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A highly selective alkaloid uptake system in vacuoles of higher plants*

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Abstract. Vacuoles were isolated from different plant cell cultures and the transport mechanism for alkaloid uptake at the tonoplast membrane, as well as the compartmentation of enzymes and products inside the cells were investigated. While serpentine, the major alkaloid of *Catharanthus roseus* cells, is definitely located inside the vacuole, two key enzymes of the indole-alkaloid pathway, strictosidine synthase and a specific glucosidase, are located in the cytosol. Transport of alkaloids across the tonoplast into the vacuolar space has been characterized as an active, engergy-requiring mechanism, which is sensitive to the temperature and pH of the surrounding medium, stimulated by K^+ and Mg^{2+}, and inhibited by N,N′-dicyclohexylcarbodiimid and Cu^{2+}. The alkaloids accumulate inside the vacuoles against a concentration gradient, and the uptake system is specific for alkaloids indigenous to the plant from which the vacuoles have been isolated.

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

Introduction

The vacuole, as the largest organelle of plant cells, plays a fundamental role in the compartmentation of enzymes (Boller and Kende 1979; Leigh et al. 1979), and inorganic ions (Leigh and Deri Tomos 1983), as well as the storage of primary and secondary metabolites (Matile 1978; Sasse et al. 1979). Regulatory processes in the cytoplasm and in the tonoplast are obviously necessary for the accumulation of natural compounds inside the vacuole. Furthermore, the storage capacity of the vacuole may be a limiting factor in the production of a compound by plant cells (Zenk 1978). For alkaloids, the localization inside the vacuole has been experimentally proven only in few cases. Matile et al. (1970; Matile 1976) reported that vacuoles in *Chelidonium majus* latex contain about 70% of the major isoquinoline alkaloids of this species. Similarly, morphine seems to be mainly located in the vacuoles of *Papaver somniferum* latex (Fairbairn et al. 1974), and isolated vacuoles of *Nicotiana rustica* mesophyll cells contained high concentrations of nicotine (Saunders 1979).

Transport across the tonoplast membrane has been investigated mainly for amino acids (Ohsumi and Anraku 1981) and various sugars (Suzuki 1982; Willenbrink and Doll 1979; Guy et al. 1979; Thom et al. 1982; Komor et al. 1982). The mechanisms by which alkaloids are transported into and deposited within the vacuole are widely unknown.

Within our general effort to increase the alkaloid yield of plant suspension cultures and to obtain information on the underlying transport and accumulation mechanisms, we have studied the characteristics of alkaloid uptake by vacuoles isolated from cultured plant cells as well as the compartmentation of enzymes and secondary products inside these cells.

Materials and methods

Plant materials. Cells of *Catharanthus roseus* (L.) G. Don were routinely transferred every 8 d into 100 ml of fresh medium according to Linsmaier and Skoog (1965) with 2,4-dichlorophenoxy acetic acid (10^{-6} M) and 1-naphthalene acetic acid (10^{-6} M), pH 6.0, in 300 ml Erlenmeyer flasks and cultivated on a gyratory shaker (100 rpm) at 23° C in continuous light

* Dedicated to Professor Dr. Hubert Ziegler on the occasion of his 60th birthday

Abbreviation: DCCD = N,N′-dicyclohexylcarbodiimid

(650 lx). Six-day-old cultures were used in the experiments. All other cell cultures used were provided by our cell-culture laboratory.

Chemicals and radiochemicals. Adenosine 5′-triphosphate (sodium salt) and N,N′-dicyclohexylcarbodiimide (DCCD) were provided by Sigma (München, FRG). [^{3}H]vindoline ($5.18 \cdot 10^8$ Bq μmol^{-1}), [^{3}H]ajmaline ($2.78 \cdot 10^8$ Bq μmol^{-1}), and [^{3}H]catharanthine ($5.37 \cdot 10^8$ Bq μmol^{-1}) were custom synthesized by Amersham-Buchler, Braunschweig (FRG). [^{3}H]codeine ($1.04 \cdot 10^9$ Bq μmol^{-1}) and [^{3}H]morphine ($1.04 \cdot 10^9$ Bq μmol^{-1}) were purchased from Radiochemical Centre, Amersham (UK). [2-^{3}H]Tryptamine was from NEN (Boston, Mass., USA). [^{3}H]Ajmalicine ($3.70 \cdot 10^8$ Bq μmol^{-1}), [^{3}H]serpentine ($2.44 \cdot 10^7$ Bq μmol^{-1}), [^{3}H]scopolamine ($2.48 \cdot 10^7$ Bq μmol^{-1}), [^{3}H]nicotine ($9.25 \cdot 10^7$ Bq μmol^{-1}), and [^{3}H/^{14}C]strictosidine ($1.44 \cdot 10^{12}$ Bq μmol^{-1} ^{3}H; $2.64 \cdot 10^{11}$ Bq μmol^{-1} ^{14}C) were synthesized in our laboratory. All other chemicals used were standard commercial products of analytical grade.

Determination of serpentine content. A known amount of cells, protoplasts, or vacuoles was extracted with 80% ethanol under reflux for 30 min. The serpentine content of the extracts was measured with the radioimmunoassay described by Arens et al. (1978) and by the following chromatographic analysis. Aliquots of 100 μl were applied to thin-layer-chromatography plates (silica-gel with fluorescence-indicator 254 nm, Riedel-De Haen AG, Seelze-Hannover, FRG) along with serpentine standards. After development in chloroform-acetone-diethylamine (5:4:1, by vol.) serpentine fluorescence was measured with a Chromatogramm-Spektralphotometer KM 3 (Carl Zeiss, Oberkochen, FRG) and with filter combination FL 43/FL 56, and the serpentine content was calculated by comparison with the fluorescence intensity of the standard.

