Compartmentation of Secondary Metabolites and Xenobiotics in Plant Vacuoles

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I. Introduction ................................................................................................................................. 141
   A. Secondary Metabolites as Defence and Signal Compounds of Plants ................................ 141
   B. Fate of Xenobiotics in Plants ............................................................................................. 143
   C. Aims and Scope .................................................................................................................... 145

II. Vacuolar Storage of Secondary Compounds and Xenobiotics .............................................. 145
   A. Secondary Compounds ........................................................................................................ 145
   B. Xenobiotics ......................................................................................................................... 153
   C. Mechanisms Underlying Vacuolar Sequestration ................................................................ 154

III. Conclusions ............................................................................................................................. 159
   Acknowledgements ................................................................................................................... 160
   References ................................................................................................................................. 160

I. INTRODUCTION

A. SECONDARY METABOLITES AS DEFENCE AND SIGNAL COMPOUNDS OF PLANTS

Plants need to protect themselves against herbivores (mostly insects and grazing vertebrates) and microorganisms. Various defence strategies can be observed in plants (for reviews, see Levin, 1976; Swain, 1977; Wink, 1988, 1993a; Harborne, 1993; Rosenthal and Berenbaum, 1991), which are not
independent and which may operate cooperatively and synergistically. The strategies include:

- Physical protection by thorns, spikes, trichomes, or glandular or stinging hairs ("weapons"), by thick bark or cuticles of roots and stems or by robust seed coats ("armour").
- The production and storage of defence chemicals (or allelochemicals), which are abundant and a typical trait of all plants. The following situations can be distinguished:

1. Plant surfaces are usually covered by a hydrophobic cuticle consisting of antibiotic and deterrent/repellent cuticular waxes which may contain other biologically active allelochemicals such as flavonoids.

2. Plants can synthesize inhibitory proteins (e.g. lectins, protease inhibitors, toxalbumins) or enzymes (e.g. chitinase, glucanases, hydrolases, nucleases) which could degrade microbial cell walls or other microbial constituents, or peroxidase and phenolase, which could help to inactivate microbial toxins ("xenobiotics", see below) produced by pathogens.

3. As a most important trait, plants can produce and store secondary metabolites (of which more than 50,000 compounds have been described so far) with deterrent/repellent or toxic properties against microorganisms, viruses and/or herbivores. These products are often stored at strategically important sites: epidermal tissues or in cells adjacent to an infection, or in plant parts that are especially important for reproduction and survival (flowers, fruits, seeds, bark or roots). Considering their synthesis, three situations can be distinguished:
   - allelochemicals are often produced constitutively;
   - some may be activated by wounding or infection ("preformed defence chemicals"), such as cyanogenic glycosides, glucosinolates, coumaryl glycosides, alliin, ranunculin, etc.);
   - in some instances, a de novo synthesis induced by elicitors (so-called phytoalexins), infection or herbivory can be observed.

In addition to defence, secondary metabolites are employed by plants to attract pollinating insects or seed-dispersing animals, e.g. by coloured compounds such as betalains (within the Centrospermae), anthocyanins, carotinoids, flavonoids or fragrances, such as terpenes, amines and aldehydes. In this case we can consider the secondary metabolites as attracting signal substances. Also, physiological roles, such as ultraviolet protection (by flavonoids or coumarins), nitrogen transport or nitrogen storage (some alkaloids, non-protein amino acids, lectins and protease inhibitors), or photosynthetic pigments (carotinoids) may be additional features.

Although the biological functions of most plant-derived secondary metabolites have not been studied experimentally in depth, it is now generally assumed that many compounds are important for the survival and fitness of
a plant (Fraenkel, 1959; Wink, 1988, 1993a) and that they are not useless waste products, as was suggested earlier in this century. Secondary metabolites are often not directed against a single organism, but generally against a variety of potential enemies, or they may combine the roles of both deterrents and attractants (e.g. anthocyanins and many essential oils can be attractants in flowers, but are also insecticidal and antimicrobial). Thus, many natural products have dual or even multiple functions.

It might be argued that the defence hypothesis cannot be valid since most plants, even those with extremely poisonous metabolites (from the human point of view), are nevertheless attacked by pathogens and herbivores. However, we have to understand and accept that chemical defence is not an absolute process. Rather, it constitutes a general barrier which will be effective in most circumstances, i.e. most potential enemies are repelled or deterred. However, plants with chemical defences also represent an ecological niche for potential pathogens and herbivores. During evolution a few organisms have generally been successful in specializing towards that niche, i.e. with respect to a particular toxic plant in that they found a way to sequester the toxins or become immune to them. This is especially apparent in the largest class of animals, the insects (with several million species on our earth), which are often highly host plant-specific (Bernays and Graham, 1988; Bernays and Chapman, 1994). The number of these “specialists” is exceedingly small for a given plant species as compared to the number of potential enemies that are present in the ecosystem. These specialists are the exception to the general rule, similar to the situation of some viruses and bacteria which have overcome our powerful immune system.

A prerequisite for both the defence and the signal functions is the sequestration of critical amounts of the respective active secondary metabolites, otherwise the necessary effect (which is usually dose-dependent) could not be achieved. Whereas lipophilic material (e.g. many terpenoids) is stored in resin ducts, oil containers, dead cells or trichomes (Wiermann, 1981), hydrophilic compounds (e.g. alkaloids, non-protein amino acids, organic acids and glycosides) are usually confined to the vacuole, which seems to be especially adapted to the bulk storage of allelochemicals (the term “defence compartment and signal compartment” was coined to emphasize this vacuolar function; Wink, 1993b). As mentioned before, the vacuoles employed for defence or signalling can be expected in cells that are positioned in strategically favourable positions, such as epidermal tissues, or in flowers or fruits, i.e. they show a high degree of cell- and tissue-specificity.

