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Accumulation of alkaloids in plant vacuoles does not involve an ion-trap mechanism*

B. Deus-Neumann and M.H. Zenk

Lehrstuhl Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-8000 München 2, Federal Republic of Germany

Abstract. Alkaloid uptake into vacuoles isolated from a *Fumaria capreolata* L. cell suspension culture was investigated. The uptake is carrier-mediated as shown by its substrate saturation, its sensitivity to metabolic inhibitors and especially by its exclusive preference for the (*S*)-forms of reticuline and scoulerine while the (*R*)-enantiomers which do not occur in this plant species were strictly discriminated. The carrier has a high affinity for (*S*)-reticuline with a $K_m = 0.3$ µM. The rate of alkaloid uptake was 6 pmol$\cdot h^{-1} \cdot \mu l^{-1}$ vacuole, and 0.03 mg alkaloid$\cdot mg^{-1}$ vacuolar protein were taken up. Transport was stimulated five- to seven-fold by ATP and was inhibited by the ATPase inhibitors N,N′-dicyclohexylcarbodiimide and 4-4′-diisothiocyanatostilbene-2,2′disulfonic acid, as well as by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. A number of alkaloids did not compete with labelled (*S*)-reticuline for uptake into vacuoles. The uptake system is absolutely specific for alkaloids indigenous to the plant from which the vacuoles were isolated. Slight modifications of the topography of an alkaloid molecule even with full retention of its electrical charge results in its exclusion. Alkaloid efflux was also shown to be mediated by a highly specific energy-dependent carrier. These results contradict the previously proposed ion-trap mechanism for alkaloid accumulation in vacuoles. A highly specific carrier-mediated and energy-dependent proton antiport system for alkaloid uptake and release is postulated.

Key words: Alkaloid (vacuole, uptake) – *Fumaria* – Compartmentation (alkaloids) – Stereospecific uptake (alkaloids) – Vacuole (alkaloid uptake).

Introduction

The vacuoles of higher plants are storage compartments for alkaloids (e.g. Matile 1978; Saunders 1979; Deus and Zenk 1982; Neumann et al. 1983; Homeyer and Roberts 1984). The synthesis of alkaloids takes place in the cytoplasm (Deus-Neumann and Zenk 1984; Zenk 1985) or, in special cases, in plastids (Wink and Hartmann 1982) or vesicles (Amann et al. 1986). The transport of alkaloids from the cytosol across the vacuolar membrane, the tonoplast, into the vacuole is crucial for the deposition of alkaloids inside the vacuolar space (Matile 1984). A simple model for alkaloid accumulation in vacuoles of plant cells is based on the fact that the vacuolar cell sap has an acidic pH. According to this model, alkaloids can freely pass the tonoplast by diffusion in their lipophilic forms. In the acidic vacuole, they are protonated and therefore trapped as cations for which the tonoplast is slightly or not permeable at all (Müller et al. 1976; Guern et al. 1982; Neumann et al. 1983; Matile 1984). In addition, phenolic and other components of the cell sap may contribute to the immobilization of alkaloids by salt and complex formation (Matile et al. 1970; Matile 1976). These mechanisms are supported by the classical observation that vacuoles can be stained by neutral red. The mechanism of the vacuolar accumulation of this basic stain is regarded analogous to the mechanism of alkaloid accumulation (Matile 1984). The phenomenon has been called the ion-trap mechanism.

* Dedicated to Professor Harry Beevers, Santa Cruz, on the occasion of his 60th birthday

Abbreviations: CCCP = carbonylcyanide *m*-chlorophenylhydrazone; DCCD = N,N′-dicyclohexylcarbodiimide; DIDS = 4-4′-diisothiocyanatostilbene-2,2′disulfonic acid

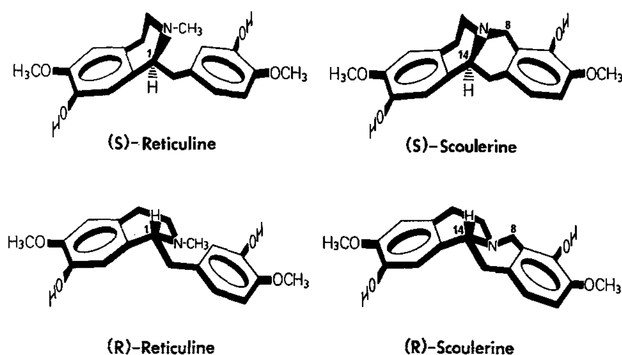

Fig. 1. Enantiomeric forms of alkaloids used in the vacuolar transport experiments

First doubts about the ion-trap mechanism as an explanation for alkaloid accumulation in vacuoles arose when it was demonstrated that isolated vacuoles of an alkaloid-containing plant (*Catharanthus*) take up only those alkaloids in substantial amounts which are specific for this particular plant. In contrast, vacuoles of an alkaloid-free plant (carrot) did not take up these alkaloids (Deus and Zenk 1982). This was the first demonstration of a highly sensitive and selective control mechanism for the transport of alkaloids across the tonoplast. This observation has further been investigated (Deus-Neumann and Zenk 1984), and the vacuolar alkaloid transport was characterized as an active, probably energy-requiring mechanism which is sensitive to temperature and the pH of the surrounding medium. The alkaloids tested accumulated inside the vacuoles against a concentration gradient. Some of these observations were later confirmed by Homeyer and Roberts (1984). For a further characterization of this highly specific vacuolar transport system, we decided to investigate the transport behaviour of two alkaloids in either their "natural" (*S*)- or in their "unnatural" (*R*)-configuration which are not found to occur in *Fumaria capreolata*, the plant species under investigation (Tanahashi and Zenk 1985). The respective enantiomers differ only in their chirality at one C-atom but not in their charge (Fig. 1). These experiments were expected to give a definitive answer as to the validity of the ion-trap mechanism.