Microscopy. A Zeiss-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. The size of vacuoles was determined with a measuring ocular (Carl Zeiss, Kpl. 8×). Condensor III/RS was used together with filter combination G 365–FT 395–Lp 420 for selective fluorescence stimulation at 365 nm.

Crude enzyme extracts. Isolated protoplasts were sedimented, frozen with liquid nitrogen, stirred in double the amount of 0.1 M potassium phosphate buffer, pH 6.5 (in the case of glucosidases 0.1 M borate buffer, pH 7.6 was used) for 30 min and then centrifuged (48000 *g*, 10 min, 4° C). Supernatants were used for the enzyme assay. Isolated vacuoles were frozen in the isolation medium and, after thawing, stirred for 30 min. The homogenates were directly used for the enzyme assays.

Enzyme assays. The activity of strictosidine synthase was measured according to Treimer and Zenk (1979) and Pfitzner and Zenk (1982). Specific glucosidase was determined as described by Hemscheidt and Zenk (1980), unspecific glucosidases according to Hemscheidt (Institut für Pharmazeutische Biologie, University of Munich, FRG) and acid phosphatase according to Bergmeyer (1962). The protein content of crude extracts was determined as described by Bensadoun and Weinstein (1976).

Isolation of protoplasts. Protoplasts were isolated as described by Schieder (1975) using the following enzymes: Meicellase, Onozuka SS and Macerozyme (all Japan Biochemicals Co. Ltd. Nishinomiya, Japan), Pectinol D (Röhm GmbH, Darmstadt, FRG), Cellulysin (Calbiochem, La Jolla, Calif., USA), and Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan). The composition of the enzyme solutions for the degradation of cell walls depended on the plant material used. Cultivated cells of *Catharanthus roseus, Rauwolfia serpentina, Datura meteloides,* and *Papaver somniferum* were treated with a solution of 3.0% Meicellase and 1.5% Macerozyme in 0.5 M mannitol, pH 5.8, at 30° C for 4–6 h. Protoplasts from *C. roseus* mesophyll cells were prepared in a solution of 0.4% Onozuka SS, 0.3% Driselase and 0.2% Macerozyme in 0.4 M mannitol, pH 5.6, at 30° C for 6 h. For *Nicotiana sylvestris* cells, a 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 was used, for 7 h at 30° C. For *Daucus carota* cells, 4.0% Cellulysin and 2.0% Macerozyme in 0.4 M mannitol, pH 6.0 were used for 5 h at 30° C.

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 0.5 M NaCl was used. Isolated vacuoles were purified by a Urografin density gradient (sodium salt of N,N′-diacetyl-3,5-diamino-2,4,6-triiodo benzoic acid) exactly according to the method of Grob and Matile (1979). The purity of the vacuolar fraction was determined microscopically. Only those vacuole suspensions were used for the experiments which were free of cytoplasmic material. Urografin was a gift of Schering AG, Berlin, West Germany.

Uptake experiments. A modification of the medium of Doll et al. (1979) was used for the determination of alkaloid uptake by isolated vacuoles. One part of vacuole suspension was mixed with three parts of the following incubation medium: NaCl (0.7 M), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes)/NaOH buffer (40 mM), pH 7.6, EDTA (4 mM), $MgCl_2$ (4 mM) and ATP (4 mM). Radioactively labelled alkaloids were then added to the suspension. For controls, vacuoles were destroyed by freezing and thawing and subsequently incubated under the same conditions. The accumulation of the alkaloids within the vacuoles was measured by using the centrifugation method developed by Heldt and Sauer (1971).

Results

Alkaloid compartmentation. Catharanthus roseus cell suspension cultures are one of the best characterized plant materials with regard to alkaloid metabolism (e.g. Zenk 1980). This cell culture produces representatives of the main indole-alkaloid classes (Stöckigt and Soll 1980; Kohl et al. 1982; Kurz et al. 1982; Petiard et al. 1982), among these the heteroyohimbine alkaloid, serpentine, as the major product (Zenk et al. 1977) which shows bright blue fluorescence in UV light. Using a fluorescence microscope, it could already be observed during the isolation of vacuoles from protoplasts that the highly blue-fluorescent serpentine was restricted to the vacuoles. To verify this observation, quantitative determinations of the vacuolar serpentine content were necessary. For this purpose, cells, protoplasts, and isolated vacuoles from *C.*

Table 1. Comparison of serpentine contents in cells, protoplasts, and isolated vacuoles of *Catharanthus roseus*

	Serpentine content (nmol per 10^6 cells, protoplasts, or vacuoles)
Cell	0.50
Protoplast	0.54
Vacuole	0.49

Table 2. Activities of strictosidine synthase, specific glucosidase, unspecific glucosidase, and acid phosphatase in protoplasts and vacuoles isolated from cells of *Catharanthus roseus*

Enzyme	Enzyme activity		
	pkat μg^{-1} protein	pkat $nmol^{-1}$ serpentine	pkat (10^6 protoplasts, vacuoles)$^{-1}$
Strictosidine synthase			
Protoplast	0.77	129.5	64.0
Vacuole	0	0	0
Specific glucosidase			
Protoplast	1.63	275	550
Vacuole	0.05	0.1	0.5
Unspecific glucosidase			
Protoplast	0.01	1.90	1.00
Vacuole	0	0	0
Acid phosphatase			
Protoplast	5.3	884	450
Vacuole	5.2	946	542

roseus were extracted, and serpentine concentrations in the extracts were measured using a radioimmunoassay as well as quantitative thin-layer chromatography for crosscheck. It was clearly established that within experimental error serpentine was exclusively located within the vacuoles (Table 1). These results extend the general observation that secondary plant products are stored within the vacuolar compartment.