B. FATE OF XENOBIOTICS IN PLANTS

Xenobiotics can be characterized as compounds that are not indigenously present in a particular plant species. In nature, xenobiotics can be secondary
metabolites which are produced by other plants and released to the environment either via the rhizosphere or by leaching from aerial parts. These compounds often interfere with the germination and seedling growth of plants of the same or other species which compete for light, water or nutrients. These interactions have been called “allelopathic” (for reviews, see Rice, 1984; Waller, 1987; Inderjit et al., 1995). When plants are infected by fungi or bacteria, the pathogens often produce secondary compounds (“phytotoxins”) to weaken the defence of the host plant. A more recent exposure of plants to xenobiotics is due to agrochemicals and industrial pollutants.

Mechanisms have evolved in plants during evolution to cope with secondary metabolites produced either by competing plants or by pathogens. These mechanisms can also be used by plants when exposed to man-made xenobiotics (the so-called “green liver” concept: Sandermann et al., 1985; Dodge, 1989; Hathway, 1989). The handling of xenobiotics shows some similarities between animals and plants (for reviews, see Cole, 1994; Sandermann, 1994). As an initial reaction, lipophilic compounds are often oxidized, reduced or hydrolysed (Jacoby and Ziegler, 1990) in order to reveal or introduce a functional group which will enhance reactivity and polarity of the molecules (“phase 1” reaction in the pharmacological literature). In a second step these compounds are conjugated with more polar molecules, such as sugars, amino acids, acids or glutathione (“phase 2”). In “phase 3”, these conjugates are eliminated or sequestered in a safe place.

Although obvious similarities exist, marked differences are also apparent (for reviews, see Baldwin, 1977; Menn, 1978) (Table I):

**Phase 1.** Whereas cytochrome-P-450 hydroxylases have broad and overlapping substrate specificities in animals (in order to cope with a wide variety of dietary secondary metabolites), plant cytochrome-P-450 enzymes with their multiple isoforms (Donaldson and Luster, 1991) are more selective and naturally function to catalyse specific reactions in the biosynthesis of secondary metabolites (Cole, 1994). Any modification of a xenobiotic would be more a side-reaction. However, Sandermann (1994) has argued that the specificity can also be due to special enzymes involved only in the metabolism of xenobiotics. In addition to cytochrome-P-450 hydroxylases, xenobiotics can be modified by demethylases and peroxidases, which are abundant in plants.

**Phase 2.** Whereas conjugation reactions with sulfate, amino acids and glucuronic acid are common in animals, plants often add sugar moieties to xenobiotics with aid of UDP-\(O\)-glucosyltransferases and UDP-\(N\)-glucosyltransferases. The resulting glycosyl derivatives are often further acylated with malonic acid by \(O\)- or \(N\)-malonyltransferases. For plants, special importance is attributed to glutathione transferases, of which different isoforms with varying substrate specificities exist (Timmermann, 1989);
TABLE I
Fate of xenobiotics in plants and animals

<table>
<thead>
<tr>
<th>Plants</th>
<th>Animals</th>
</tr>
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<tbody>
<tr>
<td><strong>Phase 1</strong> (transformation)</td>
<td></td>
</tr>
<tr>
<td>Hydroxylation, reduction, hydrolysis</td>
<td>Hydroxylation, reduction, hydrolysis</td>
</tr>
<tr>
<td><strong>Phase 2</strong> (conjugation)</td>
<td></td>
</tr>
<tr>
<td>Glucosylation + malonylation</td>
<td>Glucuronic acid conjugates</td>
</tr>
<tr>
<td>Glutathione S-conjugation</td>
<td>Conjugation with amino acids</td>
</tr>
<tr>
<td></td>
<td>Glutathione</td>
</tr>
<tr>
<td></td>
<td>S-conjugation</td>
</tr>
<tr>
<td></td>
<td>Conjugation with sulfates</td>
</tr>
<tr>
<td><strong>Phase 3</strong> (excretion/compartmentation)</td>
<td></td>
</tr>
<tr>
<td>Binding to lignin/polysaccharides</td>
<td>Excretion with urine/faeces</td>
</tr>
<tr>
<td>Vacuolar sequestration</td>
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</table>

these glutathione S-transferases conjugate xenobiotics with reduced glutathione.

**Phase 3.** Whereas in animals conjugates of xenobiotics are eliminated from the body tissues via optimized excretory systems (involving a specific ATPase), other mechanisms are required in plants, which do not have an active excretory system: conjugates are either bound to insoluble constituents, e.g. lignin and polysaccharides (Langebartels et al., 1986), or they are sequestered in the vacuole.

C. AIMS AND SCOPE

In this review the experimental evidence for the vacuolar sequestration of secondary metabolites and xenobiotics is summarized. Because the questions of how these compounds pass the tonoplast and how they are accumulated against a concentration gradient in the vacuole are discussed in other chapters of this volume, these topics will be treated more briefly in this chapter.

II. VACUOLAR STORAGE OF SECONDARY COMPOUNDS AND XENOBIOTICS

A. SECONDARY COMPOUNDS

It had been observed by light microscopy as early as the last century that many of the coloured flower pigments (e.g. anthocyanins, flavonol glycosides) or the red betalaines of Beta roots are exclusively sequestered in the vacuole.
Modern microspectrometric investigations (Schnabl et al., 1986; Gonnet and Hieu, 1992) and direct determinations in isolated vacuoles (Table II) have confirmed these early observations. The fate of the non-coloured metabolites remained almost unknown for many decades. Progress came when it was possible to prepare protoplasts from a wide number of plants in the 1970s (Ruesink, 1980) and to produce intact vacuoles without a significant efflux of vacuolar contents. Several protocols, which employ the lysis of protoplasts in slightly hypotonic media and centrifugation on sucrose or Ficoll gradients, often result in pure and intact vacuoles (for reviews, see Marty et al., 1980; Wagner, 1982; Leigh, 1983; Ryan and Walker-Simmons, 1983; Mäder, 1984; Willenbrink, 1987). In order to separate the vacuoles from the surrounding medium, centrifugation of the vacuoles through a silicone oil layer was another important step forward (Wagner, 1982; Mende and Wink, 1987).