Material and Methods

Plant material. Callus cultures of *Fumaria capreolata* L. were established from seeds in 1978 on Linsmaier and Skoog (1965) medium supplemented with 2,4-dichlorophenoxyacetic acid (10^{-6} M) and 1-naphthaleneacetic acid (10^{-6} M). Subsequently, callus cells were taken in suspension culture and cultivated in the same medium (75 ml) in 300-ml Erlenmeyer flasks on a gyratory shaker (100 rpm) at 23° C in continuous light (650 lx). Six-day-old cultures were used in the experiments. Other cell cultures were provided by our cell-culture laboratory.

Chemicals and radiochemicals. Adenosine 5′-triphosphate (sodium salt), N,N′-dicyclohexylcarbodiimide (DCCD), 4-4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and *p*-hydroxymercuribenzoate (CMB) were provided by Sigma (München, FRG). [U-^{3}H]Ajmaline ($2.78 \cdot 10^8$ Bq·μmol^{-1}), and [U-^{3}H]vindoline ($5.18 \cdot 10^8$ Bq·μmol^{-1}) were custom-synthetized by Amersham-Buchler (Braunschweig, FRG). [N-C^{3}H$_3$](*S*)-Reticuline ($2.67 \cdot 10^8$ Bq·μmol^{-1}), and [N-C^{3}H$_3$](*R*)-reticuline ($1.19 \cdot 10^8$ Bq·μmol^{-1}) were synthetized (Steffens et al. 1985) from the optically pure norreticuline enantiomers and [C^{3}H$_3$]S-adenosylmethionine in a reaction catalysed by norreticuline-N-methyltransferase. [8-^{3}H](*S*)-Scoulerine ($1.78 \cdot 10^8$ Bq·μmol^{-1}) was synthetized from [N-C^{3}H$_3$](*S*)-reticuline using the berberine bridge enzyme (Steffens et al. 1984). [8-^{3}H](*R*)-Scoulerine ($0.57 \cdot 10^8$ Bq·μmol^{-1}) was prepared by chemical racemisation of (*S*)-scoulerine through dehydroscoulerine and subsequent reduction by BH_4^-, followed by the specific oxidation of the (*S*)-enantiomer by prolonged incubation with (*S*)-tetrahydroprotoberberine oxidase (Amann et al. 1984). [^{3}H]Scopolamine ($2.48 \cdot 10^7$ Bq·μmol^{-1}) and [^{3}H]nicotine ($9.25 \cdot 10^7$ Bq·μmol^{-1}) were synthetized in our laboratory using standard methods. Catharanthine was provided by Eli-Lilly (Indianapolis, USA), codeine was from Boehringer (Ingelheim, FRG), morphine from Merck (Darmstadt, FRG), and serpentine from Boehringer (Mannheim, FRG). Our thanks are due to Professor N. Nagakura (Kobe, Japan) who synthetized unlabelled optically pure (*S*)- and (*R*)-reticuline as well as (*S*)- and (*R*)-norreticuline. All other chemicals used were standard commerical products of analytical grade.

Isolation of protoplasts. Protoplasts were isolated as described by Schieder (1975) using the following enzymes: Meicellase (All Japan Biochemicals Co., Nishinomiya, Japan) and Pectinol D (Röhm, Darmstadt, FRG). For the degradation of cell walls, 30 g cells were incubated for 4 h at 23° C in 200 ml solution of 5% Meicellase and 3% Pectinol D in a mixture of equal volumes of 0.5 M mannitol and 0.2 M $CaCl_2$, pH 5.8.

Isolation of vacuoles. Vacuoles were prepared as described by Grob and Matile (1979) with a slight modification of the standard isolation medium. For lysis of protoplasts, a solution containing 2-amino-2-(hydroxymethyl)-1,3-propanediol(Tris)-HCl (50 mM, pH 7.6), ethylenediaminetetraacetic acid (EDTA, 5 mM), and 0.475 M NaCl was used. Isolated vacuoles were purified as described previously (Deus-Neumann and Zenk 1984).

Uptake experiments. The uptake of alkaloids by isolated vacuoles was investigated as previously described (Deus-Neumann and Zenk 1984). The accumulation of alkaloids within the vacuoles was measured using the centrifugation method developed by Heldt and Sauer (1971). The uptake of labelled alkaloids could be determined directly by measuring the radioactivity of the vacuole sediment. In the case of non-labelled alkaloids, the vacuole sediment was extracted with 100 μl 80% ethanol at 40° C for 30 min. The amount of alkaloids taken up by the vacuoles was measured with specific radioimmunoassays.

Determination of alkaloid content. A known amount of cells, protoplasts or vacuoles was extracted with 80% ethanol for

30 min. The unlabelled alkaloids in the extracts were measured after appropriate dilution with the ultrasensitive, enantiomer-specific radioimmunoassay (RIA). Codeine, morphine, (*R*)- and (*S*)-reticuline were determined by separate RIAs (unpublished results); (*S*)-nicotine RIA (unpublished results); catharanthine RIA (unpublished results); serpentine RIA (Arens et al. 1978); vindoline RIA (Westekemper et al. 1980).

Microscopy. A Zeiss (Oberkochen, FRG) photomicroscope II was used throughout. The number of cells, protoplasts and vacuoles was counted in a haemocytometer (Neubauer, Walter Graf, Wertheim, FRG). Ten individual samples were counted for one determination.

