaloid fractions slightly declined (Table 9). The specific activity of [14C]acetylleptinidine isolated from the leaflet of rep 1 of the 48-h incubation was not consistent with that isolated from the leaflet of rep 2 (Table 8). However, rep 1 of the 48-h incubation also had more radioactivity (16 X, 47,462 dpm) remaining in the reservoir than did the companion replicate of this treatment, which may have indicated that translocation and distribution of radiolabel was different in rep 1 than in rep 2. This interpretation is supported by the low incorporation of radiolabel into acetylleptinidine (0.1%) by the leaflet of rep 1 relative to the other leaflets (Table 8), despite nearly 90% of the administered [14C]leptinidine being absorbed and potentially available for metabolism.

For the 48-h incubations, radioactivity associated with the Rf of solanidine was only about 1 to 2 X background levels, and therefore was not purified further. For the 96-h incubation, repetitive (2 X) PTLC of solanidine failed to yield product that was radiochemically homogeneous. Even though the possibility that solanidine was a metabolite of leptinidine or a product derived from leptinidine catabolites cannot be excluded, conclusive support for this possibility was not found in these experiments.
Table 8. Radioactivity recovered as leptinidine (LD) and acetylleptinidine (ALD) following incubation of mid-leaf leaflets for 48 and 96 h with [14C]leptinidine·HCl *

<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>Rep</th>
<th>Leaflet Fr. Wt. (g)</th>
<th>DPM Recovered as LD</th>
<th>% Recovered as LD</th>
<th>DPM as ALD</th>
<th>DPM/µmole as ALD</th>
<th>DPM/g Fr. Wt. as ALD</th>
<th>% Incorporation into ALD</th>
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</tbody>
</table>

* 451,557 dpm of [14C]leptinidine·HCl/rep; DPM adjusted for steroidal alkaloid recoveries.
Of interest is the apparent metabolism of [14C]leptinidine to a metabolite(s) corresponding to radioactivity at an Rf of about 0.6 to 0.7 (Figs. 15A and 16A). This Rf is similar to that of a product that appeared in one instance during the final steps of leptinidine purification from S. chacoense. The compound was apparently an alkaloid since it reacted positively with Dragendorff's reagent. Since no other chemical or radiochemical impurities were detected during chemical purity and radiochemical purity assays, this 'metabolite(s)' was likely formed during the incubation period by either chemical or biochemical events.

Metabolism of steroidal glycoalkaloids, their aglycones, and related compounds has been observed before. Solasodine content in fruits of S. mammosum increases in green fruit until the time of maturity and then decreases during ripening (Telek et al., 1977). Extensive metabolism of the steroidal sapogenin, diosgenin, by Dioscorea composita has also been reported by Bennett et al. (1970). Metabolism of α-tomatine, a steroidal glycoalkaloid of tomato, has been observed in excised tomato fruits of all developmental stages, with the rate and extent of degradation depending on developmental stage (Eltayeb and Roddick, 1985). In addition, degradation of radiolabeled α-tomatine was dependant upon the length of incubation in the fruits. Longer incubation periods resulted in less recovery of radiolabel in α-tomatine and higher recoveries of radiolabel in metabolic products. These results corroborate those reported here for the metabolism of [14C]leptinidine, which indicate more extensive metabolism in leaflets incubated for 96 h versus those incubated for only 48 h.

In addition, following PTLC of acetylleptinidine in ethyl acetate:hexane (1:1, v/v) radioactivity was also found near the origin and low Rfs (Figs. 15B and 16B), which corresponded to the migrational distances of extracted pigments. These data indicate that leptinidine degradation products may be utilized in the biosynthesis of plant pigments. Similar observations have been made by Eltayeb and Roddick (1985),
who administered radiolabeled α-tomatine to excised tomato fruits and found radioactivity associated with chlorophylls, xanthophylls, and carotenes. The authors suggested that this metabolic activity may reflect the existence of an "isoprenoid-scavenging" pathway.