As a general rule, hydrophilic and water-soluble secondary metabolites are sequestered in the vacuole (Table II), although their site of synthesis is usually the cytoplasm. A few compounds are synthesized in the chloroplast, such as quinolizidine alkaloids (Wink and Hartmann, 1982) or the piperidine alkaloid coniine (Roberts, 1981). Protoproberine alkaloids, such as berberine, are synthesized in small vesicles which later fuse with the tonoplast to release their alkaloidal content into the vacuole (Sato et al., 1990, 1993, 1994). Several glycosides become malonylated by O-malonyltransferase in the cytoplasm. O-malonylglucosides are very labile and thus difficult to isolate; it is likely that many more compounds exist in this form in vivo (Sandermann, 1994). The charged malonyl residue appears to act as a signal for transport into the vacuole (Matern et al., 1986; Mackenbrock et al., 1992), and malonylglucosides of isoflavones, isoflavanones and pterocarpanes were exclusively located in vacuoles from Cicer arietinum cell suspension cultures (Mackenbrock et al., 1992).

Sometimes quite high concentrations of secondary metabolites are reached in vacuoles. For example, in latex vacuoles (vesicles) of Chelidonium majus, concentrations of sanguinarine, chelidone and berberine up to 500–1000 mM were found (Hauser and Wink, 1990), and latex vacuoles of Papaver somniferum sequester up to 500 mM morphine (Pham and Roberts, 1991). Since many of these compounds are toxic and dangerous to the plant producing them, Matile (1984) considers the vacuole mainly as a site for detoxification, and coined the term "toxic compartment". This is a plausible description from the point of view of plant biochemistry. But as explained in the introduction, "defence and signal compartment" (Wink, 1993b) would better describe the functional role and corresponding ecological importance.

The close integration of vacuolar sequestration into the defence strategy of a plant is exemplified in the following.

In Sorghum, the cyanogenic glycosides are stored in epidermal vacuoles which, however, lack β-glucosidase, which is localized in chloroplasts of
### TABLE II
Vacuolar sequestration of defence and signal compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenolics</strong></td>
<td></td>
</tr>
<tr>
<td>Bergenin</td>
<td>Taneyama (1992)</td>
</tr>
<tr>
<td>Coumaroylglycosides (esculin)</td>
<td>Oba <em>et al.</em> (1981), Werner and Matile (1985)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>Taneyama (1992)</td>
</tr>
<tr>
<td>7-Glucosylpleurostemin</td>
<td>Harborne <em>et al.</em> (1993)</td>
</tr>
<tr>
<td>Isoflavanone malonylglycosides</td>
<td>Mackenbrock <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>Isoflavone malonylglycosides</td>
<td>Mackenbrock <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>Kaempferol 3,7-O-glycoside</td>
<td>Schnabl (1986)</td>
</tr>
<tr>
<td>Orientin C-glycosides</td>
<td>Harborne <em>et al.</em> (1993)</td>
</tr>
<tr>
<td>Pterocarpan malonylglycosides</td>
<td>Mackenbrock <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>Quercetin 3-triglucoside</td>
<td>Weissenböck <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>7-Rhamnosyl-6-hydroxyluteolin</td>
<td>Harborne <em>et al.</em> (1993)</td>
</tr>
<tr>
<td>Shikimic acid</td>
<td>Holländner-Czytko and Amrhein (1983)</td>
</tr>
<tr>
<td>Tricin 5-gluco side</td>
<td>Harborne <em>et al.</em> (1993)</td>
</tr>
<tr>
<td><strong>Terpenoids</strong></td>
<td></td>
</tr>
<tr>
<td>Convallatoxin and other cardenolides</td>
<td>Löffelhardt <em>et al.</em> (1979)</td>
</tr>
<tr>
<td>Gentiopicroside</td>
<td>Keller (1986)</td>
</tr>
<tr>
<td>Oleanolic acid (3-O-glucoside)</td>
<td>Szakiel and Janiszowska (1993)</td>
</tr>
<tr>
<td>Oleanolic acid (3-O-glucuronide)</td>
<td>Szakiel and Janiszowska (1993)</td>
</tr>
<tr>
<td>Primary cardiac glycosides lanatoside A, C; purpureaglycoside A</td>
<td>Kreis and Reinhard (1987), Christmann <em>et al.</em> (1993)</td>
</tr>
<tr>
<td>Saponines (avenacosides)</td>
<td>Urban <em>et al.</em> (1983)</td>
</tr>
<tr>
<td><strong>Oligosaccharides</strong></td>
<td></td>
</tr>
<tr>
<td>Gentianose</td>
<td>Keller and Wiemken (1982)</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>Keller and Wiemken (1982)</td>
</tr>
<tr>
<td>Stachyose</td>
<td>Keller and Matile (1985)</td>
</tr>
<tr>
<td><strong>Nitrogen-containing compounds (excluding alkaloids)</strong></td>
<td></td>
</tr>
<tr>
<td>Cyanogenic glycosides (linamarin)</td>
<td>Saunders and Conn (1978)</td>
</tr>
<tr>
<td><strong>Sinapoylglucosides</strong></td>
<td>Sharma and Strack (1985)</td>
</tr>
<tr>
<td><strong>Alkaloids</strong></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>Mende and Wink (1987)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>Saunders (1979); Renaudin and Guern (1987)</td>
</tr>
</tbody>
</table>
TABLE II (continued)