Results

Alkaloid compartmentation. Cell suspension cultures of *Fumaria capreolata* are most remarkable in that they synthetize and accumulate isoquinoline alkaloids belonging to five different structural types; among these are the benzylisoquinoline alkaloid, (*S*)-reticuline, and the tetrahydroprotoberberine alkaloid, (*S*)-scoulerine (Tanahashi and Zenk 1985). While reticuline is normally regarded as a biosynthetic intermediate for a great number of isoquinoline alkaloids (e.g. Cordell 1981), it accumulates in this cell culture in substantial amounts. At the onset of this study the intracellular compartmentation of this alkaloid had to be elucidated. For this purpose, cells, protoplasts, and isolated vacuoles from *F. capreolata* were examined for (*S*)- as well as (*R*)-reticuline by the enantiomer-selective radioimmunoassays. It was clearly established that within experimental error (*S*)-reticuline was exclusively located within the vacuolar compartment (Table 1). Furthermore no evidence was found for the occurrence of the (*R*)-enantiomer within this species. These results extend the general observation that alkaloids are stored within the vacuolar compartment, even if, such as in this case, the alkaloid in question must be regarded as an intermediate.

Table 1. Comparison of (*S*)- and (*R*)-reticuline contents in cells, protoplasts and isolated vacuoles of *Fumaria capreolata* as determined by stereospecific radioimmunoassays (detection limit: 10^{-7}%)

Compartment	Reticuline enantiomer (nmol per 10^6 cells, protoplasts or vacuoles)	
	(*S*)	(*R*)
Cell	0.060	0.00
Protoplast	0.055	0.00
Vacuole	0.054	0.00

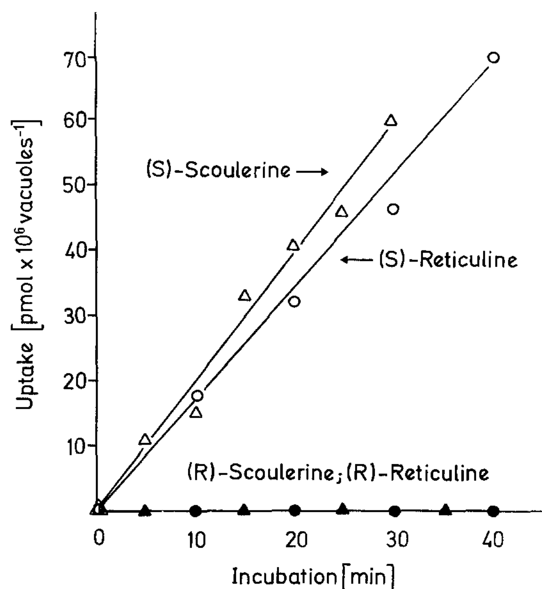

Fig. 2. Time course of uptake of (*S*)-reticuline (o—o), (*R*)-reticuline (●—●), (*S*)-scoulerine (△—△), and (*R*)-scoulerine (▲—▲) into isolated vacuoles of *Fumaria capreolata*. Vacuoles were suspended in the medium of Doll et al. (1979) containing 1 mM $MgCl_2$ and 1.5 mM ATP, and 500 pmol ^3H-labelled alkaloids were added per ml of vacuolar suspension ($4 \cdot 10^5$ vacuoles$\cdot ml^{-1}$) in a total volume of 3 ml. At the times indicated, samples were taken from the suspension and alkaloid uptake by the vacuoles was terminated by filtration centrifugation

Stereospecificity of alkaloid uptake by isolated vacuoles. According to the ion-trap mechanism hypothesis the hydrophobic alkaloid diffuses freely through the tonoplast membrane and is subsequently trapped in the acidic sap of the vacuole in its protonated form. In the case of *F. capreolata*, a vacuolar pH of at least 5 was indicated by neutral red staining. Therefore, the physical conditions for testing the ion-trap mechanism were present. The uptake of alkaloids by isolated *Fumaria* vacuoles was studied first with the tritium-labelled pair of benzylisoquinolines, (*S*)- and (*R*)-reticuline. The same batch of vacuoles was used in the two separate experiments. To a total of $1.2 \cdot 10^6$ vacuoles suspended in 3 ml of incubation medium, 1.5 nmol labelled (*S*)- or (*R*)-reticuline were added. At different time intervals, samples were taken from the suspension and subjected to filtration centrifugation (Heldt and Sauer 1971). The amount of (*S*)- or (*R*)-reticuline taken up by the vacuoles was calculated from the radioactivity measured in the vacuolar fraction after centrifugation. As shown in Fig. 2, only (*S*)-reticuline was taken up by the vacuoles while its (*R*)-enantiomer was absolutely discriminated. Uptake of (*S*)-reticuline was linear for about 40 min, then levelled off and reached a maximal value at 50 min. Subsequently, the vacuoles

started to lyse, reflected by a decline in the net accumulation of radioactivity. The experiment was repeated under the same experimental conditions as above but using unlabelled (*S*)- and (*R*)-reticuline. Recovered vacuolar fractions were analysed in triplicate by specific radioimmunoassays for both enantiomers.

Computing for the endogenous (*S*)-reticuline inside the vacuoles, the uptake of exogenous alkaloid corresponded exactly with the values obtained using labelled alkaloids. Again, only (*S*)-reticuline was taken up by the vacuoles, while the (*R*)-enantiomer was not. This result was checked by using an enantiomeric pair of tetrahydroprotoberberines. Only (*S*)-scoulerine has previously been shown to be present in *F. capreolata* suspension cells (Tanahashi and Zenk 1985). Uptake of (*S*)-scoulerine was linear over a period of at least 30 min, while the (*R*)-enantiomer was not taken up at all. In all subsequent experiments with isolated vacuoles an incubation period of 40 min was used in the reticuline, and 30 min in the scoulerine experiments.