Incorporation of \([^{14}\text{C}]\text{leptinidine}\) into acetylleptinidine and other metabolites pools could have been affected by a variety of physical and physiological events, however, two possibilities seem most likely. Firstly, the relatively low specific activities of \(\text{in vivo}\)-generated radiolabeled precursor preclude incorporation as efficient as would be expected from commercially-available, radiolabeled precursors of higher specific activity. Secondly, since steroidal alkaloids are almost exclusively found as glycosides (Heftmann, 1983), and since glycosylation events appear to be the final steps in glycoalkaloid biosynthesis (Jadhav and Salunkhe, 1973; Osman, 1980), a significant percentage of radiolabeled leptinidine is likely to be directed toward glycosylation events leading to the synthesis of leptinines (Fig. 14), thus making it unavailable for metabolism to, for example, acetylleptinidine.

In conclusion, evidence has been presented indicating that leaflets of \textit{S. chacoense} can incorporate radiolabel from \(^{14}\text{C}]\text{leptinidine}\) into acetylleptinidine and other unidentified metabolites, which may include plant pigments. These data, in conjunction with evidence for the conversion of solanidine to leptinidine (Osman et al., 1987), support the hypothesis that acetylleptinidine is synthesized from solandine via leptinidine (Fig. 14).
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GENERAL DISCUSSION

Research was initiated with the objective of testing the hypothesis that acetylleptinidine was synthesized from solanidine, via leptinidine in Solanum chacoense. This hypothesis was formulated based upon the structural relationships between these steroidal alkaloids (Fig. 14), which were established based upon HR-MS and high-field $^1$H- and $^{13}$C-NMR of the steroidal alkaloids isolated from S. chacoense, PI 458310-1 (Chapter II).

HR-MS of all three alkaloids exhibited diagnostic fragments characteristic of a solanidane carbon skeleton, and also provided evidence for the localization of the hydroxyl of leptinidine and the acetoxy of acetylleptinidine to the E or F ring. Substitution of the hydroxyl and acetoxy groups at C-23 was indicated by the chemical shifts of the 23-H to $\delta$ 3.78 (leptinidine) and $\delta$ 5.30 (acetylleptinidine), respectively. Critical evidence for the $\beta$-orientation of the C-23 hydroxyl of leptinidine was obtained using $^{13}$C-NMR, which revealed downfield shifts for the $\alpha$- and $\beta$-carbons and upfield shifts for $\gamma$-carbons that were in agreement with the expected substituent effects imposed by axial hydroxyl groups (Eggert et al., 1976). Therefore, these data established structural relationships between the isolates that were consistent with the hypothesis that solanidine is stereospecifically hydroxylated at C-23 to yield leptinidine (23-$\beta$-hydroxysolanidine), and that the 23-$\beta$-hydroxyl of leptinidine is acetylated to yield acetylleptinidine (23-O-acetylleptinidine).
Since steroidal alkaloids appear to be under developmental control (Lampitt et al., 1943; Sinden et al., 1986a), tissues may vary in their capacity for steroidal alkaloid biosynthesis. Therefore experiments were initiated to test the hypothesis that leaf physiological age affects steroidal alkaloid concentrations and biosynthesis in *S. chacoense* (Chapter III). Data from these experiments would assist in selecting the tissue most suitable for subsequent biochemical work.

This work was facilitated by the development of an analytical GC procedure that used an internal standard for accurate quantitation of steroidal alkaloids from *S. chacoense* (Chapter I). In general, individual and total steroidal alkaloid concentrations were highest when leaves were young and decreased with maturation. Relative rates of \[^{14}\text{C}\]mevalonate incorporation into individual steroidal alkaloid pools, indicated that the effects of leaf physiological age on alkaloid concentrations were due to differences in relative rate of net biosynthesis. Mid-leaves from plants and the 12- to 14-leaf stages of development appeared to be particularly suitable for subsequent biochemical work seeking to elucidate acetylleptinidine biosynthesis and steroidal alkaloid biosynthesis in general in *S. chacoense*, since they demonstrated a high capacity for the synthesis of steroidal alkaloids and individual alkaloid concentrations were distinctly different from each other in these leaves.