Vacuolar sequestration of defence and signal compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secondary metabolites</strong></td>
<td></td>
</tr>
<tr>
<td>Berberine</td>
<td>Sato et al. (1993, 1994)</td>
</tr>
<tr>
<td>Betaine</td>
<td>Matoh et al. (1987)</td>
</tr>
<tr>
<td>Betalaines</td>
<td>Leigh (1983)</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>Fujiwake et al. (1980)</td>
</tr>
<tr>
<td>Catharanthine</td>
<td>Deus-Neumann and Zenk (1984)</td>
</tr>
<tr>
<td>Codeine</td>
<td>Pham and Roberts (1991), Roberts (1987)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Homeyer and Roberts (1984), Roberts (1987)</td>
</tr>
<tr>
<td>Lupanine</td>
<td>Mende and Wink (1987)</td>
</tr>
<tr>
<td>Morphine</td>
<td>Pham and Roberts (1991), Roberts (1987)</td>
</tr>
<tr>
<td>Noscapine</td>
<td>Pham and Roberts (1991)</td>
</tr>
<tr>
<td>Papaverine</td>
<td>Pham and Roberts (1991), Roberts (1987)</td>
</tr>
<tr>
<td><strong>Polyamines</strong></td>
<td></td>
</tr>
<tr>
<td>(5)-Reticuline</td>
<td>Deus-Neumann and Zenk (1986)</td>
</tr>
<tr>
<td>Sanguinarine</td>
<td>Matile et al. (1970)</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>Deus-Neumann and Zenk (1984)</td>
</tr>
<tr>
<td>(5)-Scoulerine</td>
<td>Deus-Neumann and Zenk (1986)</td>
</tr>
<tr>
<td>Senecionine N-oxide</td>
<td>Ehmke et al. (1987, 1988)</td>
</tr>
<tr>
<td>Serpentine</td>
<td>Deus-Neumann and Zenk (1984), Blom et al. (1991b)</td>
</tr>
<tr>
<td>Solanidine</td>
<td>Han et al. (1989)</td>
</tr>
<tr>
<td>Thebaine</td>
<td>Pham and Roberts (1991), Roberts (1987)</td>
</tr>
<tr>
<td>Vindoline</td>
<td>Deus-Neumann and Zenk (1984), Brisson et al. (1992)</td>
</tr>
<tr>
<td><strong>Defence proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Phytohaemagglutinin</td>
<td>Chrispeels (1991), Chrispeels and Raikhel (1992)</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>Ryan and Walker-Simmons (1983)</td>
</tr>
<tr>
<td>Chitinase</td>
<td>Mackenbrock et al. (1992)</td>
</tr>
<tr>
<td>β-1,3-Glucanase</td>
<td>Mackenbrock et al. (1992)</td>
</tr>
</tbody>
</table>

adjacent cells (Saunders and Conn, 1978; Kojima et al., 1979; Wajant et al., 1995). Upon wounding, for example by a herbivore, the cellular integrity breaks down, and both vacuolar contents and β-glucosidase come into contact (Fig. 1). The cyanogenic glycosides are hydrolysed. Hydroxynitrile lyase, which releases HCN, is found in the cytoplasm of both epidermal and mesophyll cells. The HCN generated is a strong inhibitor of mitochondrial respiration and thus a strong toxin. In *Hevea brasiliensis* leaves, the cyanogenic glycoside linamarin is also exclusively stored in the vacuole, and the linamarase in the apoplastic space (Gruhnert et al., 1994), indicating that
the spatial compartmentation of cyanogenic glycosides and their metabolizing enzymes have evolved several times during evolution.

In root tissues of horseradish (Armoracia rusticana), the glucosinolates in addition to ascorbate (an activator of myrosinase) are also stored in vacuoles (Fig. 2), but the hydrolysing enzyme, the myrosinase, is localized in the cytoplasmic membranes and in cell walls (Matile, 1980, 1984; Lüthy and Matile, 1984). Upon wounding, all components come into contact, and mustard oil is released, which is a strong animal deterrent, a membrane-destabilizing agent and an antibiotic.

The storage and activation of coumaroylglycosides follows a similar strategy
Fig. 2. Compartmentation of glucosinolates and myrosinase in the roots of horseradish (*Armoracia rusticana*). (After Matile (1984) and Wink (1993b).)

(Oba *et al.*, 1981; Alibert *et al.*, 1985). In *Melilotus alba*, *trans*-* and 2-cis*-2-hydroxycinnamic acid is sequestered in the vacuoles of epidermal and mesophyll cells; because of the abundance of mesophyll cells, they contain 90% or more of the glucosides present in leaves. The corresponding \(\beta\)-glucosidase appeared to be localized in the extracytoplasmic space. When leaves of *Melilotus* are wounded, the compartmentation breaks down, and both glucosides and \(\beta\)-glucosidase come into contact. As a result, coumarins are generated, which function as active defence compounds.

Quinolizidine alkaloids, which figure as the characteristic secondary metabolites of many legumes, especially within the Papilionoideae (Wink,
1993c; Käss and Wink, 1995), are produced by chloroplasts of leaf mesophyll cells (Wink and Hartmann, 1982). After synthesis they are exported to the vacuole, apparently by means of a tonoplast proton antiport carrier (Mende and Wink, 1987). Some of the alkaloids are transported via the phloem (Wink and Witte, 1984) to all other parts of a lupin plant, especially the stems and fruits. In stems and petioles, the alkaloids enter the epidermal cells and are taken up into the vacuole against a concentration gradient by a transport system (Wink and Mende, 1987) (Fig. 3). Concentrations of quinolizidine alkaloids in epidermal vacuoles can be as high as 200 mM. Since these alkaloids serve as defence compounds against herbivores (e.g. insects) the epidermal localization can be interpreted as a means to place them at a strategically important site, where they can ward off intruders just when they start nibbling at a plant (Wink, 1985, 1987, 1988, 1992).