Compartmentation of alkaloid biosynthesis. It is known that a variety of enzymes are located inside the vacuolar space (Wiemken et al. 1979; Nishimura and Beevers 1979; Butcher et al. 1977). The vacuolar localization of serpentine prompted us to determine the localization of the enzymes involved in the biosynthesis of this alkaloid. For this purpose, the compartmentation of the activities of two key enzymes of the early indole-alkaloid pathway, i.e. strictosidine synthase (Treimer and Zenk 1979) and the specific glucosidase (Hemscheidt and Zenk 1980) was determined. Furthermore the activity of an unspecific glucosidase not capable of catalyzing the initiation of indole-alkaloid synthesis (Hemscheidt and Zenk 1980) was measured. As a vacuolar marker enzyme (Buser and Matile 1977; Butcher et al. 1977; Mettler and Leonard 1979), the activity of acid phosphatase was determined. As shown in Table 2, *C. roseus* protoplasts, as well as vacuoles derived from these protoplasts, exhibited identical acid-phosphatase activities indicating an exclusive vacuolar localization of this enzyme in the vacuole and allowing its use as a vacuolar marker enzyme in the case of *C. roseus* too. No vacuole-associated activities of strictosidine synthase and unspecific glucosidase were detected. Of the activity of specific glucosidase only 0.1% of the total activity of the protoplasts was found in the vacuoles and is thus likely to be due to a slight contamination of the vacuolar preparation. Since acid phosphatase was retained in the vacuoles, loss of vacuolar protein during the isolation procedure had not occurred and a vacuolar localization of the other enzymes can be excluded.

To prove the metabolic capacity of isolated protoplasts, they were administered precursors of indole-alkaloid biosynthesis. Protoplasts were suspended in the protoplast medium of Durand et al. (1973) at a density of $2 \cdot 10^6$ protoplasts ml^{-1}. [2-^{14}C]Tryptamine ($1.85 \cdot 10^4$ Bq) and double-labelled strictosidine ($9.25 \cdot 10^4$ Bq ^{3}H; $1.85 \cdot 10^4$ Bq ^{14}C), respectively, were added to 2 ml of the protoplast suspension. After incubation for 14 h at 30° C, protoplasts were separated from the incubation mixture and the reaction terminated by the addition of ethanol. Of the administered radioactivity, 22% was recovered in the protoplast extract in the case of [^{14}C]tryptamine, and 34% in the case of [^{3}H/^{14}C]strictosidine. Aliquots of the protoplast extracts were subjected to thin-layer chromatography. Labelled ajmalicine, 19epi-ajmalicine, and tetrahydroalstonine were identified in both cases. During incubation with [^{3}H/^{14}C]strictosidine the three isomers were formed in a ratio of 2:1.5:1. These results are comparable with those obtained with cell-free systems of *C. roseus* suspension cultures (Stöckigt et al. 1976). Incubation of vacuolar preparations from *C. roseus* with the two labelled precursors under identical conditions did not result in the formation of radioactive heteroyohimbine alkaloids. Our results show that isolated protoplasts of *C. roseus* take up the added precursors and utilize them for the production of species-specific compounds. The site of biosynthesis within

the cell seems to be the extravacuolar space whereas the products of biosynthesis are stored within the vacuole. Thus the alkaloidal end products must pass from the cytosol through the tonoplast membrane into the vacuole, and to get insight into the nature of the transport mechanism we studied the uptake of alkaloids by isolated vacuoles.

Alkaloid uptake by isolated vacuoles. In several reports, uptake into and storage of various compounds in the vacuoles have been demonstrated (Matile 1976; Saunders and Conn 1978; Martinoia et al. 1981; Leigh and Deri Tomos 1983). The properties of the tonoplast may be of considerable importance for product accumulation in a plant cell and may affect the extent to which products can be produced and stored by an intact plant or a plant cell culture.

Time course of vindoline uptake. The uptake of alkaloids by isolated vacuoles of *C. roseus* cells was studied with vindoline as substrate. Vindoline was identified as a natural constituent of the differentiated *C. roseus* plant (Svoboda and Blake 1975), but was not detectable in cell cultures of *C. roseus* even by radioimmunoassay (Stöckigt and Zenk, data not shown). For the experiment, 400 pmol vindoline ($2.07 \cdot 10^8$ Bq) were added to isolated vacuoles ($3.5 \cdot 10^5$ ml^{-1}) of *C. roseus* suspended in the incubation medium described above. At different intervals, samples were taken from the suspension and subjected to filtration centrifugation. The amount of vindoline taken up by the vacuoles was calculated from the radioactivity measured in the vacuolar fraction after centrifugation. As shown in Fig. 1, uptake of vindoline was almost linear for 60 min, and maximal uptake had occurred after 90 min. Longer incubation periods caused a decrease of the vindoline content of the vacuoles. Obviously the stability of vacuoles was limited during long incubation periods, and the vacuolar preparation lost vindoline already taken up by lysis. An incubation period of 90 min seemed optimal for the alkaloid uptake by isolated vacuoles of *C. roseus* and this time period was used for all subsequent experiments.