Dependency of alkaloid uptake on pH. Uptake of (*S*)-reticuline and (*S*)-scoulerine into *F. capreolata* vacuoles as a function of the pH of the incubation medium was determined. Fig. 3 clearly demonstrates that accumulation of both, (*S*)-reticuline and (*S*)-scoulerine, was highly sensitive to external pH. Interestingly, maximal uptake of (*S*)-reticuline was observed at pH 7.5 while maximal uptake of (*S*)-scoulerine occurred at a 10-fold higher H^+ concentration. Within the tested pH range (pH 5.0 to pH 8.0) no uptake of the respective (*R*)-enantiomers was observed. The uptake experiments were subsequently carried out at pH 7.5 for reticuline, and at pH 6.5 for scoulerine.

The effect of ATP on alkaloid uptake. In our previous experiments on indole-alkaloid uptake into *C. roseus* vacuoles (Deus-Neumann and Zenk 1984) we were unable to detect any stimulation of alkaloid uptake by the addition of ATP to the medium. We presented, however, indirect evidence (positive effect of K^+ and Mg^{2+} on transport; inhibitor effects) which indicated that alkaloid transport into *C. roseus* vacuoles is an active, energy-requiring process and that the energy source may be ATP. To test the influence of ATP on isoquinoline transport into *Fumaria* vacuoles, the incubation medium was complemented with ATP in concentrations from 0 to 2 mM. Uptake of labelled isoquinolines was determined after filtration centrifugation. As shown in Fig. 4, the addition of

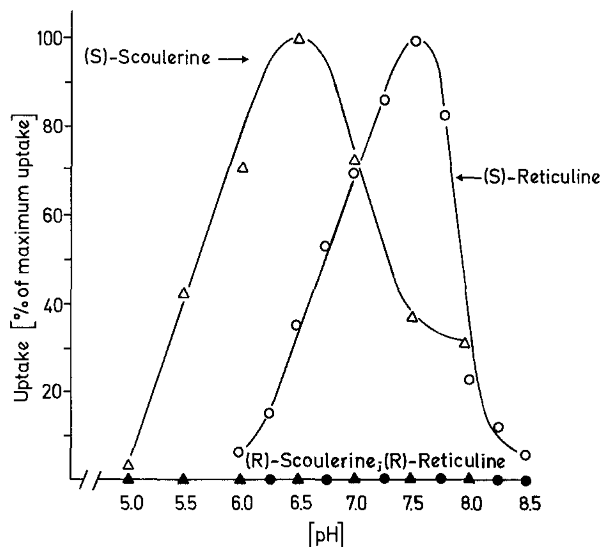

Fig. 3. Effect of pH on uptake of ^3H-labelled (*S*)-reticuline (○——○), (*R*)-reticuline (●——●), (*S*)-scoulerine (△——△), and (*R*)-scoulerine (▲——▲) by isolated vacuoles of *Fumaria capreolata*. Incubation media contained 0.5 mM and 1.5 mM ATP in the reticuline and scoulerine experiments, respectively. The alkaloid uptake was determined after 40 min (reticuline) and 30 min (scoulerine) incubation by filtration centrifugation (for experimental data see Fig. 2)

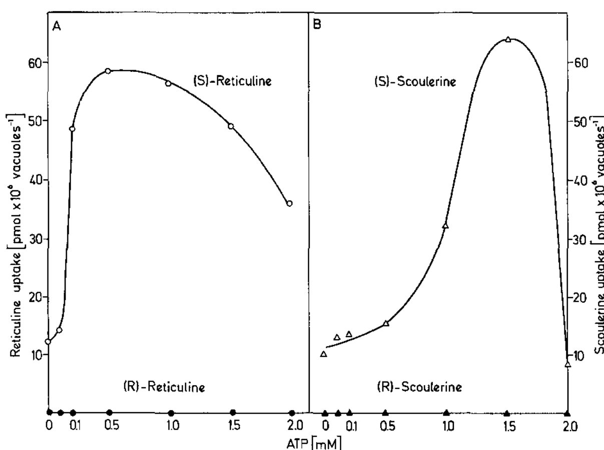

Fig. 4A, B. Effect of ATP concentration on uptake of [^3H]reticuline at pH 7.5 (**A**), and of [^3H]scoulerine at pH 6.5 (**B**) by isolated *Fumaria capreolata* vacuoles (for further experimental details see Fig. 2)

ATP stimulated the uptake of (*S*)-reticuline 5-fold, and that of (*S*)-scoulerine 7.5-fold, at the respective optimal ATP concentration which was 0.5 mM for (*S*)-reticuline and 1.5 mM for (*S*)-scoulerine. These concentrations were used in subsequent experiments. At none of the different ATP concentrations was any uptake of the (*R*)-enantiomers observed. Thus, the stereospecific transport of alkaloids into vacuoles has, for the first time, been

Table 2. Uptake of (*S*)-reticuline by isolated vacuoles of *Fumaria capreolata* as affected by ATP, ATPase inhibitors, and a proton-gradient dissipator. Additions were made to 1-ml samples of *Fumaria capreolata* vacuole suspensions ($4 \cdot 10^5$ ml^{-1}) in medium with 1 mM $MgCl_2$, pH 7.5, prior to the addition of (*S*)-reticuline (500 pmol). ATP (0.5 mM and 5 mM) was added to two DCCD-treated samples after 20 min of incubation. Incubation of all samples was terminated after 40 min by filtration centrifugation

Compounds added	(*S*)-Reticuline uptake	
	pmol per 10^6 vacuoles	% of control
ATP (0 mM)	37.8	53
ATP (0.5 mM) control	70.8	100
DCCD (3 μM)	3.0	4
DCCD (30 μM)	0	0
DCCD (30 μM)+ATP (0.5 mM)	0	0
DCCD (30 μM)+ATP (5 mM)	0	0
CCCP (0.1 μM)	18.7	26
CCCP (1 μM)	9.4	13
CCCP (3 μM)	0	0
DIDS (5 μM)	2.7	4
DIDS (10 μM)	0	0
Na-Vanadate (0.1 mM)	67.1	95
p-Hydroxymercuribenzoate (0.5 mM)	6.1	9