Cardiac glycosides are produced in a number of unrelated plant families, such as the Scrophulariaceae, Apocynaceae, Ranunculaceae, Brassicaceae and Asclepiadaceae. In Digitalis lanata it could be shown that cardenolides are synthesized in green tissue. Following synthesis, the primary glycosides (such as lanatoside A and C) with a terminal glucose in the sugar side-chain (but not the secondary glycosides) are accumulated against a concentration gradient in the vacuoles of the source tissue, or after phloem transport in those of the sink tissues (Holz et al., 1992; Christmann et al., 1993). Cardiac glycosides inhibit Na\(^+\),K\(^+\)-ATPase, and are thus strong toxins in animals. Although cardiac glycosides are usually very effective in defence, a number of insects are known which have overcome this barrier and which use the dietary toxins for their own defence. A well-studied example is the monarch (Danaus plexippus), which became insensitive to cardiac glycosides through a single point mutation in the ouabain-binding site of Na\(^+\),K\(^+\)-ATPase (Holzinger et al., 1992; Holzinger and Wink, 1996).

Peptides and proteins which are employed in defence are also often localized in the vacuole, and include protease inhibitors, lectins, enzymes or other toxalbumins. In the case of the toxic proteins, which are typical for many seeds, we can see a dual function: besides their role as defence compounds, they serve at the same time as nitrogen stores. Upon wounding or infection, the synthesis of these defence proteins (such as chitinase or protease inhibitors) can be enhanced, and the newly made proteins may end up in the vacuole or the extracellular space/cell wall, depending on their sorting signals (Chrispeels, 1991). Some storage proteins have very low abundances of essential amino acids, such as methionine in seeds of legumes or lysine in Gramineae. These deficiencies can also be interpreted as an antiherbivore strategy, because a herbivore will show symptoms of ill health and growth retardation when reared on such a diet.

These examples clearly demonstrate the intricate but cooperative interplay between different compartments, tissues and their integration in the overall defence strategy of a plant.
B. XENOBIOTICS

As mentioned in the introduction, xenobiotics are metabolized in plants to form glycosides which may further be acylated by malonic acid; other conjugates are with glutathione and cysteine. As a general rule xenobiotics enhance their hydrophilic properties, i.e. water solubility, by these procedures, which take place in the cytoplasm.

It has been shown for a number of xenobiotics, especially for some pesticides, that the corresponding conjugates are sequestered in the vacuole (Coupland, 1991). For example, the plant growth regulator (2,4-dichlorophenoxy)acetic acid (2,4-D) diffuses into plant cells (e.g. in *Phaseolus vulgaris*), where it becomes hydroxylated in the 4-position. The hydroxylation occurs via the "NIH shift" mechanism and involves the displacement of the Cl group (Thomas *et al*., 1964). 4-OH-2,5-D is then glucosylated and malonylated; O-(malonyl)glucosyl 4-OH-2,4-D is stored in the vacuole (Schmitt and Sandermann, 1982; Sandermann, 1987). In the soya bean, 2,4-D forms amide conjugates with various amino acids instead (especially glutamyl and aspartyl derivatives) (Mumma and Davidonis, 1983). In addition, the precursor of ethylene is stored as 1-(malonylamino)-cyclopropane-1-carboxylic acid in the vacuole (Bouzayan *et al*., 1989). The herbicide paraquat slowly accumulates in the vacuoles of root cells of maize seedlings, but is also translocated to the shoot (DiTomaso *et al*., 1993).

Xenobiotics and secondary metabolites stored in the vacuole can be modified by some enzymes, for example peroxidases which are often also sequestered in the vacuole (for a review, see Wink, 1993b). It was shown that anthocyanins, anthocyanidins, alkaloids and many other compounds were oxidized by vacuolar peroxidases (Calderon *et al*., 1992; Wink, 1994). It should be recalled that peroxidases may have evolved for the detoxification of microbial toxins.

In addition to vacuolar storage, some conjugated xenobiotics, such as the O-(malonyl)glucoside of pentachlorophenol and N-(malonyl)-3,4-dichloroaniline, are released from the cells and stored in the apoplast (Winkler and Sandermann, 1989). In plant cell cultures, an export of secondary metabolites and other compounds into the medium ("extracellular lytic and storage compartment") can be regularly observed (Sandermann, 1994; Wink, 1985, 1994). For example, in cell cultures of *Lupinus polyphyllus* we even observed a malonyl derivative of lupanine (which was hitherto unknown from quinolizidine alkaloids) in the medium (Wink, 1994).

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Fig. 3. Compartmentation of quinolizidine alkaloids in stems of *Lupinus polyphyllus*. (a) Vacuolar sequestration in epidermal cells. Recall that the synthesis of these alkaloids takes place in the chloroplasts of leaf mesophyll cells (Wink, 1987, 1993c). (b) Schematic drawing of lupanine transport across the tonoplast (Mende and Wink, 1987), employing a lupanine proton antiport mechanism.
C. MECHANISMS UNDERLYING VACUOLAR SEQUESTRATION

1. Uptake across the tonoplast
Biomembranes are semipermeable, and small and lipophilic molecules, such as O₂, CO₂, N₂ or benzene, can pass membranes rapidly by free diffusion. Polar metabolites, such as sugars, ions and charged molecules (e.g. amino and other acids), will diffuse freely at a very low rate. The tonoplast does not appear to differ it its semipermeability from other membranes, such as the plasmalemma. For example, lipophilic synthetic dye probes, such as methyl red, methylene blue, auramine O and acridine orange, pass both membranes readily by diffusion (Wink, 1990). Dyes that carry charged sulfate groups or are glycosides, such as procion blue, carminic acid, alizarin red and indigocarmine, cannot pass either membrane by free diffusion (Wink, 1990). For the charged or polar molecules, the tonoplast and the plasmalemma thus form penetration barriers. As discussed by Martinoia and Ratajczak and Blumwald and Gelli (this volume), the uptake of ions, sugars and amino acids is achieved with aid of special channels, carriers and pumps (Hediger, 1994), some of which have already been purified and reconstituted in artificial membranes, such as the carriers for glutamine or arginine (Thune and Dietz, 1991) and malate (Martinoia et al., 1991). In a few cases the genes for corresponding plasma membrane carriers have been isolated and expressed (Sauer and Tanner, 1989; Riesmeier et al., 1992; Hsu et al., 1993; Tsay et al., 1993).