pH-Dependency of alkaloid uptake. For our initial experiments with isolated vacuoles, the incubation medium of Doll et al. (1979), having a pH of 7.6, was routinely used. On the other hand Doll et al. (1979) demonstrated that the uptake of sucrose into vacuoles from red-beet tissue was a function of the pH of the incubation medium with optimal uptake occurring at pH 5.6. The pH-dependent

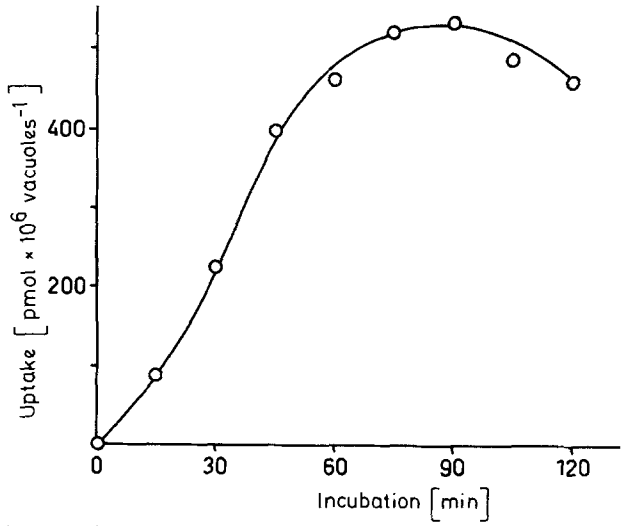

Fig. 1. Time course of vindoline uptake by isolated vacuoles of *Catharanthus roseus*. Vacuoles were suspended in the medium of Doll et al. (1979) and 400 pmol [^{3}H]vindoline ($1.9 \cdot 10^4$ Bq) were added per ml to a *C. roseus* vacuole suspension ($3.5 \cdot 10^5$ vacuoles ml^{-1}) in a total volume of 3 ml. At the times indicated samples were taken from the suspension and further alkaloid uptake by the vacuoles terminated by filtration centrifugation

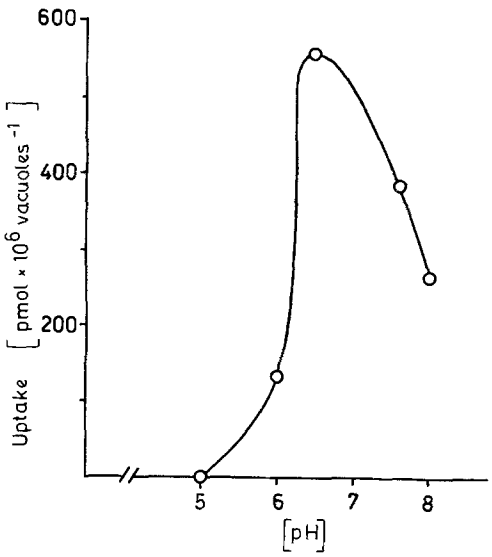

Fig. 2. Effect of pH on vindoline uptake by isolated vacuoles of *C. roseus*. The alkaloid uptake was determined after 90 min (experimental data see Fig. 1)

profile of vindoline uptake into *C. roseus* vacuoles shown in Fig. 2 clearly demonstrates that vindoline accumulation was highly sensitive to external pH. The pH of the initially used incubation medium was apparently suboptimal for this system. Subsequent uptake experiments were therefore carried out at pH 6.5.

Effect of temperature on alkaloid uptake. For protoplasts of *C. roseus*, Suzuki (1982) had already

Table 3. Effect of temperature on vindoline uptake by isolated vacuoles of *C. roseus*. The pH of the incubation medium was adjusted to 6.5 and the alkaloid uptake was measured after 60 min; for other experimental data see Fig. 1

Temperature (°C)	Vindoline uptake	
	pmol $(10^6$ vacuoles$)^{-1}$	% of maximum uptake
2	42	17.5
23	160	66.7
30	240	100

shown that sugar transport at the plasmamembrane increased with temperature up to 40° C. Vindoline uptake by isolated vacuoles of *C. roseus* was also clearly temperature dependent (Table 3). During our experiments, maximal uptake of vindoline after 60 min of incubation was found at 30° C. At room temperature (23° C) the uptake was 66.7% and at 2° C only 17.5% of that determined at 30° C. The vacuoles seemed to be less stable at 30° C during the 90 min incubation period. Therefore, further experiments were carried out at room temperature.

The effect of medium composition on alkaloid uptake. Doll et al. (1979) reported that uptake of sucrose by red-beet vacuoles depended significantly on the presence of both, Mg^{2+} and K^+, in the medium. Komor et al. (1982) concluded from their observations with vacuoles isolated from sugarcane that K^+ in the incubation medium created an electric potential at the tonoplast membrane. The composition of the incubation medium is, therefore, critical in uptake experiments with isolated vacuoles. Surprisingly, when isolated vacuoles of *C. roseus* were incubated in the medium of Doll et al. (1979) with different combinations of ATP (1 mM; Fig. 3), $MgCl_2$ (1 mM), and KCl (50 mM), vindoline uptake in the absence of ATP was consistently higher than in the presence of ATP, and KCl and $MgCl_2$, separately or in combination, enhanced vindoline uptake up to nine-fold.