Having structural confirmation of solanidine, leptinidine, and acetylleptinidine, and having identified tissue with a potentially high capacity for steroidal alkaloid/acetylleptinidine biosynthesis, experiments were initiated to test the hypothesis that leptinidine is a biosynthetic precursor to acetylleptinidine (Chapter IV). \[^{14}\text{C}\]Leptinidine was isolated to chemical and radiochemical purity from leaflets incubated with R-[2-\[^{14}\text{C}\]mevalonic acid, and the hydrochloride salt of \[^{14}\text{C}\]leptinidine was then administered to mid-leaf leaflets. Incorporation of radiolabel into acetylleptinidine was observed in both the 48- and 96-h incubation treatments, which
supported the hypothesis that leptininidine is a precursor for acetylleptininidine biosynthesis in *S. chacoense*. In conjunction with previous work (Osman et al., 1987), which demonstrated the conversion of solanidene to leptininidine, these data support the hypothesis for a two-step pathway for the synthesis of acetylleptininidine from solanidene.

**Limitations to Approach**

The single, largest impediment to a thorough investigation of steroidal alkaloid biochemistry in *S. chacoense* is the unavailability of high specific activity radiochemical precursors. Higher specific activities are particularly desirable when working with plant secondary metabolism for which metabolic rates are generally lower than those corresponding to primary metabolism or metabolism in bacteria or animal cells. Therefore higher specific activity precursors would increase total incorporation of radiolabel into metabolic products, and minimize the possibility of disrupting normal metabolic events by reducing the amount of precursor added to the system. Specific activities of radiolabeled alkamine precursors could be improved by first synthesizing the 3-oxo-derivatives using, for example, pyridinium chlorochromate, and then tritiating at C-3 by sodium borotritide reduction. The water insoluble nature of steroidal alkamines, which poses a potential limitation on efficient incorporation of radiolabeled precursor, was largely circumvented by using the hydrochloride salts.

**Future Directions**

**Biosynthesis**

Having provided supporting evidence for acetylleptininidine biosynthesis from leptininidine in *S. chacoense*, biochemical research should proceed toward using cell-free systems and [3H]solanidene and [3H]leptininidine (see above) to develop assays that could be used during enzyme purification or for determining subcellular localizations of
individual steps. Purification and sequencing of the enzymes for each of the two steps will permit the isolation of cDNA coding for these enzymes, which could then be used for transformation of commercially-important *S. tuberosum* cultivars using standard molecular biology techniques (e.g., *Agrobacterium* mediated transformation). This approach, unlike that of traditional breeding, will permit specific incorporation of the genes necessary for acetylleptinidine biosynthesis while eliminating undesirable traits that may be associated with the nuclear and organellar genomes of the wild parent.

However biochemical work should not be limited to only investigating alkamine biosynthesis. The importance of the glycoside for biological activity (Bushway et al., 1987; Friedman et al., 1991; Roddick and Drysdale, 1984; Roddick and Rijnenberg, 1986) emphasizes the need to understand glycosylation reactions in the synthesis of glycoalkaloids. A solanidine UDP-glucose glucosyltransferase has been purified from potato sprouts, which had specificity for the solanidane-type structures (Stapleton et al., 1991). Therefore it is likely that this enzyme would catalyze the same reaction for chacotriose addition to acetylleptinidine and leptinidine. Subsequent reactions in the stepwise addition of carbohydrate units remain to be elucidated.