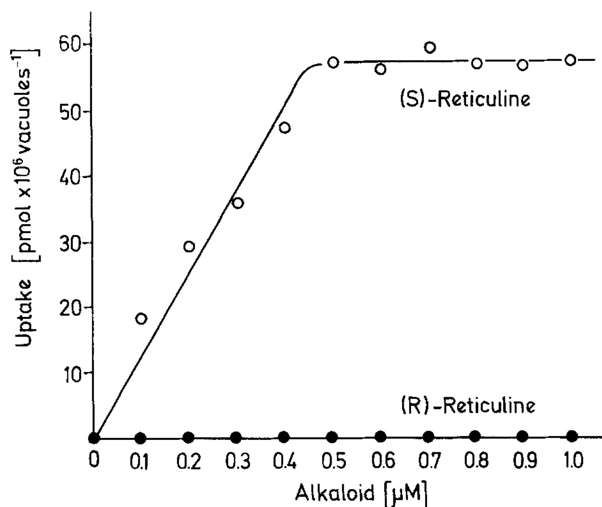

Fig. 5. Uptake of (*S*)-reticuline (o—o) and (*R*)-reticuline (•—•) by isolated vacuoles of *Fumaria capreolata* as a function of concentration. The incubation medium of Doll et al. (1979), pH 7.5, containing 0.5 mM ATP and 1 mM $MgCl_2$ was used. The experiment was terminated after 40 min by filtration centrifugation. The alkaloid uptake was determined using enantiomer-specific radioimmunoassays, while taking the endogenous (*S*)-reticuline level of the vacuoles into calculation

found to be directly driven by an energy source and appears to be ATP linked. To corroberate the energy requirement, the influence of suitable inhibitors on the transport system was determined. As in the case of indole-alkaloid uptake by *C. roseus* vacuoles, the ATPase inhibitor DCCD strongly inhibited isoquinoline-alkaloid uptake (Table 2), as did DIDS which is known to be rather specific for tonoplast ATPase (e.g. Sze 1984). The protonophore CCCP, which had previously been successfully employed by Werner and Matile (1985) to block [^{3}H]esculin (a coumarin glucoside) uptake into barley mesophyll vacuoles, also strongly inhibited (*S*)-reticuline uptake. The system was not inhibited by vanadate which was expected since this inhibitor is specific for the plasmalemma ATPase (e.g. Sze 1984). 4-Chloromercuribenzoate, which in aqueous solution hydrolyses spontaneously to 4-hydroxymercuribenzoate, clearly inhibited strongly the uptake reaction into the *Fumaria* vacuoles.

All of our results are in accordance with the assumption that alkaloid transport into the vacuole proceeds via a proton antiport system similar to the one recently described for sugar transport in sugarcane vacuoles (Thom and Komor 1984a).

Saturation experiments. The high-affinity alkaloid-transport systems which we discovered in isolated vacuoles of several plant species were clearly saturable by the respective species-specific alkaloid substrate (Deus-Neumann and Zenk 1984). Saturation kinetics were also observed for alkaloid uptake by *F. capreolata* (Fig. 5). Saturation of the system is reached with a concentration of approx. 0.5 μM (*S*)-reticuline in the medium. The K_m value for the uptake was calculated as 0.3 μM and was exactly in the range of K_m values determined for other alkaloids in several plant species (Deus-Neumann and Zenk 1984).

Specificity of alkaloid uptake. An extraordinary substrate specificity of the vacuolar alkaloid-transport system had been noted previously (Deus and Zenk 1982; Deus-Neumann and Zenk 1984) for seven different plant species including *Papaver somniferum*. For the latter species, this observation was confirmed by Homeyer and Roberts (1984). Vacuoles take up only those alkaloids which are indigenous to the plant species from which they were isolated. In order to extend these observation, *F. capreolata* vacuoles were exposed to 0.5 μM concentrations of different alkaloids and the uptake quantitated by radioimmunoassay except for scoulerine which was added in tritiated form. In confirmation with the previous results, vacuoles from *F. capreolata* took up only the species-specific alkaloids, (*S*)-reticuline and (*S*)-scoulerine, while

vacuoles from suspension cells of *Rosa canina,* a plant known to contain no alkaloids at all, did not take up any of the alkaloids.

(*R*)-Reticuline is a biosynthetic precursor of the isoquinoline alkaloids morphine, codeine, and thebaine. In *Papaver somniferum*, (*S*)-reticuline is assumed to be the immediate precursor of (*R*)-reticuline, and both enantiomers are indeed present in this plant. Vacuoles isolated from *P. somniferum* cell suspension cultures were therefore incubated with either (*S*)- or (*R*)-reticuline. As shown in Table 3, both enantiomers are taken up by the *Papaver* vacuoles. In addition, (*S*)-scoulerine, a precursor of the benzophenanthridine alkaloids found in poppy, is also taken up, while (*R*)-scoulerine which so far has not been found in nature, is, as to be expected, discriminated. Using radioimmunoassays as analytical tools it was determined that none of four indole alkaloids (ajmaline, catharanthine, serpentine, vindoline) or of two *Solanaceae* alkaloids (nicotine, scopolamine) were taken up by vacuoles of the three species listed in Table 3. In addition, *F. capreolata* vacuoles discriminated even the isoquinoline alkaloids, codeine and morphine, in spite of the fact that the morphinane alkaloid, pallidine, which belongs to the (*S*)-series, occurs in this species (Tanahashi and Zenk 1985). Codeine and morphine are, however, readily taken up by *Papaver somniferum* vacuoles and latex particles (Deus-Neumann and Zenk 1984; Homeyer and Roberts 1984).

