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RICE UNIVERSITY

ANALYSIS OF METABOLIC FLUX OF SECONDARY METABOLITE PATHWAYS IN CATHARANTHUS ROSEUS HAIRY ROOT CULTURES

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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To

My Wife and Son
ABSTRACT

ANALYSIS OF METABOLIC FLUX OF SECONDARY METABOLITE PATHWAYS IN CATHARANTHUS ROSEUS HAIRY ROOT CULTURES

John A. Morgan

*Catharanthus* *roseus*, a tropical plant, produces the valuable anti-cancer compounds, vincristine and vinblastine in extremely low amounts. My research objectives were to examine the response of plant secondary metabolism to various metabolic perturbations, and indicate limitations in the reaction network.

In hairy roots, tabersonine is an important intermediate in the synthesis of vindoline, a monomer in the formation of the anti-cancer compounds that are generally absent from cell and hairy root cultures. To understand how much metabolic flux is directed toward the tabersonine branchpoint, transient profiles of lochnericine and hörhammericine in relation to tabersonine in both dark and light -adapted cultures were quantified. The results demonstrated that the accumulation of lochnericine was growth related, similar to tabersonine, and that light repressed the formation of all three alkaloids. Using enzyme inhibitors, the involvement of separate P-450 monooxygenase dependent enzymes in the biosynthesis of hörhammericine and lochnericine was demonstrated. Furthermore, hörhammericine and lochnericine were observed to be turned over.

A search for rate limiting regions of the pathway was accomplished through precursor feeding studies. By identifying precursors, which after feeding, significantly enhance the production of alkaloids, the specific precursor branch that is limiting flux
to alkaloids can be elucidated. Precursors fed from the terpenoid portion of the pathway at 21 days in the culture cycle were found to enhance the specific yield of tabersonine. This result suggests that during the early stationary phase period (21-24 days) flux limitations may occur upstream of geraniol. On the other hand, the rate-limiting pathway could not be identified during the late growth phase (17-21 days). In part, this was due to the fact that tryptophan served as a precursor of indole acetic acid (IAA), a plant growth regulator. Therefore, changes in indole alkaloid accumulation and root growth due to tryptophan feeding were similar to those induced by exogenously added IAA. Since feeding tryptamine or terpenoid precursors did not significantly enhance indole alkaloid accumulation the rate-limitation may be downstream of loganin.

The metabolic flux distribution between separate alkaloid branches was quantified by monitoring the transient profiles of multiple alkaloids. The flux towards the Iboga alkaloids decreased while the flux to the Aspidosperma alkaloids increased between 12 and 26 days. In contrast, the flux distribution between the Corynanthe branch and the sum of the Iboga and Aspidosperma branches remained unchanged during this period. Despite significant changes in extracellular nutrient levels during this period, the total flux to the alkaloids remained constant between 12 and 26 days.

Through the precursor feeding and biogenetic flux analysis studies, flux limitations to alkaloids likely exist in the terpenoid pathway leading to secologaninin. With the tools developed to quantify metabolic flux through a simple model, metabolic flux in metabolically engineered plant cell and tissue cultures can be routinely analyzed.
ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

Natural products are chemicals produced by living organisms. An extraordinary number of chemical entities that have value as pharmaceuticals were discovered through screening natural product libraries from microorganisms and plant extracts (Shu, 1998). More than half of the current clinically important anti-infective and anti-tumor drugs have natural products as their source (Cragg et al., 1997). A key to the development of naturally derived drugs is generating enough of the compound to meet the supply needs for clinical trials as well as commercial production.

1.1 Plant Secondary Metabolites

Plants are the source of incredible diversity of secondary metabolites. Secondary metabolites are often classified as compounds which are not directly linked to the development, growth, or respiration of plants. In nature, an exact role has not been assigned for most of these compounds. However, several activities of plant secondary metabolites are protection against fungal and bacterial pathogens, anti-feeding compounds, attractants, and chemical signals (Bennett and Wallsgrove, 1994). These products find use in the food, fragrance, and health care industries. To emphasize their importance, several examples of clinically important drugs obtained from plants are shown in Table 1. The natural products listed may not be in use themselves, but have served as leads for compounds that have better efficacy or less side-effects. For example, derivatives of camptothecin are in use against several types
Table 1-1. Plant derived natural products and their clinical application.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plant Genus/Species</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgolides</td>
<td><em>Ginkgo biloba</em></td>
<td>dementia</td>
</tr>
<tr>
<td>Digoxin</td>
<td><em>Digitalis lanata</em></td>
<td>Cardiatomic</td>
</tr>
<tr>
<td>Quinine</td>
<td><em>Cinchona ledgeriana</em></td>
<td>anti-malarial</td>
</tr>
<tr>
<td>Artemisinin</td>
<td><em>Artemisia annua</em></td>
<td>anti-malarial</td>
</tr>
<tr>
<td>Scopolamine, Hyoscyamine</td>
<td><em>Duboisia sp., Atropa belladonna</em></td>
<td>anti-cholinergic</td>
</tr>
<tr>
<td>Ajmaline</td>
<td><em>Rauwolfia serpentina</em></td>
<td>anti-arrhythmic</td>
</tr>
<tr>
<td>Ajmalicine, Serpentine</td>
<td><em>Rauwolfia serpentina</em></td>
<td>anti-hypertensive</td>
</tr>
<tr>
<td>Vincristine, Vinblastine</td>
<td><em>Catharanthus roseus</em></td>
<td>anti-tumor</td>
</tr>
<tr>
<td>Camptothecin</td>
<td><em>Camptotheca acuminata</em></td>
<td>anti-tumor</td>
</tr>
<tr>
<td>Taxol</td>
<td><em>Taxus sp.</em></td>
<td>anti-tumor</td>
</tr>
<tr>
<td>Tripodiolide</td>
<td><em>Tripterygium wilfordii</em></td>
<td>anti-inflammatory</td>
</tr>
<tr>
<td>Codeine</td>
<td><em>Papaver somniferum</em></td>
<td>anti-tussive</td>
</tr>
<tr>
<td>Morphine</td>
<td><em>Papaver somniferum</em></td>
<td>analgesics</td>
</tr>
</tbody>
</table>
of cancer (Shu, 1998).

Despite the diversity of plant secondary metabolites, they are connected to primary metabolism through relatively few pathways. Figure 1-1 shows the source of chemical structures for various classes of secondary metabolites, and how the pathways are linked to primary metabolism. Knowledge about the pathways is not always complete. For example, Schwender et al. (1996) have recently shown that the synthesis of some isoprenoids in a green alga occurs not by the mevalonate pathway, but from an alternative pathway beginning from pyruvate and glyceraldehyde 3-phosphate(Schwender et al., 1996). Furthermore, the pathways, as pictured in Figure 1-1 are not simple irreversible reactions with accumulation of the end products. Many fundamental features of metabolism are not pictured, such as reversible reactions (principally restricted to primary metabolism), feedback regulation, and degradation of the final products. Compartmentation of these reactions into different subcellular compartments, specialized cells, or tissues is an important means by which the regulation of secondary metabolism is controlled.

Typical yields of secondary metabolites from plants are usually quite low. Furthermore, the growth of certain plants is slow and subject to unpredictable environmental pressures. Alternative systems for production of valuable secondary metabolites are chemical synthesis, or the use of plant cell and tissue cultures. As many secondary metabolites are of moderate molecular weight and possess multiple
Figure 1-1. Schematic of the connections of the biochemical pathways that lead from primary metabolism and proceed to secondary metabolism in plants.
chiral centers, total chemical synthesis is currently not an economically feasible alternative for production of these complex metabolites (DiCosmo, 1990). In addition to the commercial production of plant secondary metabolites (Banhorpe, 1994; DiCosmo and Misawa, 1995), plant cell and tissue cultures have been an invaluable tool used widely for studying metabolism (Zenk, 1991). Choices of culture systems range from cell suspension cultures to a differentiated tissue culture such as shoots or roots (Payne et al., 1992).

1.2 Metabolic Engineering

Metabolic engineering has recently emerged as a field that has the purposeful, rational alteration of metabolism as a goal (Bailey, 1991; Stephanopoulos and Sinskey, 1993). Due to the low levels of plant secondary metabolite production, metabolic engineering may be used as a tool to enhance levels of secondary metabolites (Nessler, 1994; Verpoorte et al., 1996). Advantages of metabolic engineering over traditional methods of plant breeding are the ability to transfer enzymes (and hence metabolites) to species that previously lacked them. Metabolic engineering also offers an advantage that the transformation may be relatively rapid compared to the time scale of the plant reproductive cycle. Even within the same plant species, metabolic engineering could allow the expression of an enzyme in a specific amount, tissue, and subcellular location. Compared to a mutational strategy, metabolic engineering can provide both a targeted gain as well as a loss of function.
Metabolic engineering may be used to increase accumulation of compounds in either the whole plant or in in vitro plant cell and tissue culture. Investigators are seeking alternative means of enhancing secondary metabolite yields in plant cell culture, as the majority of strategies have not enhanced production to commercially viable levels (Dörnenburg and Knorr, 1995). Numerous empirical methods such as medium optimization, elicitation, and precursor feeding have not resulted in significant enough levels of improvement to economically produce most secondary metabolites in vitro. Through specific modifications of the cell or tissues capabilities to synthesize and accumulate secondary metabolites, metabolic engineering offers the brightest prospects for increasing production.

Current challenges in the metabolic engineering of secondary metabolites from plants and in vitro plant cultures result from several aspects that make these compounds unique. The extraordinary diversity of plant species and their metabolites is both a blessing and a curse. The variety of compounds may benefit the plant by having molecules ‘tailored’ to ward off specific pathogens, and also provides humanity with a diverse library of potential drug leads. However, with a vast number of interesting metabolites there are gaps in knowing the complete “metabolic map” for several pathways, and even less is known regarding pathway regulation (Kutchan, 1995; Rhodes et al., 1997). A second factor which hinders progress is the enzymes of secondary metabolism are often present in low amounts, making the purification of sufficient quantity for reverse genetics difficult (Kurz, 1989). Thus, to be able to
conduct metabolic engineering in plant cell and tissue cultures, substantial increases in knowledge of enzymology, metabolite identification, and regulation of metabolism are necessary. The goal of this thesis is to use techniques that probe the flux responses to perturbations and develop a technique for the quantification of metabolic flux. From an increased understanding of the flux distribution through the biosynthetic pathways, strategies to optimize production of desired targets can be formulated.

1.3 Thesis Outline

The goal of this project is to increase the understanding of secondary metabolic pathways in the tropical plant, *Catharanthus roseus* (L.) G. Don. *C. roseus* has been widely studied, because it produces the anti-neoplastic compounds vincristine and vinblastine (Cragg et al., 1997), as well as the anti-hypertensive compounds serpentine and ajmalicine (Hamill et al., 1987). Researchers have been studying plant cell and tissue cultures for the production of these compounds (Verpoorte et al., 1994). Hairy root cultures were selected as an appropriate system, since it has been previously characterized in this laboratory (Bhadra and Shanks, 1997; Shanks and Bhadra, 1997). Chapter 2 provides a review of recent developments in the enzymology and regulation of the indole alkaloid biosynthetic pathways in *C. roseus*. Details of factors, such as hormones, signaling molecules that are involved in the regulation of the pathway will included.
Chapter 3 concerns a bioprocessing aspect of plant cell culture that has previously received little attention. The extracellular pH of plant cell cultures is known to vary between 3 and 4 pH units. The drastic changes may lead to altered nutrient uptake, sucrose catabolism, and regulation of internal pH by proton pumping. Obviously each of these parameters may have a significant impact on the growth of the hairy root cultures. Chapter 3 examines the effect of using biological buffers to control the pH on growth and alkaloid accumulation in C. roseus hairy root cultures.

A primary focus of this thesis is the response of the metabolites around the tabersonine branchpoint to various environmental and molecular perturbations. The tabersonine branchpoint is a key branchpoint which can lead to vindoline or other products (see Chapter 2). Specifically, Chapter 4 examines the effects of initial sucrose concentration and light-adaptation of hairy root cultures on the transient accumulation of metabolites including and derived from tabersonine. Furthermore, a search was conducted for metabolites between tabersonine and vindoline. Chapter 5 analyzes the response of the tabersonine branchpoint to enzyme inhibitors. Oxygenase inhibitors were used to determine the enzyme classes involved in the conversion of tabersonine. The pathway was investigated under normal and elicited conditions, using a combination of inhibitors and jasmonic acid. Additionally, the ‘turnover’ of specific secondary metabolites was examined.

Typically, production of secondary metabolites from plant cell and tissue culture are limited to low levels. Similar to primary metabolism, it is hypothesized
that certain reactions limit the flux to secondary metabolism. This concept is explored in Chapter 6 by probing rate limitations through precursor feeding. Two pathways from primary metabolism converge to create strictosidine, the central precursor of all C. roseus alkaloids (see Chapter 2). Precursors from each of these pathways were fed to determine which of the two upstream pathways was limiting. The effects of precursor dosage as well as the timing of feeding were examined.

Substantially more knowledge exists regarding the identity and levels of secondary metabolites, than for the enzymes responsible for indole alkaloid biosynthesis. By applying techniques of metabolic analysis, information regarding critical branchpoints in secondary metabolism can be elucidated solely from metabolite measurements. The quantification of metabolite levels when combined with a model for growth leads to estimates of flux. Chapter 7 develops the process by applying the biogenetic relationship of the indole alkaloids to organize them around enzymatically uncharacterized branchpoints. From this model, the productivity of several branchpoints can be monitored transiently.

Once targets for metabolic engineering have been selected, and a gene of interest has been cloned, the next step is to introduce the gene. At the time this work began the only successful transformations of C. roseus were oncogenic. To have a well characterized line as a control, a procedure for re-transformation was necessary. Chapter 8 presents results from experiments designed to re-transform a hairy root line.

Chapter 9 concludes with recommendations for future work.
CHAPTER 2: BACKGROUND

*C. roseus* is the sole source of the bis-indole vinca alkaloids, derivatives of which are currently used in chemotherapy. The biochemical pathways leading to these compounds have been studied for several decades. Several excellent recent reviews detail the enzymology and regulation of the indole alkaloid biosynthetic pathways (Meijer et al., 1993; Moreno et al., 1995; Misra et al., 1996; Verpoorte et al., 1997). In addition to the biosynthesis of these compounds, *C. roseus* is capable of synthesizing over 80 alkaloids (Taylor and Farnsworth, 1975). However, even the pathways leading to the valuable anti-neoplastic agents are still not fully characterized. This chapter will briefly review the pathways, and update the above cited reviews with recently published work.