Most of the defence and signal compounds and conjugated xenobiotics found in the vacuole (Table II) are hydrophilic, polar (such as sugars and glycosides) or even charged molecules, such as non-protein amino acids, alkaloids, some glycosides or proteins. Since these compounds have not been synthesized in the vacuole, they have to pass the tonoplast first in order to accumulate in the vacuole.

Considering the structural diversity of secondary metabolites, we cannot assume that the biochemical mechanisms which lead to their sequestration in vacuoles are identical. More lipophilic metabolites will pass the tonoplast by simple diffusion, as was shown for several weakly basic alkaloids, such as nicotine, ajmalicine, colchicine, vinblastine, ergotamine, sanguinarine, vindoline, quinine and cinchonamine (Kurkdjian, 1982; Renaudin and Guern, 1987; McCaskill et al., 1988; Renaudin, 1989; Hauser and Wink, 1990; Blom et al., 1991a). Also, ascorbate and dehydroascorbate appear to reach the vacuole of Hordeum vulgare protoplasts without a carrier, although transport across the plasmamembrane was carrier mediated (Rautenkranz et al., 1994).

Other alkaloids, which are charged species under cytosolic pH conditions or polar glycosides, appear to pass the tonoplast with aid of a carrier mechanism. Examples are the alkaloids (S)-scoulerine, (S)-reticuline, catharanthine (Deus-Neumann and Zenk, 1984, 1986), atropine, lupanine,
sparteine, 13-hydroxylupanine (Mende and Wink, 1987), senecionine-\(N\)-oxide (Ehmke et al., 1987, 1988), dopamine (Homeyer and Roberts, 1984) and polyamines (Pistocchi et al., 1988). Examples for glycosides and other polar metabolites include primary cardiac glycosides (Kreis and Reinhard, 1987; Kreis and Hölz, 1991), coumaroylglycosides (Alibert et al., 1985; Rataboul et al., 1985; Werner and Matile, 1985), acylated anthocyanins (Hopp and Seitz, 1987), flavonoids such as apigenin-7-O-(β-d-malonylglucoside) (Matern et al., 1986; Matern, 1987), 1-(malonylamino)cyclopropane-1-carboxylic acid (Bouzayan et al., 1989) and glucosides and glucuronides of the triterpene oleanolic acid. In the case of latex vacuoles of \textit{Papaver somniferum}, morphine uptake was ATP stimulated, but the authors suggest the presence of an alkaloid channel instead of a specific morphine transporter (Roberts et al., 1991). It is remarkable that for (\(S\))- or (\(R\))-reticuline or (\(S\))- and (\(R\))-scoulerine, these carriers discriminated the naturally occurring (\(S\))-configured compounds and were thus stereoselective (Deus-Neumann and Zenk, 1986).

Whether particular carriers exist for all these allelochemicals or whether they can hijack existing carriers for primary metabolites cannot be stated with certainty at present. The transport system for 1-aminocyclopropane-1-carboxylic acid (ACC) can be classified as a neutral \(l\)-amino acid carrier with a high affinity for ACC and other non-polar aminoacids (Saftner, 1994), supporting this possibility. Oat aleurone protoplasts and epidermal cells of \textit{Allium cepa} take up a number of fluorescent membrane probes and sequester them in the vacuole. The uptake of carboxyfluorescein, lucifer yellow, cascade blue hydrazide and sulforhodamine G into vacuoles can be inhibited by probenecid, indicating that the transport is carrier mediated (Oparka et al., 1991; Wright and Oparka, 1994). It is very likely that this carrier is responsible for the transport of indigenous compounds and that these xenobiotics have hijacked it. Since the above-mentioned membrane probes have physicochemical properties similar to some phloem-mobile xenobiotics, these results have obvious implications for the detoxification and compartmentation of xenobiotics in plants (Wright and Oparka, 1994).

For other xenobiotics, evidence has been presented that derived glutathione derivatives (such as glutathione \(S\)-conjugates of \(N\)-ethylmaleimide and of metolachlor) cross the vacuolar membrane with the aid of a group-specific transporter that is widely distributed in plants (Martinoia et al., 1993; Li et al., 1995a,b, 1996) and which is remarkably similar to the glutathione \(S\)-conjugate export pumps of mammalian liver.

2. \textit{Vacuolar sequestration against a concentration gradient} The concentrations of ions, sugars, acids, signal and defence chemicals is often remarkably high in vacuoles and orders of magnitude lower in the cytoplasm (Leigh et al., 1981; Boller and Wiemken, 1986). Thus, all these molecules have to be sequestered in the vacuole against a concentration
gradient. The question to be considered next concerns, therefore, the driving force for uphill accumulation in vacuoles.

As discussed by Lüttege and Ratajczak, and Davies in this volume, the major tonoplast protein is an H⁺-ATPase (Leigh and Walker, 1980; Sze, 1985; Bremberger et al., 1988), which in addition to a pyrophosphatase (Leigh and Walker, 1980; Rea and Sanders, 1987; Bremberger et al., 1988; Zehn et al., this volume) transports protons into the vacuole (Thom and Komor, 1984; Sze, 1985; Rea and Sanders, 1987; Hedrich et al., 1989; Taiz, 1992). Thus, the vacuolar hydrogen ion concentration is orders of magnitude higher than that of the cytoplasm. This gradient (proton motive force, PMF) can be utilized for secondary active transport systems (Reinhold and Kaplan, 1984; Blumwald, 1987; Hedrich and Schröder, 1989; Kurkdjian and Guern, 1989), such as substrate proton antiport mechanisms (Hager and Hermsdorf, 1981; Lüttege et al., 1981; Thom and Komor, 1984; Blumwald and Poole, 1985a,b; Briskin et al., 1985; Blumwald, 1987; Blackford et al., 1990; Getz, 1991) as discussed by Martinoia and Ratajczak and Blumwald and Gelli in more detail in this volume.