To date, the subcellular localization of steroidal alkaloid biosynthesis remains unclear, but could be addressed with high specific activity radiolabeled precursors. Osman et al. (1987) have demonstrated metabolism of solanidine to leptinidine and another hydroxylated product by microsomal fractions prepared from *S. chacoense* leaf tissue and Roddick (1976) found α-tomatine associated almost exclusively with microsomal fractions. However, Ramaswamy et al. (1976) have demonstrated complete autonomy of *S. tuberosum* tuber chloroplasts for solanidine biosynthesis. Should future work confirm roles for both microsomal fractions (endoplasmic reticulum) and chloroplasts in steroidal alkaloid biosynthesis, then a biosynthetic scenario similar to that of isoprenoid biosynthesis would begin to emerge, for which
both plastidic and extraplastidic components have been proposed (Goodwin, 1958) and identified (Schulze-Siebert and Schultz, 1987).

**Metabolism**

The metabolism of leptinidine into products other than steroidal alkaloids also deserves further investigation, and raises questions about the metabolic fate of *S. chacoense* steroidal alkaloids. All three of these alkaloids are likely to be metabolized in similar ways because of their chemical similarity. In particular, the metabolism of *S. chacoense* steroidal alkaloids and glycoalkaloids into plant pigments should be verified and confirmed (Chapter IV). Eltayeb and Roddick (1985) suggested that the metabolism of the α-tomatine carbon skeleton into plant pigments may reflect the existence of a "isoprenoid-scavenging" pathway. If such a pathway were functional, then one may expect that the isoprenoid products may be re-introduced into triterpene biosynthesis and may become re-incorporated into steroidal alkaloids. Thus, a cyclical pathway for steroidal alkaloid metabolism could be envisaged. Catabolism of steroidal alkaloids into plant pigments becomes an even more intriguing concept in the context of the localization of steroidal alkaloid biosynthesis in chloroplasts (Ramaswamy et al., 1976). Therefore, it may be possible that a cyclical pathway for steroidal alkaloid metabolism, which includes both synthesis and catabolism, may occur completely in plastids. A biochemical understanding of the catabolic pathways for steroidal glycoalkaloids and alkamines may contribute to potato cultivar improvement by reducing glycoalkaloid concentrations or maintaining concentrations at or below safety thresholds during physiological or mechanical stresses, under which glycoalkaloid concentrations can increase significantly.
Conclusions

The overall objective of experiments reported here was to test the hypothesis that acetylleptinidine biosynthesis in *S. chacoense* proceeds from solanidine with leptinidine as an intermediate. However, this objective was not addressed until (1) appropriate analytical techniques were developed for the quantitation of *S. chacoense* steroidal alkaloids; (2) steroidal alkaloids isolated from *S. chacoense* had been structurally confirmed; and (3) a fundamental understanding of the effects of leaf physiological age on the concentrations and biosynthesis of solanidine, leptinidine, and acetylleptinidine had been attained. The proposed hypothesis for acetylleptinidine was supported in part by the observation that \[^{14}\text{C}]\text{leptinidine was converted to }\[^{14}\text{C}]\text{acetylleptinidine in detached leaflets of }S. \text{ chacoense, PI 458310-1. From these experiments and from previous work (Osman et al., 1987), evidence has been presented for a biosynthetic relationship between solanidine, leptinidine, and acetylleptinidine, which can be used in future experiments leading to the incorporation of Colorado potato beetle resistance into commercially-important potato cultivars.}
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APPENDIX A