2.1 Biosynthetic Pathways

2.1.1 Strictosidine Precursor Pathways

Strictosidine, the central precursor of the indole alkaloids is formed by the condensation of one tryptamine and one secologanin unit (Figure 2-1). The enzyme responsible for this formation is strictosidine synthase (SSS). SSS has been found to be localized to the vacuoles of *C. roseus* cells (McKnight et al., 1991). A cDNA for SSS was first cloned from cell cultures of *R. serpentina* (Kutchan et al., 1988). Furthermore, it has been shown that the gene for SSS occurs as a single copy in the
Figure 2-1. The biosynthetic pathway leading from tryptophan and IPP to strictosidine, the central intermediate in indole alkaloid formation.
genome (Pasquali et al., 1992), with multiple isoforms of this enzyme due to post-translational modification of the enzyme.

The formation of tryptamine is catalyzed by tryptophan decarboxylase (TDC). TDC is localized in the cytoplasm (De Luca and Cutler, 1987). The gene for TDC has been cloned (De Luca, 1989) and there is a single genomic copy (Goddijn et al., 1994).

The pathway leading to the monoterpenoid unit, secologanin, is more complicated. Early feeding studies found that $^{14}$C labeled mevalonic acid was incorporated into vindoline in C. roseus seedlings (Money et al., 1965). However, recently, there is increasing evidence for an alternative pathway leading to isopentenyl pyrophosphate (IPP). This pathway was recently reviewed (Eisenreich et al., 1998). In a study that fed $^{13}$C labeled glucose to C. roseus cell suspensions, the synthesis of secologanin was studied, and it was concluded that the majority of flux did not come from the mevalonate pathway, but from an alternative pathway to IPP (Contin et al., 1998). The first committed precursor of this alternative pathway, 1-deoxy-D-xylose, is derived from a condensation of glyceraldehyde 3-phosphate and pyruvate, both products of the glycolytic pathway. Apparently, it is possible for communication between the mevalonate and alternative pathways, but it remains to be established through what intermediates this takes place.

IPP is isomerized by isopentenyl isomerase to dimethylallyl pyrophosphate (DMAPP). DMAPP and IPP then condense to form geranyl pyrophosphate (GPP).
Geraniol is formed by the dephosphorylation of GPP. However, it is not known if this step is enzymatic or the subcellular location where this reaction occurs. Geraniol is converted to 10-hydroxy geraniol by the enzyme geraniol 10-hydroxylase, which is a P-450 monooxygenase (Madyastha et al., 1976). G10H has been found to be localized in the membranes of vesicles that co-centrifugate with those from the pro-vacuole (Madyastha et al., 1977). More recently, the enzyme was purified to homogeneity (Meijer et al., 1993) and several attempts were made to clone the gene by similarity to other cytochrome P-450 monooxygenase enzymes. However, all of the putative clones tested showed no G10H activity (Mangold et al., 1994).

2.1.2 Indole Alkaloid Pathways

Figure 2-2 shows the formation of multiple classes of alkaloids from strictosidine. Recently, the enzyme responsible for the deglucosylation of strictosidine was purified (Luijendijk et al., 1998). The biosynthesis of the alkaloids ajmalicine and serpentine is fairly well characterized and reviewed in several articles (Meijer et al., 1993; Verpoorte et al., 1997). The final conversion of ajmalicine into serpentine occurs through the action of basic vacuolar peroxidase enzymes (Blom et al., 1991).

The two precursors of bis-indole alkaloids are vindoline and catharanthine, which when combined produce the dimeric anti-cancer compounds vincristine and vinblastine. The formation of catharanthine is completely uncharacterized at the
Figure 2-2. Indole alkaloid pathways in *C. roseus* leading from strictosidine to the bis-indole alkaloids, vincristine and vinblastine. Dotted lines represent multiple or uncharacterized steps in the pathways.
enzymatic level. In contrast, the pathway between tabersonine and vindoline (Figure 2-3) has been extensively examined. Vindoline accumulation is primarily restricted to the leaves of the whole plant, but catharanthine can be found throughout the plant as well as in cell-suspension cultures. Vindoline has been detected in tumorous cell suspension cultures and shooty teratomas, but in minute amounts (O'Keefe et al., 1997). The lack of the last four enzymes in the pathway leading to vindoline are thought not to be present in most cell and root cultures as they have been shown to be restricted to the aerial parts of the plant with light required for their expression (De Luca et al., 1988).

Recently, the first and last two steps of vindoline biosynthesis have been further characterized. The conversion of tabersonine to 16-hydroxytabersonine is mediated by a P-450 monooxygenase (St-Pierre and De Luca, 1995). The enzyme was found to be localized to the endoplasmic reticulum. The O-methyltransferase enzyme was not characterized, but its localization was reported to be in the cytosol (St-Pierre et al., 1998). The third step in the pathway remains the only uncharacterized step. The next step is catalyzed by a N-methyltransferase (NMT), that is localized to the thylakoid membrane (Dethier and De Luca, 1993). The expression of the final two steps are regulated by light and are developmental and tissue specific (De Luca, 1993). Each of the final two enzymes are localized in the cytosol. The cDNA for the 2-oxoglutarate dependent dioxygenase (D4H) that is responsible for the formation of desacetoxyvindoline was recently cloned and a single genomic copy of the gene was
Figure 2-3. Pathway leading from tabersonine to vindoline. OMT - 16-O-methyltransferase, NMT - N-methyltransferase, D4H- desacetoxyvindoline 4-hydroxylase, DAT- deacetylvinodline 4-O-acetyltransferase. The dotted line represents an uncharacterized hydrolase.
found (Vasquez-Flota et al., 1997). The activity of the enzyme is apparently under post-transcriptional control as different isoforms of the enzyme are present in etiolated and light-grown seedlings (Vasquez-Flota and De Luca, 1998). The gene for the final step in vindoline biosynthesis, which encodes an O-acetyltransferase was also recently cloned (St-Pierre et al., 1998).

The formation of the bis-indole alkaloids has been previously shown to be dependent on vacuolar peroxidases (Endo et al., 1988; Misawa et al., 1988). A specific enzyme responsible for the coupling of vindoline and catharanthine was recently purified and characterized (Sottomayor et al., 1998).

2.1.3 Compartmentation

Several subcellular compartments are known to be involved in the biosynthesis of the indole alkaloids. The location of the characterized enzymes is shown in Figure 2-4. In addition to the subcellular compartmentation, certain enzymes are tissue specific. As mentioned earlier, the enzymes responsible for the conversion of tabersonine to vindoline have the highest activity in young leaves. However, some of the earlier enzymes such as TDC and SSS have higher transcript levels in the roots than the leaves (Pasquali et al., 1992). Furthermore, the activity of G10H, a putative rate-limiting step has highest activity in the roots (Meijer et al., 1993). These facts raise the interesting possibility that the roots are responsible for some of the biosynthetic precursors that are subsequently transported to the leaves.
Figure 2-4. Subcellular localization of metabolites and enzymes involved in indole alkaloid biosynthesis (See text for abbreviations).
(adapted from Meijer et al., 1993)
2.2 Factors Regulating the Accumulation of Indole Alkaloids

2.2.1 Hormones

Plant hormones are known to be involved in a wide range of functions in plant development. The plant hormone, auxin has been found to down-regulate indole alkaloid accumulation in cell suspension cultures (Pasquali et al., 1992). Conversely, both exogenously applied ethylene and cytokinins have been found to enhance ajmalicine accumulation (Yahia et al., 1998). The mechanism was found to occur by independent pathways. Each of these findings has some ambiguities as other work has found that ethylene has little effect on ajmalicine biosynthesis (Schlatmann et al., 1994). Furthermore, when the gene responsible for cytokinin biosynthesis was overexpressed in *C. roseus* calli, alkaloid production was not enhanced, despite increased cytokinin concentrations (Garnier et al., 1996).

2.2.2 Elicitors

Elicitation is a process in which plant cells dramatically change their metabolism in order to mount a defense response. Both primary and secondary metabolism can be drastically altered. Earlier work in our laboratory examined the effects of defined elicitors upon secondary metabolism in *C. roseus* hairy roots (Rijhwani and Shanks, 1998). Jasmonic acid (JA), a signal transducer, was shown to have the most significant effects upon all alkaloids quantified. This result agrees with other work that jasmonate enhances alkaloid accumulation in *Catharanthus* seedlings.
(Aerts et al., 1994). Later work showed that JA was able to induce accumulation of new alkaloids, although these were not identified (Dymov et al., 1997). The necessity of JA for alkaloid biosynthesis is not conclusively known at this time. The octadecanoic pathway leads to signaling molecules involved in the defense response, such as jasmonic acid. A functional octadecanoic pathway, was found necessary for indole alkaloid production in cell suspension of C. roseus since inhibitors of this pathway greatly reduced alkaloid accumulation (Gantet et al., 1998). The presence of JA was able to partially overcome the inhibition of alkaloid accumulation by these inhibitors as well as by auxin. In contrast, inhibitors of jasmonic acid biosynthesis did not affect indole alkaloid accumulation in seedlings (Aerts et al., 1996). Jasmonate was also found to modulate the induction of TDC and D4H activities (Vasquez-Flota and De Luca, 1998). For these enzymes, the action of jasmonate was found to occur at the transcriptional, post-transcriptional, and post-translational levels.

2.2.3 External Environmental Parameters

Conditions in submerged cell or tissue cultures are obviously different than those present in the field grown plant. Nevertheless factors such as nutrient composition, shear stress, and the concentration of dissolved gases, can have a significant impact on growth and alkaloid productivity of cultures. For example, Schlatmann et al. found that a minimum amount of glucose was necessary to maintain
alkaloid production in production phase medium (Schlatmann et al., 1995). However, the specific productivity of ajmalicine varied inversely with glucose between 23 g/L and 75 g/L. The result was shown not to be an osmotic effect.

Gas phase metabolites are known to affect cell growth and alkaloid productivity of C. roseus cell cultures (Payne et al., 1988; Lee and Shuler, 1991; Schlatmann et al., 1994; Vani, 1996). In contrast, other work has found that the carbon dioxide and ethylene concentrations have little impact upon ajmalicine production in bioreactors (Schlatmann et al., 1994). Dissolved oxygen (DO) levels have been shown to affect ajmalicine production in high-density cultures (Schlatmann et al., 1995). Remaining above a DO of 29% was found critical to supporting ajmalicine production. Above 43% DO the ajmalicine production was constant and independent of DO. Between a DO of 29% and 43% there was a strong correlation among the DO and the specific productivity of ajmalicine (Schlatmann et al., 1995). In recent work, Schlatmann et al. proposed a model that relates the ajmalicine specific productivity to the dissolved gaseous metabolites. In contrast to their earlier work, the specific ajmalicine productivity was a strong function of the dissolved carbon dioxide (Schlatmann et al., 1997).

2.3 Transgenic Experiments

Genetic engineering experiments are limited by the availability of cloned genes, and methods to transform C. roseus. Oncogenic transformation of C. roseus calli
culture showed that overexpression of TDC increased tryptamine accumulation, but found no increase in indole alkaloid production (Goddijn et al., 1995). This result may be attributed to limitations in the terpenoid pathways or due to the oncogenic nature of the transformation. Subsequent work established that overexpression of both TDC and SSS in cell suspension cultures resulted in a wide range of phenotypes (Canel et al., 1998). Consistently, high SSS activity was found to be necessary, although not sufficient, to sustain high rates of indole alkaloid accumulation. Surprisingly, high TDC activity was detrimental to the culture growth and not beneficial to enhanced alkaloid accumulation. In a cell line overexpressing only SSS, the cultures were found to be able to support a high level of indole alkaloid accumulation even with low TDC activity (Whitmer et al., 1998). The production of alkaloids could be enhanced only by feeding both loganin and tryptamine. The authors concluded that the utilization of tryptamine appeared to enhance flux through the indole alkaloid pathway (Whitmer et al., 1998).

In conclusion, great strides in the state of the knowledge about indole alkaloid biosynthesis are being made in the C. roseus system. However, there is still much work to be done. Important accomplishments will involve cloning of several key P-450 monooxygenases, such as G-10H and tabersonine 16-hydroxylase. Furthermore, progress on identification of the signal transduction process in response to elicitors will aid in developing production strategies for the C. roseus alkaloids.
Chapter 3: Effects of buffered media upon growth and alkaloid production of *Catharanthus roseus* hairy roots

3.1 Summary

The influence of buffered media upon the growth and alkaloid productivity of *Catharanthus roseus* hairy root culture was examined. As expected, the buffers minimized shifts in the pH of the media and had slightly negative affects upon growth. The growth of the hairy roots remained optimal in unbuffered media. The specific yield of lochnericine was significantly lower in response to the addition of buffers, while tabersonine was significantly higher.

3.2 Introduction

Plant cell and tissue cultures are unique when compared to mammalian or bacterial cultures in that the external pH of the medium drastically changes over the course of batch growth. In other fermentations, pH is typically maintained over a relatively narrow range through the use of a pH controller or the addition of buffers. Numerous studies have examined the effects of initial pH on growth and secondary metabolite production from plant cell and tissue cultures (Ho and Shanks, 1992; Zhang and Furusaki, 1997). Furthermore, only a few studies have examined the effects that buffers have upon plant
culture growth (Nesius and Fletcher, 1973; Banthorpe and Brown, 1990). However, in each of these studies the effects upon secondary metabolism were not studied. Since, the extracellular pH may also have a role in cell signaling, it potentially could affect the regulation of secondary metabolism. This paper will present evidence that media pH can be used as a controllable parameter in plant cell culture for the specific enhancement or reduction of secondary metabolites.

3.3 Materials and methods

3.3.1 Chemicals: The buffers 2-[N-Morpholino]ethanesulfonic acid (MES) and 2-[(2-Amino-2-oxoethyl)amino]ethanesulfonic acid (ACES) (Sigma Chemical, St. Louis, MO) have useful pH ranges of 6.1 - 7.5, and 5.5 - 6.7 respectively.

3.3.2 Culture Conditions The hairy root line LBE 6-1 was used for all experiments (Bhadra and Shanks, 1997). The media was a filter-sterilized solution of 30 g / L sucrose, half strength Gamborg’s B5 mineral salts and full strength Gamborg’s vitamins. The initial pH of the media was adjusted to 5.7. For cultures in which buffer was added, the pH was readjusted to 5.7 before inoculation. The medium containing ACES had 20 mM of buffer, and the MES medium had 50 mM buffer. Cultures were initiated every 3 weeks by placing 5 root tips (35-40 mm) into 50 ml of media. The cultures were shaken at 100 RPM, and kept at 26º C in the dark.

3.3.3 Growth measurements: Cultures were harvested in triplicate every other day between 14 and 26 days. The pH of the external media was measured by a pH probe and
pH indicator paper. The fresh weight of the cultures was measured after thorough blotting of excess media. Cultures were then immediately frozen at -70 °C, and dry weight was determined after lyophilization.