Saturation experiments. Sucrose uptake into vacuoles of red-beet tissue was shown to be a function of sucrose concentration and was saturable. Similar observations were made by Cram (1983) for sulfate transport across the tonoplast of carrot root cells, and by Thom et al. (1982) for 3-O-methylglucose uptake by sugarcane vacuoles. These results support the concept that transport of compounds across tonoplast membranes is a carrier-mediated process. In our case, alkaloid transport

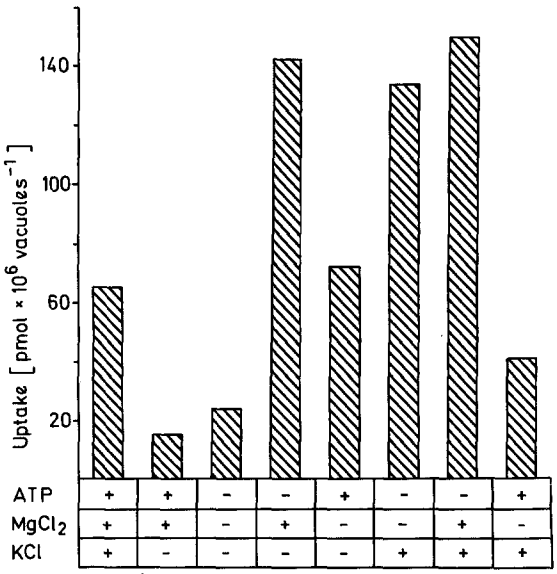

Fig. 3. Effect of medium composition on vindoline uptake by isolated vacuoles of *C. roseus*. ATP (1 mM), $MgCl_2$ (1 mM), and KCl (50 mM) were added in different combinations to the medium of Doll et al. (1979), pH 6.5. Further experimental data see Fig. 1

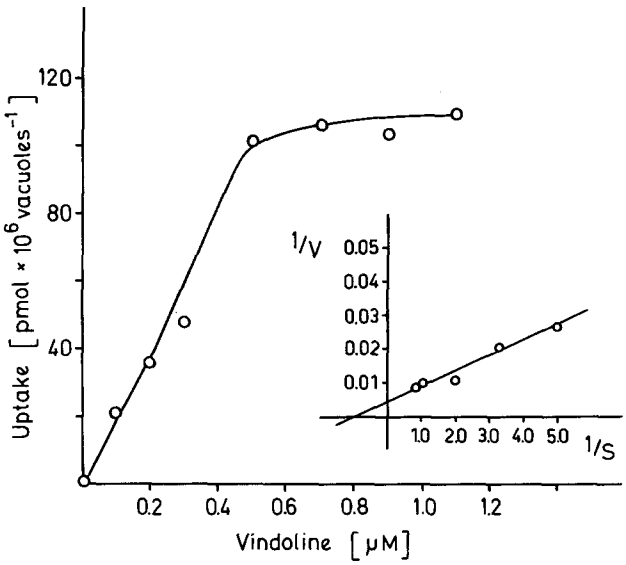

Fig. 4. Influence of alkaloid concentration in the medium on vindoline uptake by isolated vacuoles of *C. roseus*. The incubation medium of Doll et al. (1979), pH 6.5, only containing $MgCl_2$ (1 mM) as additive, was used. Further experimental data see Fig. 1

of isolated vacuoles from different plant species was clearly saturable, as demonstrated in Fig. 4 in the case of vindoline uptake by vacuoles of *C. roseus*. The K_m values for the uptake were calculated as 1.5 μM for vindoline, 2.5 μM for catharanthine, and 1.67 μM for ajmalicine by vacuoles of *C. roseus*. The K_m for nicotine uptake by isolated vacuoles of *Nicotiana sylvestris* was 1 μM, and aj-

Table 4. Ajmalicine concentration of incubation medium and within isolated vacuoles of *C. roseus*. [^{3}H]Vindoline was added in increasing amounts (0–1150 pmol ml^{-1}) to 1-ml samples of *C. roseus* vacuole suspension (medium supplemented with 1 mM $MgCl_2$, pH 6.5). After 90 min the incubation was terminated by filtration centrifugation and the alkaloid concentrations in the medium were compared with the concentrations inside the vacuoles. The average vacuolar volume was determined as 30 pl (±0.7 pl)

Ajmalicine (μmol)	
Concentration in incubation medium	Vacuolar concentration
0	0
0.06	0.24
0.24	1.04
0.42	1.93
0.60	2.71
0.78	2.40
1.15	3.58

Table 5. Uptake of vindoline by isolated vacuoles of *C. roseus* in the presence of inhibitors. Either DCCD or Cu^{2+} was added to 1-ml samples of a *C. roseus* vacuole suspension ($4.0 \cdot 10^5$ ml^{-1}) in medium with $MgCl_2$ (1 mM), pH 6.5, prior to the addition of [^{3}H]vindoline ($1.9 \cdot 10^4$ Bq); ATP (5 mM) was added to one of the DCCD-treated samples after 60 min of incubation. Incubation of all samples was terminated after 90 min by filtration centrifugation

	Uptake	
	pmol (10^6 vacuoles)$^{-1}$	% of control
Control	430	100
DCCD (3 μM)	240	55.8
DCCD (30 μM)	0	0
DCCD (30 μM) + ATP (5 mM)	0	0
Cu^{2+} (0.1 mM)	280	65.1
Cu^{2+} (1 mM)	0	0

maline was transported with a K_m of 0.23 μM from isolated vacuoles of *Rauwolfia serpentina*.