Data Relative to Chapter II
Figure 17. High-resolution electron impact mass spectrum of solanidine. See Materials and Methods for experimental details.
Figure 18. $^1$H-NMR spectrum of solanidine in CDCl$_3$ plus 1 drop of C$_5$D$_3$N. See Materials and Methods for experimental details.
Figure 19. $^{13}$C-NMR spectrum of solanidine in CDCl$_3$ plus 1 drop of C$_5$D$_5$N. See Materials and Methods for experimental details.
Figure 20. H,C COSY spectrum for solanidine in CDCl$_3$ plus 1 drop of C$_5$D$_5$N. See Materials and Methods for experimental details.
Figure 21. High-resolution electron impact mass spectrum of leptinidene. See Materials and Methods for experimental details.
Figure 22. $^1$H-NMR spectrum of leptinidine in CDCl$_3$. See Materials and Methods for experimental details.
Figure 23. $^{13}$C-NMR spectrum of leptinidine in C$_5$D$_5$N. See Materials and Methods for experimental details.
Figure 24. High-resolution electron impact mass spectrum of acetylleptinidine. See Materials and Methods for experimental details.
Figure 25. $^1$H-NMR spectrum of acetylleptinidine in $C_5D_5N$. See Materials and Methods for experimental details.
Figure 26. $^{13}$C-NMR spectrum of acetylleptinidine in C$_5$D$_5$N. See Materials and Methods for experimental details.
Figure 27. H,C COSY spectrum for acetylleptinidine C$_5$D$_3$N. See Materials and Methods for experimental details.
Figure 28. $^1$H-NMR spectrum of diacetylleptinidine in C$_5$D$_5$N. See Materials and Methods for experimental details.
Figure 29. $^{13}$C-NMR spectrum of diacetylleptinidine in C$_5$D$_5$N. See Materials and Methods for experimental details.
Figure 30. H,C COSY spectrum for diacetylleptinidine C5D5N. See Materials and Methods for experimental details.
APPENDIX B

Data Relative to Chapter III
Table 9. Time-course for R-[2-14C]-mevalonic acid (2 μCi) incorporation into *S. chacoense* steroidal alkaloids. See Materials and Methods for experimental details regarding administration of radiolabeled precursor, sample preparation, and radioactivity assays.

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<th>Mean LD b</th>
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a  Solanidine  
b  Leptinidine  
c  Acetylleptinidine
APPENDIX C

Data Relative to Chapter IV
Figure 31. Radiochromatogram of [\(^{14}\)C]acetylleptinidine purification using flash chromatography. Terminal leaflets of mid-leaves from \textit{S. chacoense} were incubated with R-[2-\(^{14}\)C]mevalonic acid and [\(^{14}\)C]acetylleptinidine was purified according to Materials and Methods. Shown here is the radioactivity of fractions eluted from a 0.8 X 25 cm silica gel (E.M. Merck, 40-60 \(\mu\)m) column using ethyl acetate:hexane (2:8, v/v). A 20 \(\mu\)l aliquot was removed from each 1-ml fraction and assayed for radioactivity by liquid scintillation counting as described in Materials and Methods. Fractions 10 to 15 contained [\(^{14}\)C]acetylleptinidine according to TLC (Merck silica gel, 0.25 \(\mu\)m; dichloromethane:methanol (8:2, v/v) developed to 8 cm; visualization with iodine vapor).
Figure 32. Radiochromatogram of [14C]leptinidine purification using flash chromatography. [14C]Leptinidine was generated by base hydrolyzing (70 °C, 30 min) [14C]acetylleptinidine and purified using flash chromatography according to Materials and Methods. Shown here is the radioactivity of fractions eluted from a 0.7 X 7.0 cm silica gel (E.M. Merck, 40-60 μm) column, which was equilibrated with dichloromethane and eluted with dichloromethane:methanol (97:3, v/v). A 20 μl aliquot was removed from each 1-ml fraction and assayed for radioactivity by liquid scintillation counting as described in Materials and Methods. Fractions 15 to 25 contained leptinidine according to TLC (Merck silica gel, 0.25 μm; dichloromethane:methanol (8:2, v/v) developed to 8 cm; visualization with iodine vapor).
Figure 33. Radiochromatogram following TLC of purified [\(^{14}\text{C}\)]leptinidine. [\(^{14}\text{C}\)]Leptinidine purified using flash chromatography (see also Fig. 32) was redissolved in chloroform (1000 µl) and assayed for radiochemical purity using TLC (Merck silica gel, 0.25 µm; 1 µl application; dichloromethane:methanol (8:2, v/v) developed to 10 cm; visualization with iodine vapor). hRf values were calculated from the center of marked zones. Leptinidine migrated to an hRf of 59. Silica gel from marked zones was assayed for radioactivity as described in Material and Methods.