3.3.4 Alkaloid Analysis Dried roots were crushed to a fine powder with a mortar and pestle. Approximately 0.1 g were placed in a 50 mL centrifuge tube, and extracted with 45 mL of MeOH by placing in a sonicating bath for 5 hours. The supernatant was clarified by centrifugation (4000 RPM) of the cellular debris for 15 min. The crude MeOH extract was evaporated to a volume of approximately 4 mL, and then passed through a 0.22 μm, 13 mm filter, of which 10 μL were injected on the HPLC column. The mobile phase was a 32:32:36 mixture of MeOH: MeCN: 5 mM (NH₄)₂PO₄ buffer. An initial flow rate of 1 ml/min was maintained for 20 min, then linearly ramped for 10 min to 1.4 ml/min. The flow rate was returned to 1 ml/min over the next five minutes, where it was held for 5 minutes. Alkaloids were identified by photodiode array detection, and were quantified as previously reported (Shanks et al., 1998).

3.4 Results and discussion

3.4.1 Culture growth

The pH profile from the control and buffered cultures are shown in Figure 3-1. The trend of the pH in the control culture agreed with the previously reported results for the same hairy root line (Bhadra and Shanks, 1997). As anticipated, the pH of the buffered media
Figure 3-1. Effect of buffers upon extracellular pH of hairy root cultures. Data are mean and standard deviation from 3 cultures.
was maintained over a much narrower range. Between 14 and 26 days of the culture cycle, the pH of the ACES containing media varied between 5.6 and 5.8, while the MES medium pH increased with time from 5.7 to 6.3. As the MES buffer has a higher working range than the ACES buffer, the pH values were controlled as expected.

The increase in dry weight of the cultures was monitored between 14 and 26 days (Figure 3-2). Between 14 and 22 days there was no significant difference in growth between the control and buffered cultures. However, on day 26, the buffered cultures had significantly less biomass than the control culture (p < 0.05). Thus, the use of these buffers provided no beneficial effect for enhancement of biomass. As a further measure of growth, the ratio of fresh to dry weight (FW/DW) was examined over the course of the experiment (Figure 3-3). The MES culture maintained the lowest FW/DW ratio for the entire culture period. This may be related to the higher osmolarity of the media since MES was used at a 50 mM concentration while the ACES buffer was present at just 20 mM.

The effect of altered plant cell growth in buffered media has been observed in other species. For example, Hahn et al., found that unbuffered plant cell (maize) suspension cultures had superior growth compared to cultures in a citrate buffer (Hahn et al., 1997). In principle, this agrees with the results presented above. However, one study that used MES in cultures of Tanacetum, Mentha, and Anethum observed an increase in growth compared to an unbuffered medium (Banthorpe and Brown, 1990). Using an alternative strategy of adding NaOH and HCl to control the pH of the media,
Figure 3-2. The accumulation of dry weight (DW) for hairy root cultures in buffered and unbuffered media.
Figure 3-3. The ratio of fresh weight (FW) to dry weight (DW) during the growth cycle of buffered and control cultures.
Martin and Rose noticed that the unbuffered control culture attained the maximum dry weight earlier than the optimal pH controlled culture (Martin and Rose, 1976). The effect of maintaining a constant pH was found to strongly influence the utilization of ammonium, nitrate, and sucrose. Using buffers to control the pH possibly had similar effects in this study. The combination of these results suggests that pH is an important parameter for controlling growth of plant cell suspension cultures, but differences in plant species, media, and buffer type used don’t allow generalizations to be determined with respect to growth.

3.4.2 Alkaloid Production

Five alkaloids, serpentine, ajmalicine, tabersonine, lochnericine, and hörhammericine, were quantified between day 14 and day 26. The specific yields of ajmalicine and serpentine were not statistically different between the control and buffered cultures (data not shown). However, there were significant changes in the specific yields of tabersonine and lochnericine in the buffered cultures compared to the controls. Figure 3-4 shows that the specific yield of tabersonine in the control cultures was significantly less than the buffered cultures. However, there was no significant difference between the MES and ACES buffered cultures. Furthermore, the qualitative trend of alkaloid accumulation in the buffered cultured cultures is the opposite of the control cultures. The specific yield of tabersonine was increasing for the buffered cultures, while tabersonine was slightly decreasing between 14 and 26 days in the control.
Figure 3-4. The specific yield of tabersonine in control and buffered cultures.
Figure 3-5. The specific yield of lochnericine in buffered and control cultures.
The specific yield of lochnericine is shown in Figure 3-5. The level of lochnericine remains significantly higher in the control cultures than in either of the buffered cultures, which is the opposite of the result noted for tabersonine. However, in the buffered cultures, the qualitative trend of increasing lochnericine specific yield with time matches the trend of tabersonine specific yield. Again, there was no significant difference between the buffered cultures. Contrary to the results from lochnericine, the specific yield of hörhammericine, another alkaloid derived from tabersonine, did not differ between controls and buffered solutions (data not shown).

In this paper we have shown that non-metabolizable buffers can be used to effectively regulate the pH of the media. However, this is not necessarily optimal for growth of plant cell or tissue cultures. Furthermore, by controlling pH, the accumulation patterns and amounts of secondary metabolites can be controlled. For example, it may be advantageous to use such a strategy to not only maximize products of interest, but also to reduce undesirable products.
CHAPTER 4: EFFECTS OF LIGHT AND SUCROSE CONCENTRATION ON
THE BIOSYNTHESIS OF TABERSONINE AND DERIVATIVES.

Portions of this chapter may be found in Bhadra, R., Morgan, J.A. and Shanks, J.V.
"Transient studies of light-adapted cultures of hairy roots of Catharanthus roseus:

4.1 Introduction

Tabersonine is a key precursor in the biosynthesis of the valuable anti-cancer
alkaloids. The biosynthetic pathway for the formation of vindoline from tabersonine
has been shown to be highly regulated and is development specific, tissue-specific and
light dependent (De Luca and Cutler, 1987; De Luca et al., 1988). However, the
biosynthesis of tabersonine and its biochemical transformation into other alkaloids has
not been studied in detail. Tabersonine is also the most likely precursor of several
alkaloids (Kutney et al., 1980) - e.g., hörhammericine, lochnericine, vindolinine, epi-
vindolinine - detected in hairy root cultures (Toivonen et al., 1989) and cell cultures
(Kutney et al., 1980; Stöckigt and Soll, 1980) of C. roseus, which are not
intermediates in the vindoline biosynthetic pathways.
In previous work by our group, the transient levels of tabersonine were quantified, (Bhadra and Shanks, 1997) but related compounds had not been quantified for hairy roots. Furthermore, compounds surrounding the tabersonine branchpoint were investigated and were quantified from bioreactor cultures to give an indication of the amount of flux through this branchpoint (Vani, 1996). At the end of the bioreactor run, the combined yields of lochnericine and hörhammericine were an order of magnitude greater than tabersonine. The importance of this fact is that a significant portion of flux is proceeding through tabersonine to lochnericine and hörhammericine, which is undesirable.

This chapter will examine the transient accumulation patterns of tabersonine, and its derivatives, lochnericine and hörhammericine at two levels of initial sucrose concentrations as well as in light-adapted cultures. The lower level of sucrose was used in the case of light-adapted cultures. The pathway will be analyzed through transient multicomponent measurements during growth and stationary phases. Furthermore, the results of a search for intermediates in the pathway between tabersonine and vindoline are presented.

4.2 MATERIALS AND METHODS
4.2.1 Culture Conditions

Hairy roots of Catharanthus roseus were previously established in our laboratory and alkaloid yields were estimated (Bhadra et al., 1993). A fast growing clone with high alkaloid yield was selected (Bhadra et al., 1993). The current study was conducted using the LBE-6-1 clone. Root tips (0.1 - 0.2 g FW) from 4-5 week old cultures were aseptically inoculated into 250-ml Erlenmeyer flasks containing 50 ml of liquid Gamborg's B5/2 medium and 30 g/L sucrose at pH 5.7 ± 0.1. The flasks were placed on an orbital shaker (110 rpm) in the dark at 26°C.

The light-adapted cultures were grown in 2% sucrose with a photoperiod of 12 hr/day (fluorescent, cool white light, 4-7 W/m²). The light-adapted cultures were maintained stably for 8 subcultures. Dark grown controls were adapted to 2% sucrose and maintained stably for 15 subculture cycles.

4.2.2 Alkaloid Analysis

Alkaloid Extraction
Freeze dried hairy roots from shake flask were crushed and then extracted with methanol under reflux for 3 hours in a Soxlet apparatus. The extraction procedure was adapted from Morris et al. (Morris et al., 1985). The methanol extract was evaporated to approximately 4 mL, and fractionated as described earlier (Bhadra et al., 1993).

**Alkaloid Quantification**

HPLC analysis was conducted with a 58:42 mixture of MeOH:5mM (NH₄)₂HPO₄ adjusted to pH 7.3. A flow gradient of 1 ml/min for 23.5 mins, 1.6 ml/min for 40 minutes and 1 ml/min for 26.5 minutes was used for optimum separation. A Waters 996 Photodiode Array (Waters Associates, Milford, MA) was used for peak detection.

Ajmalicine and serpentine were quantified at 254 nm. Hörhammericine, lochnericine and tabersonine specific yields were determined based on peak areas at 329 nm which corresponds to the λ_max for the α-methyleneindoline chromophore. The specific yield of tabersonine was quantified by a calibration obtained using authentic tabersonine. The same calibration was used to estimate the specific yields of hörhammericine and lochnericine because the three alkaloids have an identical chromophore and the extinction coefficients for these alkaloids are similar at 329 nm (ε₃₂₇₅,Loch = 16,825,
\( \epsilon_{329, \text{Tab}} = 15,200 \) (Laboratories, 1960). Peak identification was based on comparison of retention times and UV spectra to authentic standards (ajmalicine, serpentine, catharanthine, tabersonine) or purified samples (hörhammericine and lochnericine). Deacetylvinodoline was synthesized by treatment of vindoline with sodium methoxide in methanol.

4.3 RESULTS

4.3.1 Alkaloid Identification

From comparisons of extracts for light-adapted and dark grown cultures against authentic standards, neither deacetylvinodoline or vindoline were detected in the hairy roots. A search for the first two intermediates, 16-hydroxytabersonine and 16-methoxytabersonine, between tabersonine and vindoline was made by comparison of the UV-vis spectra from standards. No compounds exhibiting matching spectra were found. This could be due to the absence of the specific monooxygenase demonstrated to catalyze the formation of 16-OH tabersonine (St-Pierre and De Luca, 1995), or low activity of this enzyme relative to the enzymes converting tabersonine to lochnericine and hörhammericine.
4.3.2 Transient Alkaloid Profiles

4.3.2.1 3% Sucrose, dark

Past results for hairy root cultures grown in 3% sucrose and Gamborg’s B5/2 media (Bhadra and Shanks, 1997) show that the specific yield of tabersonine peaks at day 20-25 and then rapidly decreases. The combined specific yields of hörhammericine and lochnericine range from 5-15 times the level of tabersonine (Fig. 4-1). Tabersonine levels decline with the onset of stationary phase, while lochnericine and hörhammericine continue to accumulate. Lochnericine specific yield reaches a maximum of 3.5 mg/g DW at 4 weeks then declines. The specific yield of hörhammericine continuously increases between 20 and 65 days.

The total yields of these three compounds are plotted against the biomass to test if they are growth associated. Indeed as shown in Figure 4-2, the total yields of lochnericine and hörhammericine are linearly associated with the increase in biomass. Tabersonine total yield reaches a maximum at 0.35 g DW of biomass, and then decreases. Until its maximum is reached, tabersonine accumulation is also associated with biomass development. This indicates that the synthesis of these alkaloids is
Figure 4-1. The transient alkaloid specific yields for hörhammericine, lochnericine, and tabersonine in 3% sucrose, dark grown cultures. The data represent the mean of at least two independent flasks.
Figure 4-2. The total alkaloid yields for hörhammericine, lochnericine, and tabersonine in 3% sucrose, dark grown cultures. The data represent the mean of at least two independent flasks.
linked to the growth of the roots. In contrast, serpentine, another alkaloid in *C. roseus* was found to be non-growth associated (Bhadra and Shanks, 1997).

4.3.2.2 2% sucrose, dark

In dark grown cultures with initially 2% sucrose media, the specific yields of tabersonine, lochnericine and hörhammericine were similar to those grown in the 3% sucrose media (Figure 4-3). The trend of tabersonine specific yield decreasing with time was present in both cultures. In minor contrast to the 3% sucrose grown culture, hörhammericine specific yield remained relatively constant throughout the culture period in the 2% sucrose media. One notable difference between the 2% and 3% sucrose dark grown cultures was the specific yield of lochnericine did not increase and then decrease (Figure 4-3). Like hörhammericine, it remained fairly constant between 20 and 68 days.

The plot of total alkaloid yield against biomass again demonstrates the growth associated nature of lochnericine and hörhammericine accumulation (Figure 4-4). Observed in isolation, it is unclear that tabersonine is growth associated. However, since it is the precursor of lochnericine and hörhammericine, this explains the decrease in total yield later in the culture. The total combined yields for all three alkaloids is
Figure 4-3. The transient alkaloid specific yields for hörhammericine, lochnericine, and tabersonine in 2% sucrose, dark grown cultures.
Figure 4-4. The total alkaloid yields for hörhammericine, lochnericine, and tabersonine in 2% sucrose, dark grown cultures. The data represent the mean of at least two independent flasks.
lower than in the 3% sucrose media. The lower total yield is due to lower final levels of biomass and slightly lower specific yields of lochnericine.

4.3.2.3 2% Sucrose, light-adapted

The adaptation of hairy roots to light was performed with the goal of inducing some of the enzymes between tabersonine and vindoline. However, neither deactethylvindoline, vindoline or the dimeric indole alkaloids were detected in light-adapted cultures. Furthermore, no compound exhibiting the same chromophore as the first two intermediates between tabersonine and vindoline was detected. This suggests that light is necessary, but not sufficient as developmental cues are also necessary for the induction of the enzymes leading to vindoline biosynthesis (De Luca et al., 1988).

The effect of light upon the tabersonine branchpoint was dramatic. The specific yield of both tabersonine and lochnericine were significantly lower compared to the 2% sucrose dark cultured hairy roots (Figure 4-5). The specific yield of hörhammericine was significantly lower between 20 and 40 days. All three alkaloids displayed a period of increasing specific yield between 18 and 27 days. Despite the increased growth of the light-adapted roots, the combined total yield for these alkaloids was significantly less (Figure 4-6).
Figure 4-5. The transient alkaloid specific yields for hörhammericine, lochnericine, and tabersonine in 2% sucrose, light-adapted cultures. The data represent the mean of at least two independent flasks.
Figure 4-6. The total alkaloid yields for hörhammericine, lochnericine, and tabersonine in 2% sucrose, light-adapted cultures. The data represent the mean of at least two independent flasks.
4.4 Conclusions

The adaptation of the hairy roots to light appeared to inhibit flux to the tabersonine branchpoint. The mechanism for this is unknown at this time. The exact pathway between tabersonine, lochnericine and hörhammericine is also unknown. To determine the type of enzymes involved, further work characterizing this pathway is necessary. The next chapter examines this pathway under the presence of oxygenase inhibitors.