An indication for an energy-dependent transport across the tonoplast would be the accumulation of substances inside the vacuole against a concentration gradient. To characterize the alkaloid uptake in this regard the alkaloid concentrations in the medium and inside the vacuoles of *C. roseus* were determined by application of ajmalicine in increasing amounts (0–1150 pmol ml^{-1}). For the calculation of alkaloid concentration inside the vacuoles, their average diameter was measured microscopically and the average vacuole volume was calculated as 30 pl. As shown in Table 4, uptake of ajmalicine by isolated vacuoles of *C. roseus* not only compensated for the concentration gradient between vacuoles and medium, but the ajmalicine concentration inside the vacuoles rather exceeded the external alkaloid concentration in each case. This result clearly proved that the indole alkaloid, ajmalicine, is accumulated inside the isolated vacuoles against a concentration gradient.

In comparative studies, Willenbrink and Doll (1979) reported that sucrose was taken up into red-beet vacuoles against a concentration gradient by an active transport system in the tonoplast. What is the source of energy for the transport processes at the vacuolar membrane? The experiments with isolated vacuoles of *C. roseus* described above (Fig. 3) indicated that alkaloid transport at the tonoplast was not stimulated by external ATP. On the other hand, it has been shown that an energy-requiring transport at the tonoplast membrane was blocked by ATPase inhibitors (Suzuki 1982; Ohsumi and Anraku 1981). Similar experiments with isolated vacuoles of *C. roseus* should therefore give further information on the energy requirement of the transport system.

The effect of DCCD and Cu^{2+} on alkaloid uptake. Vindoline uptake was measured in the absence and presence of DCCD and Cu^{2+} (Table 5). Vindoline accumulation was strongly affected by DCCD. At 3 μM DCCD, the vindoline uptake was reduced 50%, and alkaloid uptake was completely inhibited by 30 μM DCCD. ATP did not restore vindoline uptake in the presence of DCCD. Copper ions were also inhibitory.

Although these results indicate that the transport into isolated vacuoles of *C. roseus* is an active, energy-requiring process, one still has to consider the possibility of an ion-trap mechanism for the accumulation of alkaloids inside the vacuoles.

The effect of vindoline addition to preloaded vacuoles. Matile (1976) and Matile et al. (1970) studied alkaloid accumulation in vacuoles of *Chelidonium majus* laticifers. They demonstrated that the vacuoles preferably took up sanguinarine and chelerythrine and accumulated these alkaloids against a concentration gradient. While their findings suggested an energy-dependent transport mechanism, no energy was apparently required for the alkaloid uptake. Further experiments indicated that the uptake of sanguinarine by vacuoles of *C. majus* is due to an ion-trap mechanism (Matile et al. 1970). The molecules pass the tonoplast by diffusion and are subsequently bound inside the vacuole by the formation of non-diffusable alkaloid salts. The capacity of vacuoles to accumulate these alkaloids is limited to the amount of ions available for bind-

ing. Therefore, the alkaloid accumulation in such a system may show saturation kinetics.

To examine whether an ion-trap mechanism is involved in alkaloid uptake by *Catharanthus roseus* vacuoles, the effect of excess alkaloid on preloaded vacuoles was tested. If the alkaloids inside the vacuole had 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. Vacuoles of *C. roseus* were incubated with labelled vindoline as substrate. Subsequently unlabelled vindoline (2 nmol) was added to the preloaded vacuoles. The radioactivity in the vacuoles was determined for a further 60 min and compared with that of a control (Fig. 5). [^{3}H]Vindoline was released from the vacuoles after the addition of unlabelled vindoline. Sixty minutes after addition of the unlabelled alkaloid the vacuoles were nearly devoid of radioactivity. This loss of radioactivity cannot be caused by rupture of the vacuoles since the non-treated vacuoles lost only a negligible amount ($0.15 \cdot 10^4$ Bq) of radioactivity after 2 h of incubation. These results strongly suggest that vindoline was not trapped inside the vacuole as a non-diffusable alkaloid salt. Therefore, the ion-trap mechanism may be excluded as an explanation for alkaloid transport into vacuoles of *C. roseus*.

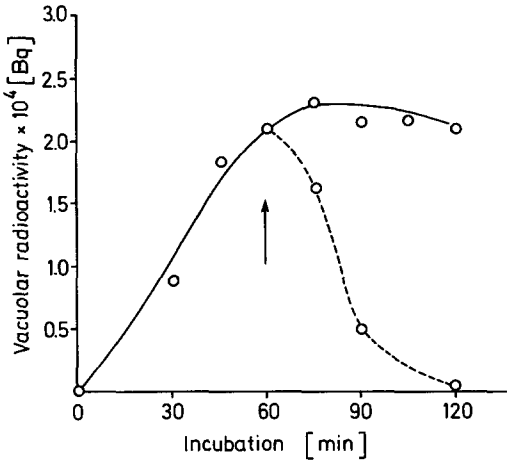

Fig. 5. The uptake and efflux of [^{3}H]vindoline. The uptake reaction (o—o) was started in 2 ml ($3.5 \cdot 10^5$ vacuoles ml^{-1}) incubation medium (1 mM $MgCl_2$, pH 6.5) with 400 pmol [^{3}H]vindoline. The *arrow* indicates the time at which 2000 pmol unlabelled vindoline were added to an aliquot (1 ml) of the incubation medium (o---o)

Specificity of alkaloid uptake. Transport mechanisms at the tonoplast have previously been described; these mechanisms were highly specific for their substrate. For instance, Willenbrink and Doll (1979) observed highly specific sucrose transport with vacuoles of red-beet tissue.