It has been reported (Wyse 1978; Willenbrink and Doll 1979; Kaiser and Heber 1984) that sucrose transport into plant vacuoles is competitively inhibited by analogues such as raffinose, glucose and maltose. These sugars apparently compete with the sucrose carrier in the tonoplast membrane. Table 4 shows data on the specificity of (*S*)-reticuline uptake by *F. capreolata* vacuoles in the presence of closely related isoquinoline alkaloids which, except for (*R*)-reticuline, also occur in this *Fumaria* species. The experimental data (Table 4) prove, within experimental error, that there is no competition nor inhibition of (*S*)-reticuline transport by other alkaloids, not even by (*R*)-reticuline; this is in full accordance with the results given above (Figs. 2, 3, 5). All these data fully support the concept of an extremely specific carrier system responsible for the transport of a given alkaloid into the vacuolar compartment.

Alkaloid efflux. If the ion-trap mechanism is operating in the vacuolar system, the alkaloids should be "trapped" in their protonated salt form in the acidic cell sap of the vacuoles and should thus be prevented from escape through the tonoplast membrane back into the cytosol, supposedly in analogy to the neutral-red staining phenomenon (Matile 1984). If the alkaloids inside the vacuoles have formed non-diffusable alkaloid salts, one should not expect that an excess of the same alkaloid added to the preloaded vacuoles would cause efflux of the alkaloid already taken up by the vacuoles as has already been shown in the *Catharanthus* system (Deus-Neumann and Zenk 1984). This type of experiment was repeated with *Fumaria* vacuoles using labelled (*S*)-reticuline as substrate. Unlabelled (*S*)-reticuline was added to these preloaded vacuoles after 20 min of incubation (Fig. 6, *arrow*). The radioactivity in the vacuoles was determined after a further incubation period of 20 min and

Table 3. Selective uptake of alkaloids by vacuoles isolated from cell cultures of three different plant species. Labelled alkaloids (500 pmol) were added to 1-ml samples of vacuole suspension ($3.0 \cdot 10^5$ vacuoles$\cdot ml^{-1}$). The incubation medium (Doll et al. 1979), pH 7.5, with 1 mM $MgCl_2$ and 0.5 mM ATP was used for reticuline uptake. For scoulerine uptake, the medium, pH 6.5, contained 1 mM $MgCl_2$ and 1.5 mM ATP. Alkaloid uptake was terminated after 40 min (reticuline) and 30 min (scoulerine) by filtration centrifugation

	Accumulated alkaloids (pmol per 10^6 vacuoles)		
	Fumaria capreolata	*Rosa canina*	*Papaver somniferum*
(*S*)-Reticuline	78	0	43
(*R*)-Reticuline	0	0	22
(*S*)-Scoulerine	59	0	65
(*R*)-Scoulerine	0	0	0

Table 4. Effect of isoquinoline alkaloids on (*S*)-reticuline transport into isolated vacuoles of *Fumaria capreolata* cell cultures. Alkaloids were added to 1-ml samples of vacuole suspension ($4.2 \cdot 10^5$ vacuoles$\cdot ml^{-1}$) in the incubation medium, pH 7.5, with 1 mM $MgCl_2$ and 0.5 mM ATP. Alkaloid uptake was terminated after 40 min by filtration centrifugation

Alkaloid added	(*S*)-Reticuline uptake	
	pmol per 10^6 vacuoles	% of control
500 pmol (*S*)-Reticuline	91	–
100 pmol (*S*)-Reticuline (control)	35	100
100 pmol (*S*)-Reticuline + 400 pmol (*R*)-Reticuline	34	97
100 pmol (*S*)-Reticuline + 400 pmol (*S*)-Scoulerine	34	97
100 pmol (*S*)-Reticuline + 400 pmol Sanguinarine	40	114
100 pmol (*S*)-Reticuline + 400 pmol Protopine	33	94

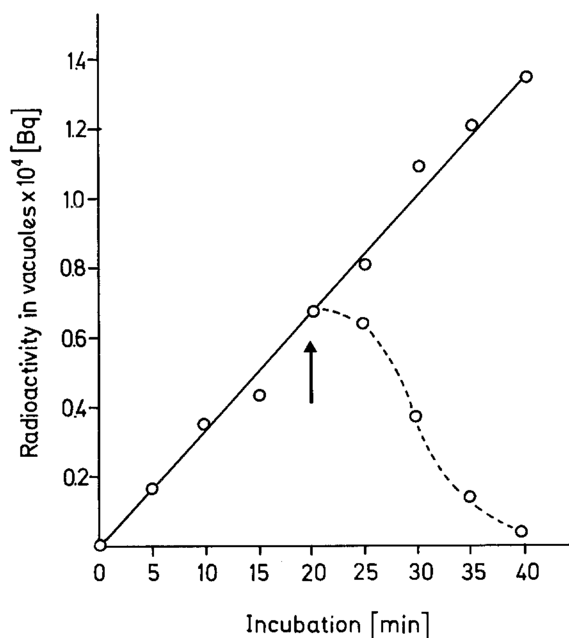

Fig. 6. Uptake and efflux of ^3H(*S*)-reticuline. The uptake reaction (o——o) was started in 4 ml ($3 \cdot 10^5$ vacuoles$\cdot ml^{-1}$) incubation medium with 500 pmol [^3H](*S*)-reticuline$\cdot ml^{-1}$. The *arrow* indicates the time at which 2000 pmol$\cdot ml^{-1}$ unlabelled (*S*)-reticuline were added to an aliquot (2 ml) of the incubation medium (o---o)

compared with that of a control (Fig. 6). Clearly [^3H](*S*)-reticuline was released from the vacuoles after the addition of excess unlabelled (*S*)-reticuline. Twenty minutes after addition of the unlabelled alkaloid the vacuoles contained only less than 10% of the original radioactivity. This loss of label cannot be caused by rupture of the vacuoles since the non-treated vacuoles accumulated labelled reticuline linearly. As in our previous report (Deus-Neumann and Zenk 1984) these results strongly indicate, in our opinion, that the alkaloid was not trapped inside the vacuole as a non-diffusable alkaloid salt.