The diversion of flux away from vindoline is an ideal target for inhibition by genetic means to prevent the ‘drain’ of tabersonine. Thus, inhibiting the enzyme(s) responsible for the conversion of tabersonine into hörhammericine and lochnericine may be a feasible approach to increase the flux of tabersonine to the vindoline biosynthetic pathway. As the enzymes for five of the six steps between tabersonine and vindoline have being characterized a future possibility is the existence of transgenic cell or tissue cultures capable of producing vindoline. Therefore, this and future studies of tabersonine and its conversion into other alkaloids are relevant towards that goal.
CHAPTER 5: Inhibitor Studies of Tabersonine Metabolism in C. roseus Hairy Roots

This chapter has been accepted and will appear in Phytochemistry, 1999.

5.1 ABSTRACT

The conversion of tabersonine to lochnericine and hörhammericicine was investigated in C. roseus hairy roots. The accumulation of lochnericine and hörhammericicine, like tabersonine, was associated with growth. Through the use of oxygenase inhibitors, 1-aminobenzotriazole (ABT), clotrimazole (CLOT), and 2,5-pyridinedicarboxylic acid (PCA), details of the metabolic pathway around tabersonine in hairy roots of C. roseus were elucidated. ABT specifically inhibited the formation of hörhammericicine, while CLOT inhibited the accumulation of lochnericine. Using jasmonic acid in combination with the inhibitors suggests an inducible P-450 enzyme responsible for the formation of hörhammericicine. The inhibitor study also revealed that both lochnericine and hörhammericicine are ‘turned over’ in hairy root cultures.

Key Word Index- Catharanthus roseus L. (G. Don), Apocynaceae, rosy periwinkle, hairy roots, P-450 inhibitors, tabersonine, hörhammericicine, lochnericine
5.2 INTRODUCTION

Plant cell and tissue cultures of *Catharanthus roseus* have been studied extensively for the production of the anti-cancer compounds, vincristine and vinblastine (see Figure 5-1) (Meijer et al., 1993). These dimeric compounds are derived from the coupling of the monomers vindoline and catharanthine. Catharanthine is routinely detected in cell and tissue cultures (Kurz et al., 1981; Drapeau et al., 1987; Vazquez-Flota et al., 1994), but the accumulation of stable and significant levels of vindoline has been elusive. Shoot (Constabel et al., 1982; Krueger et al., 1982; Endo et al., 1987) and multiple shoot cultures (Hirata et al., 1987) are capable of producing vindoline and catharanthine, and vinblastine has been detected in multiple shoot cultures (Miura et al., 1988). However, the growth rate of shoot cultures compared to the faster growing hairy root and cell suspension cultures prohibits their use in a bioprocess. Recently, stable production of vindoline was observed in cell cultures transformed with virulent *Agrobacterium* strains; although in relatively low amounts compared to the field grown plant (O'Keefe et al., 1997).

In non-transformed cell suspension cultures and hairy roots, the lack of expression of the enzymes between tabersonine and vindoline has generally been accepted to be the reason for the absence of vindoline (De Luca and Cutler, 1987). Potentially, a metabolically engineered system (Verpoorte et al., 1996; Shanks et al., 1998) in which the enzymes between tabersonine and vindoline are expressed may
Figure 5-1. Established and proposed biochemical conversions around tabersonine.

The large solid arrow represents the final five steps in vindoline biosynthesis. The leftward solid arrow represents the P-450 dependent monoxygenase characterized in whole C. roseus plants (St-Pierre and De Luca, 1995). The solid rightward arrow represents experimental evidence from Scott et al. (Scott et al., 1973). The single slashed arrows are hypothesized pathways by Kutney et al. (Kutney et al., 1980). The double arrow represents another possible conversion to hörhammericine proposed in this paper.
provide the solution to overcome this obstacle. In addition, optimal vindoline
production would require minimizing flux that is diverted away from the enzymes of
vindoline biosynthesis. Consequently, understanding of the flux through the pathways
surrounding tabersonine is important in a metabolic engineering strategy. In cell and
tissue cultures of \textit{C. roseus}, there have been numerous reports of compounds, other
than vindoline, which are likely derived from tabersonine (Scott et al., 1973; Kurz et
al., 1980; Kutney et al., 1980; Stöckigt and Soll, 1980). Previous feeding studies of
tabersonine to \textit{C. roseus} cell suspension cultures resulted in the accumulation of
lochnericine, with further conversion to lochnerininine (Furuya et al., 1992). Similarly,
in tabersonine feeding to seedlings, the major products detected were lochnericine and
vindoline (Scott et al., 1973). In the hairy root line LBE 6-1, lochnericine and
hörhammericine have been quantified in high amounts relative to tabersonine (Shanks et
al., 1998). The transient accumulation of lochnericine and hörhammericine in hairy
root cultures was previously examined from 18 to 70 days (Shanks et al., 1998).

The exact pathway and enzymes that convert tabersonine to lochnericine and
hörhammericine are presently unknown. The formation of 11-hydroxytabersonine is
the first step in vindoline biosynthesis from tabersonine; the enzyme responsible for
this conversion has been characterized as a cytochrome P-450 monooxygenase (St-
Pierre and De Luca, 1995). Similarly, lochnericine and hörhammericine also differ from
tabersonine by the incorporation of oxygen as shown in Figure 5-1. Therefore, to
investigate the epoxidation and hydroxylation of tabersonine, a dioxygenase inhibitor,
2,5-pyridinedicarboxylic acid (PCA) (Dowell, 1992 107) and two P-450 dependent monooxygenase inhibitors, 1-aminobenzotriazole (ABT) (Reichhart, 1982) and clotrimazole (CLOT) were selected. Knowledge gained from inhibitor studies about the enzyme types involved in the reactions leading to lochnericine and hörhammericine may provide clues on how to block these reactions. Jasmonic acid (JA) is known to increase flux to the alkaloids in C. roseus seedlings (Aerts et al., 1994; Aerts et al., 1996) and hairy roots (Shanks et al., 1998). Thus, JA was used in combination with these inhibitors to examine the tabersonine branchpoint under conditions of enhanced flux and restricted ability to oxygenate tabersonine.

5.3 RESULTS AND DISCUSSION

5.3.1 Culture growth and transient alkaloid profiles

Growth of the culture, as determined by increase in dry weight (DW) was remarkably linear \((r^2 = 0.99)\) over the period between 14 to 26 days (data not shown). Transient profiles of the specific yields of tabersonine, lochnericine, and hörhammericine are shown in Figure 5-2. Tabersonine specific yields which remain fairly constant, do decrease at 26 days. Hörhammericine specific yield mirrors that of tabersonine, with an increase at 26 days. Lochnericine accumulates to higher levels
Figure 5-2. Specific yields of (a) tabersonine (▲), Hörhammericine (○), and (b) lochnericine (■) from hairy root cultures. Culture conditions: Gamborg’s B5/2 media with 3% sucrose, 26°C, shaken at 100 RPM in the dark. Symbols represent the mean of at least two data points, and error bars represent standard deviation.
Figure 5-3. Total yields of (a) tabersonine (▲), horhammericine (⊙), and (b) lochnericine (■) from hairy root cultures. Culture conditions: Gamborg’s B5/2 media with 3% sucrose, 26°C, shaken at 100 RPM in the dark. Symbols represent the mean of at least two data points, and error bars represent standard deviation.
than tabersonine or hörhammericine for the entire period. Both lochnericine and hörhammericine total yields increased during the growth period of the roots (Figure 5-3). Specifically, between 14 and 22 days the accumulation of all three alkaloids were linear.

The high specific yields of lochnericine and hörhammericine reported here are apparently not unique to hairy roots of *C. roseus*. Tabersonine, lochnericine, and hörhammericine were reported as the major alkaloids in both normal and hairy root cultures of *Catharanthus trichophyllus* (Davioud et al., 1989). Tabersonine feeding to cell suspension cultures resulted in its transformation to lochnericine as the major product (Furuya et al., 1992). Thus the diversion of flux at tabersonine away from the vindoline pathway commonly occurs in plant cell and tissue cultures, and may represent an impediment to the formation of maximal levels of vindoline in a metabolically engineered system.

### 5.3.2 Oxygenase Inhibitors

Based upon the results of the transient data, the inhibitor experiments were conducted over the period of 14 to 21 days, which was a period of steady growth and alkaloid accumulation. This study was analyzed in terms of the effect of the inhibitor on specific yields of alkaloids, to reveal information about the enzyme types involved
in the oxygenation of tabersonine. Further analysis of total alkaloid yield was used to examine turnover of lochnericine and hörhammericine.

As shown in Table 5-1, the addition of the P-450 dependent monooxygenase inhibitor, ABT, severely limited the formation of hörhammericine, increased the specific yield of tabersonine, and had no apparent effect upon the accumulation of lochnericine. In contrast to ABT, the addition of the dioxygenase inhibitor, PCA, had no significant effect upon the accumulation on any of these alkaloids, which indicates that an α-ketoglutarate dependent dioxygenase may not be involved in the biosynthesis of lochnericine or hörhammericine. The lack of an effect could also be attributed to the inhibitor not reaching the compartment where the enzyme is present.

After observing that using PCA produced no significant effects, experiments were repeated using ABT and CLOT, another P-450 monooxygenase inhibitor. Table 5-2 shows the specific alkaloid yields before and after a 7 day treatment of a culture at 14 days with 1.25 μmol ABT, and 2.50 μmol CLOT. Significantly less hörhammericine was measured at 21 days in the ABT treated culture, again verifying that its synthesis is inhibited. In contrast to the effects of ABT, CLOT significantly inhibited the accumulation of lochnericine and hörhammericine. The combination of these results suggests that two different P-450 monooxygenase enzymes are involved in the formation of lochnericine and hörhammericine. Interestingly, tabersonine did not accumulate in the CLOT treated cultures as much as the ABT treatment. Perhaps this is partly due to the reduction in growth of the roots. However, as the specific yield of
lochnericine was significantly decreased, it appears that CLOT specifically inhibits the formation of lochnericine.

**Table 5-1.** Effect of addition of ABT and PCA upon specific alkaloid yields. ABT (0.5 μmol) and PCA (0.5 μmol) were added at day 10, and all cultures, including the control, were harvested at day 21. The values reported are the mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Control</th>
<th>Inhibitor ABT</th>
<th>Inhibitor PCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabersonine</td>
<td>0.95±0.16</td>
<td>2.89*±0.11</td>
<td>0.93±0.12</td>
</tr>
<tr>
<td>Lochnericine</td>
<td>4.33±0.19</td>
<td>4.39±0.24</td>
<td>4.26±0.52</td>
</tr>
<tr>
<td>Hörhammericine</td>
<td>1.27±0.07</td>
<td>0.28*±0.02</td>
<td>1.24±0.06</td>
</tr>
</tbody>
</table>

\* denotes significant difference at p < 0.01
Table 5-2. Effect of addition of ABT and CLOT upon specific alkaloid yields. 1.25 μmol ABT and 2.50 μmol CLOT were added at day 14. The control cultures and cultures with inhibitor added were harvested at day 21. The values reported are the mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Control</th>
<th>Inhibitor ABT</th>
<th>Inhibitor CLOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabersonine</td>
<td>1.21±0.04</td>
<td>2.49**±0.21</td>
<td>1.41*±0.07</td>
</tr>
<tr>
<td>Lochnericine</td>
<td>2.35±0.17</td>
<td>2.41±0.16</td>
<td>0.49**±0.07</td>
</tr>
<tr>
<td>Hörrhammericine</td>
<td>0.49±0.02</td>
<td>0.15**±0.01</td>
<td>0.25**±0.03</td>
</tr>
</tbody>
</table>

* denotes significant difference at p < 0.05; ** denotes significant difference at p < 0.01

Each of these monooxygenase inhibitors was found to be detrimental to the growth of the root cultures at the levels used. Other P-450 inhibitors were found to have similar affects on cultured root growth (Sugimoto et al., 1997). The lower total yields of tabersonine for the cultures treated with CLOT and the lower yields of lochnericine for the cultures treated with ABT at 21 days are apparently due to root
growth inhibition (data not shown) as the specific yields were relatively unaffected (Table 5-2).

Inhibitor studies also have potential to readily observe the turnover of compounds for which formation is inhibited. Since the rate of accumulation is the rate of formation of a compound minus the rate of transformation and catabolism, transformation is more readily observable if the rate of synthesis can be reduced. Figure 5-4 shows total yield of lochnericine from the same experiment reported in Table 5-2, but also includes the control at day 14. Total yields are reported, which allows comparisons of total alkaloid levels before and after the addition of inhibitor. The total yield of lochnericine accumulated at 14 days was significantly higher (p< 0.01) compared to the total yield present in the inhibited culture at 21 days, which indicates turnover of this compound. This turnover could either be in the form of transformation to hörhammericine and other products or catabolism. However, the total yield of hörhammericine did not significantly increase in cultures treated with either ABT or CLOT (data not shown). Furthermore, the presence of lochnerinine and hörhammerinine were not observed. Hörhammericine was also observed to be significantly transformed to other products or degraded during the period between 21 to 24 days in a culture treated with 5.0 μmol ABT at 21 days (p< .01) (Figure 5-5).
Figure 5-4. The total yields of lochnericine for 2 week and 3 week control cultures, and for cultures treated with 2.50 μmol of CLOT or 1.25 μmol of ABT. * indicates that the total yield of lochnericine was significantly lower (p < 0.01) at 21 days in the CLOT treated culture, than the total yield at 14 days.
Figure 5-5. The total yields of hörhammericine for 21 day and 24 day control cultures, and for cultures treated with 5.0 μmol of ABT or 5.0 μmol of PCA. ** indicates that the total yield of hörhammericine was significantly lower (p < 0.01) at 24 days in the ABT treated culture, than the total yield present at 21 days.
Thus the use of *in vivo* inhibitor studies complements other tools to investigate the important phenomenon of alkaloid turnover (Verpoorte et al., 1994).

### 5.3.3 Elicitation and Inhibition

Jasmonic acid (JA) is known to be a signal transducer which when applied results in increased accumulation of alkaloids in *C. roseus* (Aerts et al., 1994; Aerts et al., 1996). Specifically, in hairy roots, previous work has shown that the tabersonine branchpoint is highly responsive to treatment with JA (Shanks et al., 1998). Interestingly, JA application (0.25 mg/ flask at 21 days) resulted in a significant increase in hörhammericine specific yield and a significant decrease in tabersonine specific yield after 100 hours. In the experiments reported in this paper, JA was added at 14 days at the same time as ABT and CLOT. Similar to the previous study, hörhammericine specific yield was dramatically increased, and tabersonine was significantly decreased after a 1 week treatment of JA (p<0.01) (Table 5-3).