With protoplasts of *C. roseus*, Suzuki (1982) also showed a strict selectivity in sugar uptake. For a further characterization of the alkaloid transport system, the uptake of different alkaloids (Fig. 6) prepared from *C. roseus* and other plant tissue cultures was investigated (Table 6). It is quite clear that the vacuoles preferentially took up those alkaloids indigenous to the plant from which the vacuoles had been isolated. Thus, only ajmalicine and two other indole alkaloids, catharanthine and vindoline, were taken up by vacuoles from *C. ro-*

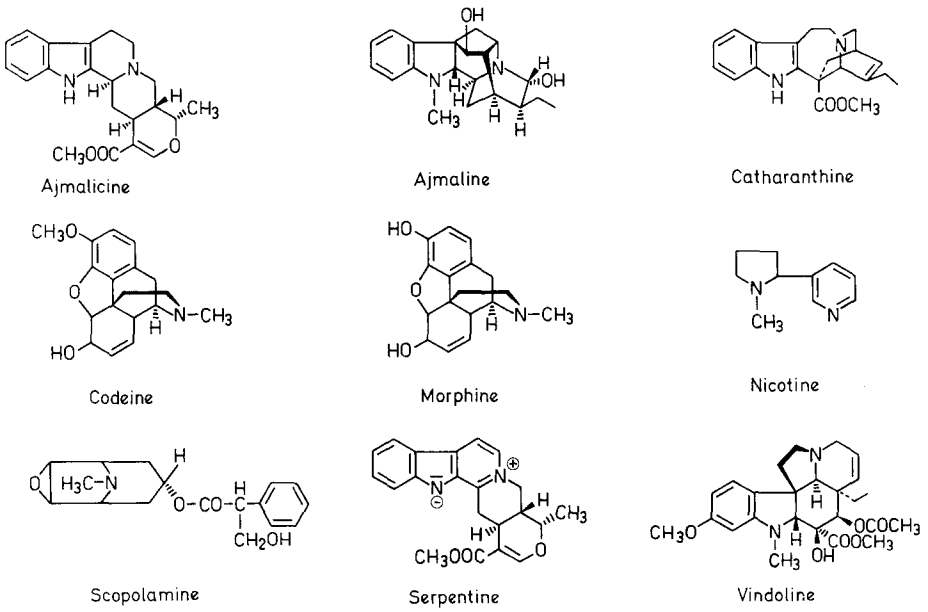

Fig. 6. Alkaloids used for the uptake experiments

Table 6. Selective uptake of alkaloids by vacuoles isolated from different plant species. Labelled alkaloid (400 pmol) was added to 1-ml samples of vacuole suspensions. The incubation medium, pH 6.5, contained $MgCl_2$ (1 mM), and KCl (50 mM). Alkaloid uptake was terminated after 90 min of incubation by filtration centrifugation (cell cultures were used if not otherwise indicated)

	Accumulated alkaloids [pmol (10^6 vacuoles)$^{-1}$]						
	Catharanthus roseus	*C. roseus* (mesophyll)	*Rauwolfia serpentina*	*Nicotiana sylvestris*	*Datura meteloides*	*Papaver somniferum*	*Daucus carota*
Ajmalicine	81	75	31	0	0	0	0
Catharanthine	31	230	211	0	2	0	0
Serpentine	0	0	0	0	0	0	0
Vindoline	199	47	44	0	0	0	0
Ajmaline	5	0	7	0	0	0	0
Nicotine	0	0	0	30	20	0	0
Scopolamine	0	1	1	15	22	0	0
Morphine	0	n.d.[a]	0	n.d.[a]	0	28	0
Codeine	0	0	0	0	0	9	0

[a] n.d. = not determined

seus, while non-species-specific alkaloids, such as codeine, morphine, or nicotine were discriminated. Similarly, vacuoles from *Papaver somniferum* cell cultures accumulated codeine and morphine, but none of the indole alkaloids or alkaloids occurring in solanaceous plants. Vacuoles from *Daucus carota*, a plant which does not produce any of these alkaloids, accumulated none of them. These results clearly demonstrate the high degree of selectivity of the transport process at the tonoplast membrane. Therefore, a sensitive and highly selective mechanism is responsible for the control of alkaloid uptake into vacuoles of plant cells.

Discussion

As a consequence of the development and refinement of techniques for protoplast and vacuole isolation, the important function of vacuoles in the compartmentation of enzymes, ions, as well as primary and secondary products of metabolism has been fully realized (Matile 1984). From his investigations with isolated vacuoles of *Chelidonium majus*, Matile (1976) concluded that the cells protect themselves against the poisonous alkaloids, sanguinarine and chelerythrine, by sequestering them inside the vacuoles. Jans (1974) had previously demonstrated that sanguinarine and chelerythrine destroy cell membranes and subsequently cause cell death. With vacuoles isolated from barley mesophyll cells, Kaiser et al. (1982) demonstrated that the photosynthesizing protoplasts avoid osmotic problems in the cytosol by transferring the bulk of newly formed photosynthetic products into the vacuole. In barley leaves, vacuoles are storage compartments also for nitrate (Martinoia et al. 1981). The vacuolar localization of nitrate in this case may be an integral part in the highly complex regulation of nitrate-reductase activity. Certain ions are abundant in vacuoles (Leigh and Deri Tomos 1983) and may function in the osmotic balance of the plant tissue. All these examples prove that a functional vacuole is required for plant cell metabolism, and it is obvious that the tonoplast as the barrier between cytosol and vacuolar space must play a decisive role in determining the composition of the vacuolar fluid (Matile 1978). Therefore, a controlled transport system must be involved in the uptake of natural products into the vacuole and may in fact be a limiting factor in the production of natural products by the intact plant or a plant cell culture. This aspect, the relationship between the productivity of a plant cell culture and vacuolar uptake and storage capacity, has scarcely been investigated.