There is some experimental evidence that some secondary metabolites and conjugates of xenobiotics are also transported into the vacuole by an H⁺ antiport mechanism, and examples include lupanine (Mende and Wink, 1987), (S)-reticine, (S)-scoulerine (Deus-Neumann and Zenk, 1986), and 1-(malonylamino)cyclopropane-1-carboxylic acid (Bouzayan et al., 1989). In all these cases, vacuolar uptake was dependent on Mg ATP and could be inhibited by reagents which dissipate proton gradients. In addition, lupanine transport was enhanced by K⁺, indicating either that the membrane potential could be additionally involved (Mende and Wink, 1987) or that K⁺ activates the H⁺-translocating pyrophosphatase as was shown for beet vacuoles (Leigh, 1983).

Conversely, although the transport of glutathione S-conjugates of xenobiotics was MgATP-dependent, transport was not inhibited by compounds which disrupt secondary activated uptake processes, i.e. proton antiport mechanisms cannot be the driving force in this case. Instead, the carrier appears to be a specific ATPase with a pronounced sensitivity to vinblastine, vanadate and verapamil, similar to the export pumps in mammalian liver (Martinoia et al., 1993; Tommasini et al., 1993; Li et al., 1995a). The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump and also shows substantial sequence homology to the human multidrug resistance-associated protein (MRP1), and might also be related to the plant glutathione S-conjugate transporter (Li et al., 1996; Tommasini et al., 1996). In plants, the transport of oxidized glutathione (GSSG) is also achieved by this pump and is competitively inhibited by the glutathione S-conjugate of the herbicide metolachlor (Tommasini et al., 1993). Once in the vacuole the conjugates are degraded by a carboxypeptidase, suggesting that glutathione S-conjugates represent the
transport form but not the storage form of xenobiotics (Wolf et al., 1996). The activity of these transporters appears to be inducible by some xenobiotics, such as herbicide antidotes (so-called safener) (Gaillard et al., 1994; Li et al., 1995b). Recently, it has been shown that the protein encoded by the Bronze-2 gene in maize, which is responsible for the deposition of anthocyanins in the vacuole, is a glutathione S-transferase (Marrs et al., 1995). Thus, anthocyanins, e.g. cyanidin-3-glucoside, as well as xenobiotics are conjugated with glutathione. Many herbicide conjugates are metabolized to cysteine conjugates and then acylated with malonic acid. Since malonlated cyanidin-3-glucoside is the major maize anthocyanin, Marrs et al. (1995) postulate by analogy that anthocyanin is transported into the vacuole as the glutathione S-conjugate with the aid of the ATP-dependent glutathione S-conjugate “export” pump. Once in the vacuole, anthocyanins are further converted to the malonyl derivative. However, Hopp and Seitz (1987) had shown that a carrier system exists for acylated anthocyanins at the tonoplast which would contradict the assumption of Marrs et al. (1995). Whether the glutathione conjugate pump also transports other secondary metabolites remains to be shown.

It has been emphasized that an apparent uphill transport can also be achieved by certain “trapping” reactions (Matile, 1978, 1984; Boller and Wiemken, 1986). Normally, passive transport or diffusion will come to a standstill when equal concentrations are reached on both sides of the tonoplast. If we assume that the molecule which enters the vacuole is trapped or otherwise changed, then it is removed from the equilibrium, and the transport process can go on until all trapping molecules are exhausted and equal concentrations of “free” molecules are reached on both sides.

The trapping reactions discussed (Table III) include “ion traps” (which could be relevant for nitrogenous compounds, which become protonated in the vacuole (Nishimura, 1982; Boller and Wiemken, 1986; Guern et al., 1987; Renaudin, 1989), e.g. alkaloids do not pass membranes as protonated molecules except when highly lipophilic (Kurkdjian, 1982; Renaudin, 1989; Hauser and Wink, 1990)). The monoterpenoid indole alkaloids ajmalicine and vindoline appear to cross the tonoplast by simple diffusion (Renaudin and Guern, 1987; McCaskill et al., 1988; Renaudin, 1989; Blom et al., 1991b) and not by carrier-mediated transport, as reported earlier (Deus-Neumann and Zenk, 1984, 1986). In the vacuole, ajmalicine is effectively converted into the more polar serpentine by basic peroxidases, which cannot leak out of the vacuole (Blom et al., 1991b). It has been suggested that intravacuolar serpentine binds to the tonoplast (Pradier et al., 1988).

Other trapping reactions include crystallization (often observed for calcium oxalate; Franceschi and Horner, 1980), conformational changes (e.g. apigenin 7-O-(6-O-malonylglicoside) (Matern et al., 1983, 1986; Matern, 1987) and cyanidin-3-O-sinapoylxylosylglycosyl galactoside (Hopp and Seitz, 1987), and isomerization (discussed for cis- and trans-coumarylglicosides;
TABLE III
Mechanisms for uptake and sequestration of defence/signal compounds and xenobiotics in vacuoles

<table>
<thead>
<tr>
<th>Uptake mechanisms</th>
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<tbody>
<tr>
<td>Membrane/vesicle fusion:</td>
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<tr>
<td>- Endoplasmic reticulum vesicles (e.g. prolamin protein</td>
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<tr>
<td>bodies)</td>
</tr>
<tr>
<td>Simple diffusion:</td>
</tr>
<tr>
<td>- Lipophilic secondary metabolites</td>
</tr>
<tr>
<td>Active/passive transport (channels/transporters):</td>
</tr>
<tr>
<td>- Ions, amino acids, organic acids, sugars, polar</td>
</tr>
<tr>
<td>secondary metabolites;</td>
</tr>
<tr>
<td>- conjugated xenobiotics;</td>
</tr>
<tr>
<td>- glutathione S-conjugates</td>
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</table>