Additionally, the accumulation of lochnericine was significantly decreased as a result of treatment with only JA (p <0.01). The combination of JA and ABT had a similar outcome as the ABT treatments (Tables 5-1,5-2, and 5-3). There was a significant reduction in hörhammericine specific yield, with significant increases in the specific yield of tabersonine (p <0.01). In contrast to the previous ABT only treatments, a significant increase in lochnericine was observed compared to controls (p <0.01).
Table 5-3. Effect of the combination of elicitation on hairy root cultures by jasmonic acid (JA), and addition of either ABT or CLOT upon specific alkaloid yields. At 2 weeks, 0.5 mg of JA was added to each treatment flask. At the same time 0.50 μmol ABT and 0.50 μmol CLOT were added to separate duplicate flasks, and the cultures were harvested at 21 days. Control cultures were harvested in triplicate. The values reported are the mean ± standard deviation.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>Control</th>
<th>JA</th>
<th>JA and ABT</th>
<th>JA and CLOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabersonine</td>
<td>0.47± 0.05</td>
<td>0.22±0.01</td>
<td>2.24±0.39</td>
<td>0.29±0.05</td>
</tr>
<tr>
<td>Lochnericine</td>
<td>3.28± 0.04</td>
<td>1.88±0.11</td>
<td>6.99±0.56</td>
<td>2.14±0.13</td>
</tr>
<tr>
<td>Hörhammericine</td>
<td>0.48± 0.05</td>
<td>5.31±0.10</td>
<td>0.71*±0.21</td>
<td>3.64±0.18</td>
</tr>
</tbody>
</table>

* no significant difference between treatment and control at p < 0.05
Notably the combination of CLOT and JA was unable to completely inhibit the increase in hörhammericine specific yield. Furthermore, the specific yield of tabersonine decreased, similar to the JA only treatment, rather than increase as expected for the CLOT only treatment. This result raises the possibility that in the elicited condition an inducible P-450 enzyme in the pathway to hörhammericine exists. Of particular relevance is a recent study that reports the cloning of a methyl jasmonate inducible cytochrome P-450 dependent monooxygenase involved in benzylisoquinoline alkaloid biosynthesis (Pauli and Kutchan, 1998).

The use of oxygenase inhibitors proved useful to study the tabersonine branchpoint. Similar studies have examined the biosynthetic pathways of taxol (Srinivasan et al., 1996), brassinolides (Winter et al., 1997) and benzylisoquinoline alkaloids (Sugimoto et al., 1997). The pathway from tabersonine to lochnericine and hörhammericine is still not conclusively defined. From the results, ABT apparently strongly inhibits the formation of hörhammericine, but not the formation of lochnericine. Since ABT is a P-450 monooxygenase inhibitor, it likely prevents the hydroxylation of tabersonine. Furthermore, as only the specific yield of tabersonine was increased it suggests that hörhammericine may not absolutely require lochnericine as an intermediate. If hörhammericine was formed from lochnericine, higher levels of lochnericine would have been expected. The results from the inhibition with CLOT are ambiguous with regard to the pathway. The suppression of both lochnericine and hörhammericine levels isn’t indicative of either a route to hörhammericine through
lochnericine or via a 19-hydroxytabersonine intermediate. Furthermore, the increase in hörhammericine yield in cultures treated with both JA and CLOT suggest the existence of a pathway inducible by jasmonic acid for the formation of hörhammericine. Therefore, the possibility of hörhammericine formation from either lochnericine or 19-hydroxytabersonine (Figure 5-1) can not be ruled out.

The inhibitor ABT had been shown to be specific in reducing the activity of the P-450 type enzyme, cinnamic acid 4-hydroxylase (C4H) (Reichhart, 1982). As ABT is also an effective inhibitor of the formation of hörhammericine, C4H and the enzyme catalyzing this hydroxylation of tabersonine may have similarity. Interestingly, geraniol 10-hydroxylase (G10H), a cytochrome P-450 involved in the formation of the indole alkaloids of C. roseus was apparently not greatly inhibited by the ABT or CLOT treatment. Evidence for this comes from the combined specific yield of tabersonine, lochnericine, and hörhammericine was not reduced in the period from 10 to 21 days for the ABT treated cultures (Table 5-1). Furthermore the combined specific yields of ajmalicine and serpentine, two alkaloids derived from a pathway where G10H is the only known P-450 type enzyme involved, were suppressed only 30% by ABT treatment, and 16% by CLOT (data not shown). This may be due to the subcellular localization of G10H or the specificity of the inhibitor for differing monooxygenases.
In conclusion, the diversion of flux at the tabersonine branchpoint may be an impediment to the production of vindoline in transgenic cell or tissue cultures of *C. roseus*. The realization of significant vindoline production from cell or tissue cultures obviously requires the expression of the enzymes involved in vindoline biosynthesis. Additionally, it may be desirable to block the enzymes leading to lochnericine and perhaps hörhammericine. Whether or not the overexpression of the enzyme for the first step in vindoline biosynthesis from tabersonine would successfully compete for tabersonine is a critical future test. Further enzymological work is also required to clearly define the pathways leading to lochnericine and hörhammericine.

5.4 EXPERIMENTAL

5.4.1 Culture Conditions  The hairy root line LBE 6-1 was used for all experiments (Bhadra and Shanks, 1997). The maintenance media consisted of a filter-sterilized 3% (w/v) sucrose solution, Gamborg’s B5/2 mineral salts and vitamins. The initial pH of the solution was adjusted to 5.7. Cultures were initiated every 21 days by placing 5 root tips (35-40 mm) in 250 ml flasks containing 50 ml of media, which were shaken at 100 RPM, and kept at 26° in the dark.

5.4.2 Alkaloid Analysis  The extraction and sample preparation procedures were simplified from those previously reported (Bhadra and Shanks, 1997). The crude
MeOH extract was rotary evaporated to a volume of approximately 4 ml, and then passed through a 0.22 μm, 13 mm filter, of which 10 μl were injected on the HPLC column. The HPLC method was modified slightly to reduce total run time. The mobile phase consisted of a 32:32:36 mixture of MeOH: MeCN: 5mM (NH₄)₂PO₄ buffer. An initial flow rate of 1 ml/ min was maintained for 20 min, and linearly ramped for 10 min to 1.4 ml/min. The flow rate was returned to 1 ml/min over the next five minutes, where it was held for 5 minutes. Lochnericine and hörhammericine were identified by comparing their UV-vis, proton NMR spectra and MS fragmentation patterns against literature values (Kohl et al., 1981). As lochnericine and hörhammericine have the same chromophore as tabersonine, they were quantified at 329 nm using the tabersonine calibration curve. The tabersonine standard was a gift from Dr. Hamada (Okayama University, Japan).

5.4.3 Oxygenase Inhibitor Study Culture conditions remained the same as described earlier. At 10 days in the culture cycle, filter sterilized inhibitors (ABT and PCA) were added to make a 10 μM solution. These cultures were harvested at 21 days. To independent cultures, 25 μM of ABT and 50 μM CLOT were added at 14 days, and these cultures were harvested at 21 days. ABT and CLOT were dissolved in a minimal amount of EtOH. All treatments were performed in triplicate, and an equal volume of filter sterilized water or EtOH was added to controls.
5.4.4 Statistical Analysis  Differences between treated and control samples were analyzed by a students t-test using Microsoft Excel v.5.0 (Redmond, WA).

5.5 ACKNOWLEDGMENTS

This work was supported in part by the Robert A. Welch Foundation (C 1197), by National Science Foundation (NSF) Young Investigator Award to J. V. S. (BCS 9257938), and by NSF grant BES-9411928. J.M. was partially supported by a National Institutes of Health Training in Biotechnology Grant No. T32-GM08362. Tabersonine was a generous gift from Dr. H. Hamada, Okayama University, Japan. We are also grateful to S. Rijhwani and R. Bhadra for their valuable comments on the manuscript.
Chapter 6: Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures.

6.1 ABSTRACT

Precursors from the terpenoid and tryptophan branches were fed to determine which of the two branches limits metabolic flux to indole alkaloids. Feeding of either geraniol, 10-hydroxygeraniol, or loganin at 21 days each resulted in significant increases in the accumulation of tabersonine. The addition of tryptophan or tryptamine had no effect. Thus, during the early stationary phase of growth the terpenoid pathway appears to be rate-limiting. In contrast, the feeding of tryptophan during the late growth phase of the culture cycle resulted in significant increases in flux to the indole alkaloids. However, the increase in alkaloids appears due to the conversion of tryptophan to the auxin, indole acetic acid (IAA). Feeding low levels of IAA was able to reproduce the increase in alkaloid accumulation without affecting root growth. Providing further evidence for this hypothesis, the addition of tryptamine did not enhance alkaloid accumulation. Conversely, feeding higher levels of auxin or tryptophan resulted in increased branching and thickening of the hairy roots and a dramatic reduction in metabolic flux to the alkaloids. However, feeding terpenoid precursors had no effect. Therefore, neither pathway tested revealed to be rate-limiting during the late growth phase. Finally, the combination of elicitation with
jasmonic acid and addition of either loganin or tryptamine did not enhance the accumulation of indole alkaloids over elicitation alone.

6.2 INTRODUCTION

*Catharanthus roseus* (L.) G. Don, the Madagascar periwinkle, synthesizes numerous terpenoid indole alkaloids. Notably, the dimeric alkaloids vincristine, vinblastine and synthetic derivatives of these natural products are powerful anti-cancer agents. The amounts of these alkaloids produced by the plant are extremely low, hence leading investigators to examine plant cell and tissue cultures as alternative means of production. Ajmalicine and serpentine are also medicinally valuable *C. roseus* alkaloids that have use as anti-hypertension agents. Plant cell and tissue cultures of *C. roseus* have been extensively studied for the intended purpose to enhance production of these valuable indole alkaloids (Verpoorte et al., 1993).

Tissue differentiation plays a significant role in the types of alkaloids produced. For example, the synthesis of vindoline is restricted to the leaves of the plant (De Luca et al., 1988), while ajmalicine and serpentine are the major alkaloids found in roots of the plant as well as cell suspension cultures (van der Heijden et al., 1989). However, in hairy roots of *Catharanthus* species, tabersonine, lochnericine, and hörhammericine are major products in addition to ajmalicine and serpentine.
(Davioud et al., 1989; Shanks et al., 1998). Since the biosynthesis and accumulation of specific metabolites are influenced by tissue type, rate limitations in hairy roots may be different than cell suspension cultures.

All of the terpenoid indole alkaloids are derived from a central intermediate, strictosidine (Figure 6-1). The indole portion of the molecule is derived from tryptamine, which is formed by the decarboxylation of tryptophan. The terpenoid portion of strictosidine, secologanin, is known to be derived from geranyl pyrophosphate, which is subsequently converted to geraniol, 10-hydroxygeraniol, and loganin by multiple steps (Meijer et al., 1993).

Based upon precursor feeding and enzyme activity studies, a current hypothesis is that the terpenoid pathway is rate-limiting in cell suspension cultures (Schiel et al., 1987; Moreno et al., 1993). However, previous feeding studies have reported contradictory results. For example, tryptophan feeding has resulted in increased growth and specific yields of serpentine in one cell line (Zenk et al., 1977), reduced alkaloid content in different study (Knobloch and Berlin, 1980), and no effect in another study (Kargi and Ganapathi, 1991). In other examples, addition of tryptophan to cells increased tryptamine levels, but had no effect on indole alkaloid levels (Merillon et al., 1986; Facchini and DiCosmo, 1991). Similarly, addition of tryptamine stimulated alkaloid production in one study (Krueger and Carew, 1978), while inhibiting alkaloid accumulation in another (Döller et al., 1976).
Figure 6-1. Proposed biosynthetic pathway leading to the indole alkaloids in *C. roseus* hairy roots. Dashed lines represent multiple or uncharacterized reactions in the pathway. Compounds in bold were fed as precursors in this study. Italicized compounds were quantified from the extracts of hairy roots.
The addition of loganin or secologanin to cell suspension cultures increased indole alkaloid levels (Merillon et al., 1989; Moreno et al., 1993), which suggests the monoterpenoid pathway may be rate limiting. Feeding of geraniol was found to have no effect on alkaloid production (Krueger and Carew, 1978). Even earlier in the pathway, the feeding of mevalonic acid had no effect on indole alkaloid production levels, which indicates the rate limiting enzyme may lie between mevalonic acid and loganin (Krueger and Carew, 1978; Moreno et al., 1993). However, recent evidence indicates that mevalonic acid is not the only precursor of the strictosidine. Instead, a novel triose phosphate/pyruvate pathway is implicated as the major provider of carbon for the monoterpenoid pathway leading to the indole alkaloids (Contin et al., 1998).

The goal of this study was to determine rate-limiting sections of the pathways leading to the formation of indole alkaloids by precursor feeding. The unique aspects of this study are the system used, as well as the wide range of precursors fed and alkaloids quantified. To our knowledge, this is the first report of a precursor feeding study for root or hairy root cultures of *C. roseus*.

6.3 MATERIALS AND METHODS

6.3.1 *Chemicals*: The precursors were of highest available purity. Geraniol (Fluka, Ronkonkoma, NY) and 10-hydroxygeraniol (Aldrich, Milwaukee, WI) were added
directly to the culture media as liquids. Mevalonic acid was prepared by mixing equal molar solution of NaHCO3 and mevalonic acid lactone (Sigma Chemical, St. Louis, MO). Loganin (Apin Chemical Ltd., Oxon, UK), tryptamine (Fluka), pyruvic acid, and L-tryptophan (Sigma) were dissolved in filter sterilized water before adding to the cultures. The hormones 1-napthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) were added from stock solutions (Sigma). When necessary a few drops of HCl were added to help dissolve the precursor. Equal amounts of distilled water, with and without HCl were added to control cultures.

6.3.2 Methods: Earlier work characterized the growth of the hairy roots into exponential and stationary phases when modeled by a simple exponential growth model (Bhadra and Shanks, 1997). According to the model, the stationary phase begins at 21 days for this culture. The timing of the precursor addition was designed to explore alkaloid productivity in late growth phase (17-21 days) as well as early stationary phase (21-24 days). Precursors were added at 17 and 21 days with the cultures harvested at 21 and 24 days respectively. In the elicitation experiments, jasmonic acid (JA) (50 mg/L) was added either on day 20 or 22 with tryptamine (20 mg/L) and loganin (40 mg/L) added on day 21. Cultures were harvested on day 24.