In order to test whether or not an exchange of preloaded alkaloid with extra-vacuolar alkaloid in the sense of the ion-trap is possible, *Fumaria* vacuoles were first preloaded for 20 min with labelled (*S*)-reticuline and then various alkaloids were added. After a further 20 min, radioactivity in the vacuolar fraction was determined. As shown in Table 5, preloaded labelled (*S*)-reticuline is rapidly lost from the vacuoles when excess unlabelled (*S*)-reticuline is supplied in the medium (see also Fig. 6). It is not surprising that (*R*)-reticuline does not cause loss of radioactivity, since it is not taken up into the vacuoles. (*S*)-Scoulerine, which is, however, transported into the vacuoles and does not compete with reticuline (Table 4), does not cause an efflux of [^3H](*S*)-reticuline from the vacuoles (four concentrations tested). Neither do sanguinarine and protopine, both alkaloids being produced by the *Fumaria* culture, cause an exchange for labelled (*S*)-reticuline. This experiment excludes the possibility of competition between externally supplied and internally stored alkaloids for binding functions (carboxyl groups, phenols etc.) within the vacuole. On the contrary, these data clearly

Table 5. Efflux of [^3H](*S*)-reticuline from preloaded isolated *Fumaria* vacuoles in the presence of various alkaloids and known transport inhibitors. Isolated vacuoles ($4.5 \cdot 10^5 \cdot ml^{-1}$) had been preloaded with 500 pmol [^3H](*S*)-reticuline ($1.3 \cdot 10^5$ Bq) for 20 min at pH 7.5 in the presence of 0.5 mM ATP, then the compounds were added and the experiment terminated by filtration centrifugation after further 20 min

Compound added	(*S*)-Reticuline (pmol per 10^6 vacuoles)	Radioactivity in vacuoles	
		Bq x 10^4	% of control
500 pmol [^3H](*S*)-Reticuline (control)	102	2.70	100
500 pmol [^3H](*S*)-Reticuline + 2000 pmol (*S*)-Reticuline	4	0.11	4
500 pmol [^3H](*S*)-Reticuline + 2000 pmol (*R*)-Reticuline	105	2.78	103
500 pmol [^3H](*S*)-Reticuline + 1000 pmol (*S*)-Scoulerine	100	2.65	98
500 pmol [^3H](*S*)-Reticuline + 2000 pmol (*S*)-Scoulerine	108	2.85	106
500 pmol [^3H](*S*)-Reticuline + 3000 pmol (*S*)-Scoulerine	103	2.73	101
500 pmol [^3H](*S*)-Reticuline + 4000 pmol (*S*)-Scoulerine	100	2.65	98
500 pmol [^3H](*S*)-Reticuline + 2000 pmol Sanguinarine	108	2.86	106
500 pmol [^3H](*S*)-Reticuline + 2000 pmol Protopine	97	2.57	95
500 pmol [^3H](*S*)-Reticuline + 30 μM DCCD + 2000 pmol (*S*)-Reticuline	35	0.92	34
500 pmol [^3H](*S*)-Reticuline + 3 μM CCCP	3	0.08	3
500 pmol [^3H](*S*)-Reticuline + 3 μM CCCP + 2000 pmol (*S*)-Reticuline	8	0.22	8
500 pmol [^3H](*S*)-Reticuline + 10 μM DIDS	90	2.39	88
500 pmol [^3H](*S*)-Reticuline + 10 μM DIDS + 2000 pmol (*S*)-Reticuline	93	2.48	92

demonstrate that a specific alkaloid can readily be transported into and out of a vacuole only by catalysis of highly specific carriers. Alkaloids which are similar but not identical in structure with a given alkaloid cannot use the same carrier system. These data support the postulate that for every alkaloid transported there must be a highly specific binding site at the carrier molecule, which then allows the transport into as well as out of the vacuole. Since tonoplast energization has been demonstrated in the present study to be required for vacuolar uptake of alkaloids, the question remained whether energization is also required for efflux. Vacuoles preloaded with [^{3}H](*S*)-reticuline were treated with the ATPase inhibitor DCCD in the presence of unlabelled (*S*)-reticuline; this resulted in some 30% inhibition of efflux, a result which is not clear cut (Table 5). However, addition of CCCP, which is known to dissipate the membrane potential, caused an almost complete loss of radioactivity from the preloaded vacuoles (Table 5) indicating that H^+ is the electrogenic ion (Sze and Churchill 1981). This effect was also shown in the absence of additional unlabelled (*S*)-reticuline. Interestingly, however, the addition of DIDS to the preloaded vacuoles prevented the efflux of labelled (*S*)-reticuline in the presence of excess exogenous unlabelled (*S*)-reticuline almost completely. It is reported that DIDS inhibits the generation of an ATP-dependent membrane potential and directly inhibits the electrogenic proton-pumping ATPase by interacting with some anion-sensitive site of the enzyme (Sze 1984). In the presence of DIDS the alkaloids can no longer be actively transported out of their compartment but remain within the vacuole.

These results, in our opinion, present convincing proof that energization of vacuolar transport of alkaloids is necessary in both transport directions; influx and efflux of alkaloids into and out of the vacuolar compartment is linked to an energy-requiring process. These findings contradict the ion-trap mechanism hypothesis.