<table>
<thead>
<tr>
<th>Mechanisms for uphill accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active transport:</td>
</tr>
<tr>
<td>- ATPase-coupled “pumps”</td>
</tr>
<tr>
<td>Secondary active transport:</td>
</tr>
<tr>
<td>- Proton gradients generated by H⁺-ATPase, pyrophosphatase</td>
</tr>
<tr>
<td>(proton motive force) used by proton antiport carriers</td>
</tr>
<tr>
<td>Trapping mechanisms:</td>
</tr>
<tr>
<td>- Protonation of alkaloids in acidic vacuoles</td>
</tr>
<tr>
<td>- Binding of secondary compounds to tannin, other</td>
</tr>
<tr>
<td>phenolics or polyphosphates</td>
</tr>
<tr>
<td>- Complexation of alkaloids with chelidonic acid,</td>
</tr>
<tr>
<td>meconic acid</td>
</tr>
<tr>
<td>- Binding of secondary metabolites to proteins?</td>
</tr>
<tr>
<td>- Crystallization (calcium oxalate)</td>
</tr>
<tr>
<td>- Conjugation</td>
</tr>
<tr>
<td>- Change of conformation or configuration</td>
</tr>
</tbody>
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Rataboul et al., 1985). Whereas the trans to cis isomerization is almost irreversible under natural conditions and would trap the coumarylglycosides permanently in the vacuole, the conformational shift observed with acylated flavonoids and anthocyanins is pH-dependent and thus reversible (Matern, 1987).

Another trapping procedure could be the binding of molecules to complexing compounds such as tannins or polyphosphates (Matile, 1978). Examples for such complexing reagents are chelidonic and meconic acids. Isolated latex vacuoles of Chelidonium majus take up sanguinarine and various other lipophilic alkaloids (Matile, 1978; Hauser and Wink, 1990). It could be shown that these vesicles contain about 660 mM (range 200–1300 mM) chelidonic acid, which readily complexes alkaloids (Hauser and Wink, 1990). The reaction of these alkaloids with chelidonic acid obviously provides the buffer for apparent accumulation against a concentration gradient. We do not know, however, the mechanism for concentrating chelidonic acid in vesicles. In the case of latex vacuoles of Papaver somniferum, the concentration of meconic acid can be as high as 250 mM,
and was considered as relevant for trapping of morphine, codeine, papaverine and other poppy alkaloids (Pham and Roberts, 1991). In cells of Coptis japonica, the berberine content of the vacuoles was correlated with their malic acid accumulation; the resulting malate-berberine complex might trap berberine in the vacuole (Sato et al., 1992). The accumulation of lupanine in epidermal vacuoles was also favoured by malate, suggesting a similar mechanism (Wink and Mende, 1987). Crystallization, which has been observed for the alkaloids berberine and sanguinarine and for calcium oxalate (Renaudin and Guern, 1990), is another, albeit rare, trapping reaction.

Summarizing, it is apparent that the uptake of metabolites into the vacuole can be directly or secondarily by energy-dependent export “pumps” or proton antiports, or can be accomplished by various trapping or binding reactions. The trapping mechanisms are compatible with both transport and diffusion processes.

III. CONCLUSIONS

A typical feature of plants and other sessile organisms is the production of secondary metabolites, which can be considered as chemical defence and signal compounds. They are mediators of plant–plant, plant–herbivore and plant–microorganism interactions, and thus important for the fitness of plants. Although chemical defence appears to be the major function, some coloured or scented metabolites play important additional roles in reproductive biology (attraction of pollinating or seed-dispersing animals). Defence and signal functions can only be achieved if local concentrations of secondary metabolites are high enough. Most of these compounds are synthesized in the cytosol or in plastids. Since many of the allelochemicals are also toxic for the producing plant, they need to be stored in a separate compartment, which is the vacuole in the case of hydrophilic compounds. The storage of vacuolar defence or signal compounds is often tissue-specific, e.g. many compounds are accumulated in a strategically favourable position, such as epidermal cells, which have to ward off small enemies in the first place. Here, the vacuoles function as “defence or signal compartments”.

Lipophilic compounds may cross the tonoplast by simple diffusion, whereas hydrophilic ones, such as amino acids, organic acids, ions and many polar allelochemicals (alkaloids, glycosides), are taken up by carrier- or channel-mediated processes. The driving force for uphill transport can be proton gradients which are generated by tonoplast-associated H⁺ ATPases and H⁺ pyrophosphatases. In other cases, diverse trapping processes within the vacuole seem to be involved.

In general, xenobiotics of plant, microbial or industrial origin are handled by plants in a similar way to secondary metabolites, although some marked differences also exist. Vacuolar sequestration is a most important trait for
both secondary metabolites and man-made pesticides; in the future, it will be important to discuss results from both fields in a closer context as reactions seen today obviously evolved under similar evolutionary constraints.

Depending on the cell type in question, a vacuole can function in a variety of ways: besides turgor regulation the vacuole can serve as a lytic, storage, defence or signal compartment (Wink, 1993b). It seems likely that some of these functions are exclusive, and not all vacuoles within one cell or within the same tissue must have identical properties. To make the picture even more complex, all these functions must be considered as dynamic and not static, being regulated in space and time (Boller and Wiemken, 1986) and under environmental constraints.

ACKNOWLEDGEMENTS

Work from our laboratory was supported by grants of the Deutsche Forschungsgemeinschaft. I would like to thank my co-workers, Dr P. Mende, Dr M. T. Hauser and Dr R. Perrey for cooperation, and Mrs C. Theuring, U. Schade, M. Weyerer and U. Dostal for technical assistance.

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