6.3.3 Culture Conditions The hairy root line LBE 6-1 was used for all experiments (Bhadra et al., 1993). The media was a filter-sterilized solution of 30 g/L sucrose, half
strength Gamborg's B5 mineral salts and full strength Gamborg's vitamins. The initial pH of the media was adjusted to 5.7. Cultures were initiated every 3 weeks by placing 5 root tips (35-40 mm) into 50 mL of media. The cultures were shaken at 100 RPM, and incubated at 26° in the dark.

6.3.4 Alkaloid Extraction: The fresh weight of the cultures was measured after thorough blotting of excess media. Cultures were then immediately frozen at -70°, and dry weight was measured after lyophilization. Dried roots were finely ground, and approximately 0.15 g were extracted with 45 mL of MeOH in a sonication bath for 5 hours. The supernatant was clarified by centrifugation of the cellular debris for 15 min.

6.3.5 Alkaloid Analysis The crude MeOH extract was evaporated to a volume of approximately 4 mL, and then passed through a 0.22 μm, 13 mm filter, of which 10 μl were injected on a Bondclone 10μ C18 HPLC column (300 mm x 3.9 mm) (Phenomenex, Inc., Torrance, CA). The mobile phase consisted of a 32:32:36 mixture of MeOH: MeCN: 5mM (NH₄)₂PO₄ buffer. An initial flow rate of 1 mL/min was maintained for 20 min, and linearly ramped for 10 min to 1.4 mL/min. The flow rate was returned to 1 mL/min over the next five minutes, where it was held for 5 minutes. Alkaloids were identified by photodiode array detection, and were quantified as
previously reported (Shanks et al., 1998). The alkaloids quantified in this study were ajmalicine, serpentine, tabersonine, lochnericine, and hörhammericine.

6.3.6 Statistical Analysis The effect of a treatment was analyzed by using a students t-test from to compare means (Microsoft Excel 4.0, Redmond, WA).

6.4 RESULTS

6.4.1 Precursor dosage determination

The first issue examined was the proper amount of precursor to add to the cultures. Feeding studies with different dosages of geraniol revealed that at and above 160 mg/L there were toxic effects on the growth of the cells. Figure 6-2 shows that lower dosages of geraniol resulted in the similar effects upon the accumulation of tabersonine. To feed a constant amount of precursor per unit biomass, 8 mg/L of geraniol was the dosage selected for feeding at 17 days, and 16 mg/L at 21 days. For the other precursors, equal molar (52 µM at 17 days or 104 µM at 21 days) amounts were fed. At these levels, no precursor had a significant effect on biomass
**Figure 6-2.** The effect of feeding various levels of the precursor geraniol on the specific yield of tabersonine. Geraniol was fed at 21 days and the cultures were harvested after 72 hours. There was no statistical difference between the treatments. The difference between feeding geraniol and the control was statistically significant (p < 0.05) for the 8 mg/L and 80 mg/L treatments. The data represent the mean and standard deviations of triplicate flasks.
accumulation. Therefore, specific yields of alkaloid accumulation will be used for comparison of treated and control cultures.

6.4.2 Early stationary phase feeding

The timing of feeding had significant effects on the accumulation of alkaloids. Results from the precursor feeding during early stationary phase (21 days) will be presented first, followed by the results during the late growth phase feeding (17 days). Neither tryptophan nor tryptamine had a positive effect on alkaloid accumulation. The only statistically significant effect (p<0.05) due to tryptophan feeding was a 29% reduction in the accumulation of tabersonine. Feeding the terpenoid precursors also significantly affected tabersonine. In contrast to tryptophan, adding geraniol, 10-hydroxygeraniol, and loganin all significantly increased the accumulation of tabersonine by more than 50% above the control (Figure 6-3). There was no statistically significant difference between the three treatments. Logarin was found to be completely removed from the media by 48 hours, and no intracellular pools of logarin were detected. Surprisingly, the specific yield of hörhammericine was reduced by 24% when either geraniol or logarin was fed (p<0.05). However, the increase in tabersonine total yield was larger than the decrease in hörhammericine (data not shown). The addition of mevalonic acid had no effect on alkaloid accumulation, which is similar to previous reports (Moreno et al., 1993, Krueger, 1978 #972).
Figure 6-3. Feeding the terpenoid precursors, geraniol, 10-hydroxygeraniol and loganin significantly (p<0.05) increased the specific yield of tabersonine in hairy root cultures (n=3). The precursors were added at 21 days and the cultures were harvested at 24 days.
The evidence for rate limitations occurring in the terpenoid pathway is rather limited, as only the specific yield of only one alkaloid was found to be increased in response to feeding, and in some cases the specific yield of hörhammericine was reduced (Table I). To support these findings, further experiments were performed. Linalool, an analog of geraniol, known not to be incorporated into the indole alkaloids was fed at the same level as geraniol. The addition of linalool had no effect on alkaloid accumulation. Therefore, the increase in tabersonine from geraniol feeding does not appear to be due to non-specific elicitation. The question why only tabersonine accumulated was investigated by feeding tabersonine (40 mg/L) at 21 days and harvesting cultures at 24 days. Tabersonine was completely uptaken during this period, but only a small amount was transformed to other products (Figure 6-4). This implies that the roots may have a limited capacity to transform tabersonine at this stage of the growth cycle. However, it could also be that tabersonine did not reach the correct cellular or subcellular location necessary for transformation. Interestingly, the specific yield of hörhammericine was significantly reduced in response to tabersonine feeding (Figure 6-4). One possibility for this decrease is that tabersonine is an inhibitor of hörhammericine formation. As a whole there is convincing evidence that the terpenoid pathway is limiting during this stage of growth for hairy root cultures.
Figure 6-4. The addition of tabersonine (40 mg/L) to hairy root cultures at day 21 resulted in the increased specific yield of tabersonine (p<0.01) and the decreased specific yield of hörhammericine (p<0.05). The data represent the mean and standard deviations of triplicate flasks.
Other feeding studies with different tissue types had generally similar results. In cell suspensions fed at a similar time in the growth phase of the cultures, the terpenoid precursors, loganin and secologanin significantly enhanced ajmalicine accumulation (Moreno et al., 1993). Later work on a cell line genetically engineered to constitutively overexpress the strictosidine synthase gene, showed that feeding loganin and tryptamine together enhanced the flux late in the growth cycle (Whitmer et al., 1998). On the contrary, in our experiments, combined feeding of tryptamine and loganin produced the same results as feeding loganin alone (data not shown).

If the terpenoid pathway is limiting upstream of geraniol, the next step would to examine which steps are responsible. The phosphorylated intermediates IPP, DMAPP, and GPP are not amenable to feeding studies since they are unlikely to be able to cross the cell membrane. In primary metabolism, pyruvate is a precursor that leads to both the mevalonate pathway or the deoxyxyulose phosphate pathway. Hence, 200 mg/L of pyruvate was added to cultures at 21 days. After three days, 50% of the pyruvate remained in the media. Not surprisingly, pyruvate feeding had no significant effect on alkaloid accumulation. As more details arise from the deoxyxyulose phosphate pathway perhaps precursors leading to IPP will become available for future feeding studies.

6.4.3 Late growth phase feeding
The results from precursor feeding at 17 days were both quantitatively and qualitatively different. Contrary to feeding at 21 days, tryptophan enhanced accumulation of the alkaloids tabersonine, hórhammericine, and serpentine (Table 6-1). This is significant in that the enhancement was in alkaloids in two downstream pathways (Fig. 6-1). However, tryptamine feeding did not have any statistically significant effect on alkaloid accumulation as would be expected if tryptophan was limiting. No intracellular tryptamine pools were detected in the culture extracts.

To further test the enhancement of alkaloid accumulation, a larger amount of tryptophan (100 mg/L) was fed to cultures at 12 days in the growth cycle. The morphology of the roots began to change after 4 days, with pronounced thickening of the roots and proliferation of root branching. However, even though there was accelerated growth, the total yields of alkaloids were found to be significantly lower. Based on these findings, an explanation for the influence of tryptophan on growth and alkaloid accumulation were sought. We hypothesized that the effects may be auxin mediated as the morphology of the roots was similar to that reported when exogenous auxin is added (Aerts et al., 1992). Indeed feeding NAA (1 μM) at 14 days and harvesting the root cultures at 21 days enhanced growth compared to controls grown over the same period and also reduced the total yield of the predominant alkaloid of the roots, lochnericine (Figure 6-5).
Figure 6-5. A comparison of the effects feeding NAA (1μM) and tryptophan (500μM) on the accumulation of biomass and the total yield of lochnericine. NAA was added at 2 weeks and tryptophan was fed at 12 days. Each treatment and corresponding control cultures (n=3) were harvested at 21 days.
The results from feeding high levels of tryptophan and auxin, led us to test if lower levels of auxin feeding would be similar to lower levels of tryptophan feeding. With the addition of 10 nM IAA, an increase in alkaloids from the two downstream pathways was seen without an effect on growth (Table 1). In cell suspensions of *C. roseus*, ajmalicine and serentine contents were enhanced by the addition of low concentrations of IAA or NAA (Merillon et al., 1989). However, it is generally found in cell suspensions that auxin represses alkaloid accumulation (Whitmer et al., 1998). Similar to the results presented here, alkaloid production in transformed root culutures of *Hyoscyamus muticus* was significantly enhanced by the addition of auxin without affecting the root growth.

Interestingly, auxin has been reported to affect the activity of TDC, a key enzyme in alkaloid biosynthesis, in opposite directions. In a hairy root culture of *C. roseus*, tryptophan decarboxylase (TDC) activity was down-regulated by auxin at the transcriptional level (Goddijn et al., 1992). The levels of auxin used by Goddjin *et al.* were higher than those used in the portion of this study which resulted decreased alkaloid yields. In another study, TDC activity was enhanced in radicles of seedlings (Aerts et al., 1992). The levels of auxin used in this study similar to the study by Goddjin *et al.*, but the difference in results could be due to the difference in tissues examined. Since it is known that hairy roots are more sensitive to auxin than non-
transformed tissues perhaps the response of TDC activity is a function of the tissue type and hormone concentration (Shen et al., 1990).

Table 6-1. Percent change compared to control in alkaloid specific yield cultures fed during late growth phase (day 17). Cultures were harvested 96 hours after addition of precursor.

<table>
<thead>
<tr>
<th>Alkaloid</th>
<th>IAA (10 nM)</th>
<th>Tryptophan</th>
<th>Tryptamine</th>
<th>Geraniol</th>
<th>10-hydroxy geraniol</th>
<th>Loganin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabersonine</td>
<td>-4</td>
<td>41*</td>
<td>23</td>
<td>13</td>
<td>5</td>
<td>+3</td>
</tr>
<tr>
<td>Lochnericine</td>
<td>18*</td>
<td>11</td>
<td>-1</td>
<td>31*</td>
<td>-5</td>
<td>47</td>
</tr>
<tr>
<td>Hörhammermicine</td>
<td>7</td>
<td>22*</td>
<td>-8</td>
<td>-5</td>
<td>-2</td>
<td>-29</td>
</tr>
<tr>
<td>Ajmalicine</td>
<td>16*</td>
<td>11</td>
<td>36</td>
<td>8</td>
<td>-14*</td>
<td>-1</td>
</tr>
<tr>
<td>Serpentine</td>
<td>13</td>
<td>50*</td>
<td>-14</td>
<td>-8</td>
<td>2</td>
<td>-23*</td>
</tr>
</tbody>
</table>

* indicates statistically significant difference (p < .05) between treatment and control (n=3).

The hypothesis that tryptophan feeding increases the levels of IAA, was not completely unexpected. In hairy roots derived from agropine Agrobacterium strains, the genes for the synthesis of IAA from tryptophan are inserted (Cardarelli et al., 1985). LBE 6-1 is derived from A. rhizogenes 15834 (Bhadra et al., 1993) which is an agropine strain. Furthermore, even in untransformed roots of Arabidopsis thaliana, a
model dicotyledon plant, tryptophan was reported to be the precursor of IAA in roots (Müller et al., 1998). The low levels of auxin that were found to affect metabolism are likely due to hairy roots enhanced sensitivity to auxin (Shen et al., 1990).

The earlier studies and the results reported herein show the importance of considering differences in tissue type and amounts of hormone applied when interpreting results from hormone treatments. Furthermore, since tryptophan has multiple biochemical fates, such as protein, alkaloid, and hormone synthesis, the effects of metabolic manipulations on all of these pathways must be considered. For example, *C. roseus* cell lines engineered with high levels of TDC were found to be detrimental to normal growth of the cultures (Canel et al., 1998). It was hypothesized that tryptophan necessary for growth was diverted into tryptamine, thus causing these negative effects. Another example of how converting tryptophan to tryptamine has interactive effects is the participation of tryptophan in the regulation of the phenylpropanoid pathway (Yao et al., 1995). Finally, in this study, the probable conversion of tryptophan into IAA caused significant effects on metabolism.

6.4.4 Elicitation and Precursor Feeding

Earlier work from our laboratory had shown that JA significantly increases the flux to alkaloids when hairy root cultures are elicited at 3 weeks (Rijhwani and Shanks, 1998). was to combine elicitation with precursor feeding To understand which precursors are limiting under elicited conditions, an experiment analyzed the
combination of elicitation with JA with precursor feeding. Elicitation alone produced a 2.5 fold increase in the specific yield of the five quantified alkaloids (Table 6-2). Adding the precursors a day before elicitation resulted in no significant difference between this treatment and only JA treated cultures. Surprisingly, adding the precursors a day after elicitation significantly reduced the specific yields of tabersonine, ajmalicine, and serpentine (Table 6-2). This finding was consistent whether tryptamine or loganin was added. Similarly, earlier work on combined elicitation and precursor feeding found that elicitation and feeding secologanin or loganin resulted in lower increases in ajmalicine than precursor feeding alone (Moreno et al., 1993). One difference between that study and the present work was for the elicitation only control, we observed large increases in flux to several alkaloids (Table 6-2), while in the work of Moreno et al ajmalicine levels remained unchanged (Moreno et al., 1993). This is likely due to the use of different elicitors. In this study, we used JA a known potent elicitor of secondary metabolism, while Moreno et al. used a cell free extract from a Pythium (Moreno et al., 1993) fungal culutre.

The issue of which precursor pathway is rate-limiting during the late growth phase is still unresolved. Since feeding tryptamine had no positive effect on alkaloid accumulation, one possibility is a limitation in the terpenoid branch after loganin, such as secologanin. However, this would be in contrast to what was found with cell
suspension cultures. During the growth phase of the SSS overexpressing cell line, loganin feeding alone enhanced flux to total alkaloids (Whitmer et al., 1998).