Discussion

It is now general knowledge that secondary plant products are accumulated and stored in the vacuolar compartment of higher plants to serve their ecochemical function. One of the largest group of secondary products are the alkaloids, of which about 6000 different molecular structures are known today. In a most stimulating recent article, Matile (1984) states that the mechanisms by which secondary products are removed from the cytosol, pass through the tonoplast and accumulate in the vacuole are more interesting than the fact of compartmentation. He elaborates that "a specific transport system does not seem to be required for the permeation of alkaloids through the tonoplast, since the uncharged alkaloids are so hydrophobic, that they can easily diffuse through membranes" (translated from the German original). Once they are inside the vacuole, within the acidic environment of this compartment, the alkaloids will be protonated, and it is assumed that they cannot permeate the tonoplast in this form. The hypothesis described above is called the "trap" mechanism, a term coined by Brooks and Brooks (1941) and later extended to "ion-trap" mechanism by Höfler (1947) for this phenomenon. This ion-trap mechanism for alkaloid accumulation in plant vacuoles has been favoured by Neumann, Müller and their colleagues (Neumann et al. 1983 and literature cited therein) for two decades. Recent experiments in their laboratory seem to verify this mechanism (Neumann et al. 1983). If, along with Matile and Neumann et al. , one excludes a carrier-mediated active transport for alkaloids, then two factors must be responsible for the trap mechanism; first, the permeability of the tonoplast towards alkaloids, and second, the storage capacity of the vacuole.

In this paper we have tested the permeability properties of the tonoplast for alkaloids. Isolated intact vacuoles were used to investigate the transport of two stereoisomeric forms, each of reticuline as well as of scoulerine, which have exactly the same electrical charge, the same pK and other chemical properties, but differ in their stereochemistry at C-1 and C-14, respectively (Fig. 1). Both, (*S*)-reticuline and (*S*)-scoulerine, but not the (*R*)-enantiomers, occur naturally in *F. capreolata* (Tanahashi and Zenk 1985). It was shown that (*S*)-reticuline is located in the vacuole of the *Fumaria* cells (Table 1), and we assume that (*S*)-scoulerine is stored there too. Uptake experiments with isolated *Fumaria* vacuoles, using two different analytical methods for alkaloid quantitation, showed that the vacuolar system transported exclusively the (*S*)-forms of the alkaloids. This differential transport behaviour of stereoisomeric forms of alkaloids is inconsistent with the ion-trap mechanism. The trap mechanism requires that the tonoplast membrane is highly permeable for the hydrophobic neutral forms of the alkaloids, which applies to both, the (*S*)- and (*R*)-enantiomers of the alkaloids. Since vacuolar transport sharply discriminates between the stereoisomers, one of the prerequisites for the validity of the trap mechanism

is lost. Furthermore, the transport system is clearly energy dependent as evident from the seven fold stimulation of vacuolar transport in the presence of ATP (Fig. 4). High ATP concentrations (>2 mM) inhibited the vacuolar uptake process, and this might be explained by the observed inhibition of ATPase by high concentrations of ATP. These enzymes have several ATP-binding sites which fulfill regulatory functions and, at nonphysiological ATP concentrations, might get blocked (Thom and Komor 1984b, and literature cited).

Both the ATPase inhibitors, DCCD and DIDS, as well as the proton-gradient dissipator CCCP inhibited vacuolar alkaloid transport (Table 2), indicating the existance of pumps generating electrical membrane potential. In contrast to the reported 200% stimulation of morphine uptake by 1000-*g* organelles of *Papaver somniferum* in the presence of *p*-hydroxymercuribenzoate (CMB, Homeyer and Roberts 1984), this inhibitor clearly reduced strongly the uptake into *Fumaria* vacuoles. Homeyer and Roberts (1984) observed an artifact in the supposed transport stimulation by CMB. Uptake of (*S*)-reticuline in *Fumaria* vacuoles was saturable (Fig. 5) and an apparent K_m value for the transport of (*S*)-reticuline of 0.3 μM was calculated. The high affinity of the alkaloid-transport system contrasts with the much lower affinities of sugar-carrier systems previously characterized in vacuolar preparations. Thom et al. (1982) determined a K_m of 169 μM for 3-O-methylglucose uptake into sugarcane vacuoles, while the K_m of sucrose uptake into barley vacuoles was 21 mM (Kaiser and Heber 1984). In *Fumaria* the rate of transport was $6.3\ \mathrm{pmol \cdot h^{-1} \cdot \mu l^{-1}}$ vacuole which is 100-fold lower than, for instance, the rate of the sucrose-transport system in *Saccharum* vacuoles (Thom et al. 1982). Efflux experiments with vacuoles preloaded with [^{3}H](*S*)-reticuline proved that alkaloid unloading is also mediated by a highly specific carrier system (Table 5). Alkaloid transport through the tonoplast membrane in both directions proceeds, therefore, neither by diffusion nor by a channel-protein mechanism as suggested by Homeyer and Roberts (1984).

Finally, there was a high degree of species specificity observed in the vacuolar transport system. Vacuoles took up only those alkaloids in substantial amounts which were indigenous to the plant from which the vacuoles had been isolated. Thus *Fumaria* vacuoles took up only (*S*)-reticuline and (*S*)-scoulerine, while vacuoles of *Papaver*, which, on the basis of biosynthetic considerations should contain (*R*)-reticuline, indeed took up (*R*)-reticuline as well. Vacuoles of *Rosa* did not take up any of the offered alkaloids which is intelligible since this species does not contain alkaloids. These and our previous results (Deus and Zenk 1982; Deus-Neumann and Zenk 1984) demonstrate an unparalleled specificity among known uptake systems in higher plants. Evidence is now accumulating that for every alkaloid group, maybe even for every single alkaloidal molecular species, there is a highly specific alkaloid carrier, or specific binding site of a general carrier, present in the tonoplast membrane. The binding sites of the protein carrier to the transported alkaloids must therefore involve an extreme structural diversity and topography which so far has only been known for the immunosystem in higher animals.

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