Table 6-2. The effect of combined elicitation with jasmonic acid (JA) (50mg/L) on day 20 and addition of either loganin (40 mg/L) or tryptamine (20 mg/L) on day 21. Cultures were harvested 72 hours after addition of precursor (day 24). The data represent the mean and standard deviations of triplicate flasks.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serpentine</th>
<th>Ajmalicine</th>
<th>Tabersonine</th>
<th>Hörhammericine</th>
<th>Lochnericine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.42 ± 0.04</td>
<td>0.72 ± 0.07</td>
<td>0.90 ± 0.06</td>
<td>0.64 ± 0.06</td>
<td>3.81 ± 0.28</td>
</tr>
<tr>
<td>JA only</td>
<td>1.10 ± 0.06</td>
<td>1.13 ± 0.05</td>
<td>0.92 ± 0.10</td>
<td>6.40 ± 0.75</td>
<td>6.50 ± 0.43</td>
</tr>
<tr>
<td>JA + loganin</td>
<td>0.66 ± 0.17*</td>
<td>0.90 ± 0.13*</td>
<td>0.79 ± 0.08*</td>
<td>5.36 ± 0.82</td>
<td>6.00 ± 0.74</td>
</tr>
<tr>
<td>JA + tryptamine</td>
<td>0.70 ± 0.03*</td>
<td>0.91 ± 0.13*</td>
<td>0.77 ± 0.09*</td>
<td>5.67 ± 0.47</td>
<td>5.40 ± 1.00</td>
</tr>
</tbody>
</table>

* indicates statistically significant difference (p < .05) between JA + precursor fed and JA only treatment (n=3).
In conclusion, the amount of increase in metabolic flux to total indole alkaloids due to precursor feeding was moderate compared to elicitation with JA. Several reasons may account for the limited increases in alkaloid accumulation. First, the low flux increase simply reaffirms the observation that it is difficult to enhance flux without changing the levels of several enzymes (ap Rees and Hill, 1994). Elicitation, which does change enzyme levels was more effective at enhancing overall flux to indole alkaloids from hairy roots (Rijhwani and Shanks, 1998). Although all of the precursors are known to be uptaken, it is not known if there was preferential uptake by certain regions of the roots. Furthermore, the subcellular distribution of precursors was not examined. In the future, more precise studies using genetic means will be necessary to test rate-limitations in *C. roseus* hairy root cultures.
Chapter 7: Quantification of Metabolic Flux in Plant Secondary Metabolism by a Biogenetic Organizational Approach

7.1 Abstract:

Metabolic engineering represents a promising approach to enhance the yield of valuable natural products from plants. Necessary for analysis of native and modified pathways, is a method to quantify flux through metabolite measurements. Rather than focusing on the accumulation of only the final products, analyzing a wide range of secondary metabolites has significant advantages. We propose a model that organizes the flux analysis by grouping metabolites of similar biogenetic origin. To this end, we have identified and transiently quantified metabolites from several branches of the indole alkaloid pathway in *Catharanthus roseus* hairy root cultures. By using these data to calculate instantaneous changes in total alkaloid levels, we are able to examine the distribution of flux around key branchpoints. Furthermore, this analysis provides crucial information such as an estimate of total flux to secondary metabolism.

7.2 Introduction

*Catharanthus roseus* is a tropical plant that biosynthesizes a diverse array secondary metabolites, most notably indole alkaloids. Several of these alkaloids are used as anti-cancer and anti-hypertension agents. The biochemical pathways leading to the monoterpenoid indole alkaloids were established through feeding radiolabeled precursors
followed by extensive product analysis. Secologanin was found to be a central intermediate in monoterpene indole alkaloid biosynthesis as it is incorporated in ajmalicine, catharanthine, tabersonine, and vindoline (Battersby et al., 1969). Indole alkaloids have been classified upon the level of rearrangement of the secologanin group (Atta-Ur-Rahman and Basha, 1983). Examples of the biogenetic classes found in C. roseus are given in Figure 7-1.

The evidence for the order of the branchpoints was determined by precursor feeding studies in seedlings (Qureshi and Scott, 1968; Scott et al., 1969). The proposed pathway is also based upon evidence that tabersonine is not a precursor of catharanthine (Brown et al., 1969) as was originally suggested from earlier in vitro transformation studies (Qureshi and Scott, 1968). There is evidence that no measurable in vivo conversion occurs between catharanthine and coronaridine (Kutney et al., 1973).

Metabolic engineering strategies utilize the quantification of flux as a tool to predict and evaluate the effects of specific metabolic alterations. A common technique to quantify metabolic fluxes in cell cultures is known as metabolic flux balancing, which relies on measurements of growth and external metabolite concentrations to close a mass balance (Noorman et al., 1991). However, as the entire biochemical pathways in plant cell and tissue cultures are far less characterized than microbial or mammalian cultures. Furthermore, the extracellular concentrations of nutrients such as sugars, inorganic nitrogen, and phosphate are undergoing rapid changes during the period of rapid growth (Bhadra and Shanks, 1997). Therefore, the basis for the flux estimations will be made directly from total alkaloid measurements.
Figure 7-1. Proposed alkaloid pathways leading to the different biogenetic classes in C. roseus hairy root cultures. The Corynanthe pathway contains an unrearranged carbon skeleton and produces ajmalicine and serpentine. The Iboga pathway produces
catharanthine and coronaridine, which were demonstrated not to be interconvertable
occurs (Kutney et al., 1973). Tabersonine, lochnericine, and hörhammericine all contain
the Aspidospermine chemical structure.

A first step in characterizing metabolism is to focus exclusively on a single class of
secondary metabolites such as the alkaloids. The characterization of the biochemical
pathways leading to the valuable pharmaceutical alkaloids in C. roseus is incomplete. In
addition, plant tissue cultures are not amenable to chemostat experiments, which facilitate
the quantification of flux by providing a steady-state. Consequently, estimates of fluxes
are difficult to obtain. This paper presents a formulation for calculating fluxes in
secondary metabolism of plant tissue cultures. This method is based solely on
quantification of metabolites combined with a simple growth model, and does not require
in vitro enzyme measurements. The results from this analysis quantify the distribution
of flux around branchpoints which is important when multiple pathways compete for the
desired flux. Finally, extensions of this analysis may point to sections of the metabolic
pathway responsible for control of flux or rate-limitations.

7.3 Materials and Methods

7.3.1 Culture conditions: The hairy root line LBE 6-1 was used for this study. It was
maintained on a 3 week subculture cycle in Gamborg’s B5/2 media with 3% sucrose and
an initial pH of 5.7. Cultures were initiated by placing 5 root tips (35-40 mm) in 250 ml
flasks containing 50 ml of media, which were shaken at 100 RPM, and kept at 26° in the 
dark.

7.3.2 Alkaloid analysis: Alkaloids were extracted and analyzed as previously described 
(Bhadra et al., 1998). Lochnericine and hörhammericine were quantified based on the 
response factor at 329 nm for tabersonine. Similarly coronaridine was quantified based 
upon the response factor for catharanthine at 281 nm. The HPLC method previously 
reported (Morgan and Shanks, 1998) was used to separate hörhammericine, ajmalicine, 
serpentine, lochnericine, and tabersonine. For the analysis of catharanthine and 
coronaridine, the following method was used. An injection volume of 20 µL was used. 
The mobile phase consisted of a 0.1 M buffer of CH₃COONH₄, MeCN, and MeOH 
(42:29:29). The flow rate was 1.0 mL/min until 15 min, 1.3 mL/min from 15 to 55 min 
and 1.0 mL/min from 56 to 65 min.

7.3.3 Flux Calculations: The calculations of flux were based upon the total alkaloid 
productivities of the roots, which is defined as total flux (mmol alkaloid/ day) in Eq. 1.

\[
\text{Total flux} = \frac{d \cdot (C_i \cdot X)}{dt} \tag{1}
\]

where \(X\) = biomass (g DW), \(C\) = alkaloid specific yield (mmol / g DW), \(t\) = time (days), 
and \(i\) represents each alkaloid. This choice for the definition of flux was made because the 
tissue is not evenly distributed throughout the reactor volume, and as the growth of the 
tissue is not homogeneous specific productivity seems less appropriate. The values for
the variables, \( C \) and \( X \) are taken directly from alkaloid and biomass measurements. The derivative of their product was estimated by using linear regression (KaleidaGraph 3.0) to fit the product of concentration and biomass data to either a first or second order polynomial which maximized the value of the coefficient of correlation. The function was then numerically differentiated and evaluated at times within the range of the data for the derivatives. Hence for the growth during this time equation 2 was proposed:

\[
\frac{d (X)}{dt} = X_0 + k
\]

where \( k \) = growth constant (g DW / day).

7.4 Results and Discussion:

Interestingly, the growth of the roots during the period of 12 to 26 days was linear (Figure 7-2). This implies that perhaps some nutrient is limiting growth during this period. Kanokwaree and Doran have also reported that the growth of shake flask hairy root cultures becomes linear after a short exponential growth stage and implicated oxygen limitations (Kanokwaree and Doran, 1997). Similar to Kanokwaree et al., we tested nutrient limitations by partial media replacement at 7 and 14 days in the culture cycle. No enhancement in the growth of the hairy roots was detected (data not shown). This result is consistent with limitations in the mass transport of oxygen to hairy roots.

Seven alkaloids were identified to be present in our system through the use of standards, and spectral identification. These alkaloids were ajmalicine, serpentine, tabersonine, lochnericine, hörhammericine, catharanthine, and coronaridine (Figure 7-1). Previous studies using \(^{14}\text{C}-\) labeled precursors determined that these alkaloids belonged to
Figure 7-2. The growth profile of the hairy root culture between 14 and 26 days. There is a strong linear correlation ($r^2 = 0.989$) between the dry weight and time. Each data point represents the mean ± standard deviation (n=3).
different biogenetic classes (Atta-Ur-Rahman and Basha, 1983). Each class represents a rearrangement of the carbon skeleton, thus indicating a branchpoint in the biosynthesis of the alkaloids. The alkaloids detected in the C. roseus hairy root cultures are from the Coryanthine, Aspidosperma, and the Iboga classes. Figure 7-1 shows the connection between these classes and the order that the branchpoints diverge.

The transient specific yields of catharanthine and coronaridine are shown in Figure 7-3. This is the first report of the transient yield of coronaridine from C. roseus cell or hairy root cultures. Catharanthine specific yields have been previously reported (Toivonen et al., 1989; Jung et al., 1994; Vazquez-Flota et al., 1994), but this is the first presentation of data from the hairy root line LBE 6-1. Coronaridine is important because it is biosynthetically related to catharanthine, which is one of two monomers needed to form the anti-cancer dimeric indole alkaloids.

The total productivities were calculated by analyzing the instantaneous increase in total alkaloid yields. Assuming that the pathways are linear, irreversible, and there is no turnover of the final products, then the total productivities represent flux. The total productivities for each biogenetic class of alkaloids are summed to estimate the instantaneous flux towards each branch. The fluxes were summed and normalized against a total flux. From this analysis the ratio of fluxes can be compared around key branchpoints. For example, the ratio of the flux to the Iboga compared to the Aspidosperma alkaloids decreases with time (Figure 7-4). This suggests the activity of enzymes that compete for a central metabolite are varying with time. In contrast, Figure 7-5 shows that the ratio between the flux to the Coryanthine alkaloids, and the sum if the
Figure 7-3. The specific yields of catharanthine and coronaridine (mg/g DW) between 14 and 26 days in *C. roseus* hairy root cultures. The data reported are the mean ± standard deviation (n=3).
Figure 7-4. The distribution of flux between the Iboga and Aspidosperma branches of the indole alkaloid pathways is shown between 14 and 24 days. The majority of the total flux to indole alkaloids go to the Aspidosperma alkaloids. The fraction of total flux in Iboga pathway decreases with time, while the flux toward the Aspidosperma pathway increases.
Figure 7-5. The distribution of flux between the Coryanthe branch and the sum of the Iboga and Aspidosperma branches is shown between 14 and 24 days. Relatively no change in the distribution of flux at the branchpoint connecting these two pathways occurs.
Iboga and Aspidosperma classes is essentially constant between 14 and 26 days. Hence, little control of the flux distribution seems to be exerted at this point.

Finally, by summing all of the total productivities, an estimate of total flux to alkaloids is obtained. This is a conservative estimate as other alkaloids present in our system remain to be identified and quantified. Interestingly, the total alkaloid flux of the hairy root culture remained constant over the period of linear growth (Figure 7-6). This was determined from linear regression of the data ($r^2 = 0.97$). Since no higher order or power low model led to a significant reduction in the error sum of squares, the linear model was accepted as the best fit of the data. Because the linear model was the best fit, the total productivity ($3.54 \pm 0.60$ mg/day \textbullet{} L) did not vary significantly during this experiment ($p < 0.05$). As the total flux is constant, it suggests that control of the flux to alkaloids is regulated above the nodes studied here. Several possibilities exist for this type of control, such as feedback inhibition, mass transport limitations, or rate-limiting pathways between primary and secondary metabolism.

The primary use of this analysis is in systems which have carbon skeleton rearrangements or enzymology that delineates branchpoints. As this analysis organizes metabolic pathways similar to a “top-down” approach (Brown et al., 1990), this scheme could also be applied to perturbation type studies to determine group flux control coefficients (Simpson et al., 1997). Furthermore, earlier work has shown that from transient metabolite measurements, flux control coefficients, a useful tool to quantify
Figure 7-6. The total (µmol/L) of indole alkaloids plotted against culture time. The total flux (9.45 µmol/L day) was found by calculating the slope of the line. Since, the graph shows a strong linear correlation ($r^2=0.97$) between total alkaloids and culture time, it appears that the total flux to alkaloids is constant. Each data point represents the mean ± standard deviation (n=3).
metabolic regulation, can be determined (Delgado and Liao, 1992). To represent the fluxes more precisely, the model presented here needs to be modified to include structural components. The fact that there is an age distribution of root tissue implies that such an analysis would require dissecting the root into sections, and analyzing each section for alkaloids.

The proposed scheme of calculating flux is relevant to a variety of secondary metabolite systems in different plant species. In systems with undefined enzymology, it can be applied in conjunction with $^{13}$C NMR experiments to elucidate flux control between branchpoints. Furthermore, a comparison of biosynthetic fluxes in genetically altered and wild-type cell lines can be made using this approach.

7.5 Acknowledgments

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Chapter 8: Preliminary studies of the retransformation of hairy root cultures.

8.1 Introduction

After targets for the metabolic engineering of an organism have been selected, the following step is to clone the genes. Following the cloning of the genes of interest, the next step involves introduction of the genetic material into the organism. To permanently change metabolism at the molecular level by rational means requires that the organism is amenable to genetic transformation. Furthermore, having the ability to transform a well characterized line is desirable for several reasons. First, the best control for evaluating the effects of genetic changes is a well characterized original line, which is transformed with a vector that contains no other genes other than antibiotic resistance. This control can be compared with the untransformed line to show any effects of the transformation procedure or due to antibiotic resistance. Secondly, a great deal of time and effort is required to characterize the growth and metabolism of a hairy root line.

Currently, there are several techniques available for plant transformation. The most common method for dicotyledon plants is Ti plasmid mediated gene transfer (Robins, 1994). Physical methods, such as microparticle bombardment, microinjection, electroporation, and liposome fusion are also possible (Glick and Pasternak, 1994). Particle bombardment is a process with a relatively low efficiency for generating genetically stable transformed tissues (Sanford, 1990). Furthermore, the process will produce chimeras which need to be sorted and stabilized. However, it has the advantages of being able to transform organelles, and is not limited by tissue type or plant species (Sanford, 1990). Microinjection has the disadvantage of being able to transform one cell at
a time, and involves a high degree of skill. The disadvantage of the direct transformation methods is that they are generally limited to protoplasts. Hence, they require methods for generation of protoplasts, and regeneration of plant tissue, which are often difficult.

_Catharanthus roseus_ is amenable to transformation by several strains of _Agrobacterium rhizogenes_ and _A. tumefaciens_ (Goddijn et al., 1995). The original hairy root lines developed in this laboratory were transformed by infecting seedlings with _A. rhizogenes_ 15834, a virulent strain (Bhadra et al., 1993). To transform the hairy root lines, a disarmed strain of _Agrobacterium_ should be used. This will prevent the introduction of genes which encode for enzymes responsible for plant hormone biosynthesis. This is the strategy we selected in our attempts to retransform the hairy root clone LBE 6-1.

### 8.2 Materials and Methods

#### 8.2.1 Strain and Vector

The disarmed vector, pBI121 (Clonetech, Mountain View, CA) containing a neomycin phosphotransferase II gene, with the nopaline synthase (NOS) promoter and terminator region, was maintained in _A. tumefaciens_ strain LBA4404. The vector pBI121 also contained the gene for β-glucuronidase, with expression driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminated by the NOS polyadenylation region.

Expression of β-glucuronidase was detected by X-glucuronic acid (Molecular Probes, Eugene, OR). The _Agrobacterium_ strain was grown at 28°C in LB media with 50 μg/mL kanamycin for 48-72 hours before use.
8.2.2 Transformation

Hairy roots were obtained from a 3 week subculture grown in 75 mL of filter sterilized media containing Gamborg's B5/2 salts, 3% sucrose, and Gamborg's vitamins with the initial pH adjusted to 5.7. The roots were injured by puncturing with a sterile hypodermic needle or were cut at the tip by a sterile razor blade. Only the first several millimeters of the root tips were injured, as this is region of meristematic cell division. The root tips were then immersed in the liquid suspension of the Agrobacterium. Alternatively, the needle or razor blade was dipped into the Agrobacterium solution before injuring the roots. The root tips were placed on plates containing the media stated above solidified with 6 g/L agar for 48 hours. Subsequently, the roots were transferred to plates containing 50 μg/ mL kanamycin and 100 μg/ mL timentin. The roots were transferred to fresh plates every week to prevent bacterial contamination.

8.2.3 Evaluation of transformation

Roots that appeared to grow upon the antibiotic plates were assayed for transformation by staining with X-Gluc. Untransformed but injured, and untransformed and uninjured roots were also stained as negative controls. The roots were fixed by placing tissue in a solution of 1 mM EDTA, 100 mM NaH₂(PO₄), 2% paraformaldehyde, and a pH of 7.0. The solution was placed on ice for 30 minutes. The roots were then washed in the Na-phosphate buffer thrice. To stain the roots they were vacuum infiltrated with a solution containing 2 mM X-Gluc, 0.5% Triton X-120, and 50 mM Na-phosphate buffer at pH 7 for 1-2 seconds. The tissue and solution were then incubated at 37°C for
several hours. The staining process was terminated by washing the tissue in distilled water.

### 8.2.4 Callus induction

Exogenously applied auxin and cytokinin were added to liquid media after filter sterilization or media for solid plates after autoclaving the media but before the gel set. The auxins tested were 2,4-dichlorophenoxyacetic acid (2,4-D) and α-napthaleneacetic acid (NAA), and the cytokinin used was kinetin. Combinations of hormones were added to 1 and 3 week old roots.

### 8.3 Results and Discussion

The roots which were infected with *Agrobacterium* were visually monitored for growth. Unfortunately after several attempts, no stable transformation event was detected. Out of 234 transformation attempts, only 4 roots grew significantly more than controls on the selection plates. These roots, 4 other roots that were injured and dipped in the *Agrobacterium* but did not show any growth, and 4 untransformed roots were assayed for expression. After staining for GUS activity, the transformed roots showed some blue coloration around the injured spots. Under a microscope it appeared to be several cells that were transformed. However, these cells were not proliferating.

The goal of this transformation was to transform the meristem cells, so that a root could be regenerated without having to move to a cell suspension culture. Cell suspension cultures can be genetically unstable, and we didn’t want the culturing procedure to change the phenotype of the well characterized line. Furthermore, techniques for the generation
of cell cultures from these hairy roots had not be established. From the results presented above, it became obvious that it would be necessary to induce callus at some point in the transformation procedures.

Hormone combinations, and concentrations that were optimal for producing calli from our hairy root clone LBE 6-1 were determined. The use of the hormones in liquid culture had various effects. Kinetin alone had no affect on growth of the hairy roots. Auxin alone caused the roots to thicken and increased lateral branching. Not qualitative difference was noted between NAA and 2,4-D, although NAA appeared to have stronger effects at similar concentrations. If auxin was used in high concentrations (0.5 mg/L) the roots lost structural integrity, and turned brown. This suspension of cells could not be cultured further. However, if auxin was used in combination with kinetin then root growth could be maintained in the thickened morphology. The ratio of 10:1 α-naphthaleneacetic acid (NAA): kinetin was found to be optimal for callus induction on solidified media. The absolute concentration was not critical. Callus induction took 5 weeks for the concentration of 0.05 mM NAA and 0.005 mM kinetin. For regeneration, simply transferring the callus to hormone free media regenerated the hairy roots. Roots took approximately 4-6 to regenerate from callus. No analysis was done to determine if the regenerated root lines had the same growth or alkaloid productivity.

8.4 Conclusions

The direct retransformation of roots is very labor intensive, and was not successful. Remedies for the problems encountered could be to transform callus with microparticle bombardment or Agrobacterium mediated gene transfer. Alternatively, the
root could be infected with \textit{A. tumafaciens}, and then transformed to callus from which hairy roots are regenerated. A more straightforward approach that would avoid using disorganized tissue, would be to transfer the Ti plasmid to \textit{A. rhizogenes}, which would then be used to infect seedlings or leaves. However, this does have the disadvantage of lacking a ‘true’ control. For comparison to a control, the transformed lines should be compared to several independent lines of hairy roots generated with only the antibiotic resistance gene.
Chapter 9: Future Directions

The results presented in this thesis have advanced the current knowledge of several aspects of secondary metabolism in *C. roseus*. Recommendations for future work are based upon the results from the studies presented in this thesis.

The search for metabolites between tabersonine and vindoline in dark and light-adapted cultures demonstrated that the levels of certain metabolites change, but apparently no new alkaloids were detected. The absence of significant amounts of the first two metabolites after tabersonine are currently limiting the usefulness of hairy roots as a production system for the valuable alkaloid vindoline. This has been attributed to the low activity of the tabersonine 16-hydroxylase (St-Pierre and De Luca, 1995). Potentially, there are two solutions to this problem. The first would be to establish cell suspension cultures which do have moderate activity for the first two steps between tabersonine and vindoline (St-Pierre and De Luca, 1995). The second solution would be to attempt to clone the genes for the tabersonine 16-hydroxylase. One possible approach to this problem would be to use reverse transcriptase-polymerase chain reaction (RT-PCR) to clone P-450's from mRNA pools from young leaves which express high levels of this enzyme. A similar approach was taken in an attempt to clone another P-450 enzyme, geraniol hydroxylase (G10H), from *C. roseus* (Mangold et al., 1994). Using hairy root cultures as a mRNA source, this technique
can also be used to screen for the P-450 enzymes involved in the diversion of tabersonine into lochnerinicic and hörhammericic.

Precursor feeding studies have shown the importance of tryptophan pools in regulating hormone levels during the growth of the roots. Thus an investigation of the role of the levels of the aux and rol genes in hairy roots, and how it corresponds with root morphology and alkaloid productivity would be interesting. One recent investigation has taken such an approach (Palazon et al., 1998).

Through precursor feeding, we have shown that the terpenoid pathway is likely limiting flux during the later stages of growth. One key area for future investigation would be to determine what percentage of terpenoid flux comes from the mevalonate and 1-deoxyxylulose pathways. This experiment should use $^{13}$C labeled glucose or readily uptaken glycolytic intermediates (i.e. pyruvate, acetate) followed by in vitro extraction and analysis of the metabolite labeling patterns. Related extension of the $^{13}$C labeled glucose feeding studies, could be used to identify other intermediates in the indole alkaloid pathway.

From the biogenetic analysis of the secondary metabolite pathways, a large portion of the total flux ends up in the Aspidosperma alkaloids. Further identification of alkaloids is desirable. The types of alkaloids identified would give insight into the sources and sinks of secondary metabolite flux. The complete identification of one alkaloid and partial identification of another are presented in Appendix A. With more effort, several more alkaloids could be readily identified by the purification and
spectral methods given in the Appendix. However, judging from “typical”
chromatograms, no single unknown peak contains more than 10% of the total area at
any wavelength.

A continuation of the work with the buffers may be extended to cover a wider
range of pH. This work could also be done with slightly more sophisticated controls
in a bioreactor. A relatively simple addition of a pH controller could be added to the
bioreactor configuration, that would allow pH control at any specified set point.
Furthermore, perturbation experiments could be readily performed with such an
apparatus.
Appendix A: Structural Identification of Alkaloids in *C. roseus* Hairy Roots

**A-1: Introduction.** The alkaloids that may have importance in the biosynthesis of vindoline. This appendix will provide spectral data on two alkaloids previously not identified in hairy root line LBE 6-1. The first alkaloid was found while conducting a search by UV-spectra of alkaloids that resembled vindoline or deacetylvindoline. The structure could not be completely elucidated because lack of material sufficient to obtain quality $^{13}$C-spectra. The second alkaloid was found to significantly enhanced when analyzing jasmonic elicited cultures. The cultures were elicited at 21 days and harvested at 28 days. From 2-D NMR spectroscopic data the chemical structure matches echitovenine. Echitovenine has been previously reported from normal and hairy roots of *Catharanthus trichophyllus* (Cordell, 1976; Davioud et al., 1989). To our knowledge this is the first report of echitovenine from *C. roseus* cell or tissue cultures.

**A-2: Materials and Methods**

UV-vis spectra were recorded by our Waters 996 Photodiode array detector. The $\lambda_{\text{max}}$ are reported and spectra were compared to known standards. IR-spectra were recorded on a . The mass spectra were recorded by chemical ionization (CI) and fast atom bombardment (FAB) using a Finningan Mass Spectrometer. The NMR data were recorded on a Bruker AMX 500 MHz in Fourier Transform (FT)mode. The
chemical shifts were calibrated against trimethylsilane (TMS). Subsequent to FT, the spectra were phased and baseline corrected. HSQC (heteronuclear single-quantum coherence) spectra were also acquired.

**A-3: Alkaloid A: Unknown**

UV-vis spectra: $\lambda_{\text{max}}$ 252 nm, 305 nm

IR spectra: 3350 cm$^{-1}$ br (NH), 2929 cm$^{-1}$, 2860 cm$^{-1}$, 1742 cm$^{-1}$, 1635 cm$^{-1}$, 1241 cm$^{-1}$, 1023 cm$^{-1}$, 751 cm$^{-1}$

Mass Spectra: FAB: m/e 391.4, Cl: m/e 393.0, High resolution mass spectrometry (HRMS) MH$^+$ : 393.18132

Molecular formula: C$_{23}$H$_{24}$N$_2$O$_4$ theoretical MH$^+$ : 393.18143

NMR: 1H spectra: $\delta$ 8.13 d, 8.11 d, 7.98 d, 7.66 d, 7.63 t, 7.30 t, 5.54 q, 4.45 dd, 4.24 dd, 4.13 t, 3.79 s, 3.40 q, 2.65 d, 2.47 dq, 2.17 s, 2.07 s, 2.02 s, 1.54 dq, 1.25 s, 0.9 t

COSY spectra: The following is a list of the shifts of coupled protons.

1. 7.63, 7.27 and 8.13
2. 8.11 and 7.63
3. 7.98 and 7.65
4. 5.54, 2.65 and 2.17
5. 4.13 and 2.17
A partial structure of the molecule consistent with the aromatic protons is shown below in Figure A-1.

![Chemical Structure](image)

**Figure A-1.** The four aromatic protons of the benzyl group were assigned (Note that the order may be reversed). There is also pair of doublets that only correlate to each other which are far downfield. These signals are consistent with two protons on a double bond near cyclic nitrogen.
A-4: Alkaloid B: Echitoneine

UV-vis spectra: $\lambda_{\text{max}}$ 298 nm, 329 nm

IR spectra: 3390 cm$^{-1}$ br (NH), 1715 cm$^{-1}$, 1680 cm$^{-1}$, 1242 cm$^{-1}$

Mass Spectra: Cl: m/e 397.4, HRMS: MH$^+$ : 397.212767

Molecular formula: C$_{23}$H$_{29}$N$_2$O$_4$ theoretical MH$^+$ : 397.212732

NMR: 1H spectra: $\delta$ 7.15 ddt, 7.12 td, 6.84 td, 6.80 ddd, 4.68 q, 3.76 s, 3.14 distorted ddd, 2.94 dddd, 2.68 d, 2.63 d, 2.58 ddd, 2.55 distorted ddd, 2.52 dd, 2.044 td, 1.81 distorted dddd, 1.73 ddd, 1.639 m, 1.59 distorted ddd, 1.496 distorted ddd, 1.41 s, 0.97 d

COSY spectra: Below is a list of correlated protons.

1. 6.80, 6.84, 7.12, 7.15 5. 2.63, 2.68
2. 0.97, 4.68 6. 1.50, 1.64, 1.81
3. 1.59, 1.81, 2.55, 3.14 7. 1.59, 1.64, 1.81
4. 2.94, 1.72, 2.04, 2.58 8. 2.52, 4.68

.
Table A-1. The $^{13}$C spectra of echitovene and the multiplicity of each carbon (determined from DEPT spectrum) and HSQC correlations.

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<th>Shift (ppm)</th>
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<th>HSQC correlations</th>
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<td>2</td>
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**Figure A-2.** The chemical structure of echitovenine as determined from 1 and 2-D NMR experiments, and HRMS.
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