Using a highly sensitive and specific radioimmunoassay we have shown here that serpentine is exclusively stored within the vacuoles of *C. roseus* cells. This conclusion is based on the use of acid phosphatase as vacuolar marker enzyme (Table 2). Comparative investigations of enzyme activities in protoplasts and vacuoles revealed that strictosidine synthase, specific glucosidase, as well as unspecific glucosidase have an extravacuolar localization in *C. roseus* cells. In agreement with this result, protoplasts took up the indole-alkaloid precursors, tryptamine and strictosidine, and incorporated them into their specific alkaloids while isolated vacuoles were no longer able to produce any alkaloids at all. From these results we can conclude that the key enzymes of the early indole-alkaloid pathway are not compartmentalized between cytosol and vacuolar space. Presumably the entire sequence of alkaloid biosynthesis is extra-vacuolar.

From the site of their biosynthesis within the cytoplasm to the site of their storage, the vacuolar space, the alkaloids must pass the tonoplast. In this study, vacuoles isolated from different plant cell cultures were found to be sufficiently stable to allow experiments on alkaloid transport across the tonoplast. Almost 50% of the added vindoline was taken up by isolated vacuoles from *C. roseus* within 90 min. During the first hour of incubation the vindoline content of the vacuoles increased linearly. Uptake of vindoline was strongly influenced by the pH of the medium, with an optimum at pH 6.5 (Fig. 2). The strong pH dependence of transport processes across the tonoplast has also been shown in several other systems (Schwencke and De Robichon-Szulmajster 1976; Guy et al. 1979; Ohsumi and Anraku 1981; Thom et al. 1982). Optimal transport of vindoline across the tonoplast of *C. roseus* cells at pH 6.5 is in the same range as the pH optima of enzymes of the indole-alkaloid pathway, e.g. strictosidine synthase at pH 6.8 (Treimer and Zenk 1979) or specific glucosidase at pH 6.0–6.4 (Hemscheidt and Zenk 1980). Vindoline uptake increased with temperature and was optimal in our experiments at 30° C (Table 3). In agreement with a number of other reports (Ohsumi and Anraku 1981; Suzuki 1982), we found that $MgCl_2$ and KCl stimulated alkaloid uptake into vacuoles. While sugar uptake by *Pisum sativum* vacuoles was reported to be markedly stimulated by the addition of MgATP to the medium (Guy et al. 1979), we never observed any stimulation of alkaloid uptake by the addition of ATP, which was in contrast to several other reports describing the stimulation of transport processes across the tonoplast (Doll et al. 1979; Ohsumi and Anraku 1981; Knuth et al. 1983). The lack of an effect of ATP on alkaloid uptake may be due to the high concentration of NaCl (used as osmotic stabilizer) in the incubation mixture which might have influenced the membrane potential of the tonoplast. Nevertheless, ATP appears to be the energy source for the transport process because the ATPase inhibitor, DCCD, at 30 μM completely blocked the uptake of vindoline (Table 5). These results agree with the observation of Ohsumi and Anraku (1981) that uptake of basic amino acids into yeast vacuoles was highly sensitive to DCCD. They concluded from their results that a Mg^{2+}-requiring, DCCD-sensitive tonoplast ATPase functions as an energy donor for active arginine transport. Thus, alkaloid transport into *C. roseus* vacuoles also appears to be an active energy-requiring process, and the energy source may be ATP. The positive effect of K^+ and Mg^{2+} on alkaloid uptake, also observed by Doll et al. (1979) for sucrose uptake into red-beet vacuoles may be related to a stimulation of tonoplast ATPase. Alkaloid uptake into isolated vacuoles exhibited typical saturation kinetics (Fig. 4), comparable with those observed for S-adenosyl-methionine transport into yeast vacuoles (Schwencke and De Robichon-Szulmajster 1976) as well as for other vacuolar uptake systems (Willenbrink and Doll 1979; Thom et al. 1982; Cram 1983), supporting the concept of carrier-mediated transport across the tonoplast. The K_m values determined for alkaloid uptake (ajmalicine 1.67 μM, vindoline 1.5 μM) are comparable with those of high-affinity amino-acid transport mechanisms in *Escherichia coli* (Rosen 1971). Another indication of an active alkaloid transport into isolated vacuoles was the observation that ajmalicine was accumulated against a concentration gradient (Table 4). Although accumulation of some alkaloids inside the vacuole may be mediated by an ion-trap mechanism, as reported by Matile (1976) and Matile et al. (1970), such a mechanism cannot be responsible for the alkaloid accumulation inside *C. roseus* vacuoles. Our experiments strongly indicate that vindoline was not bound inside the vacuoles as a non-diffusable salt but readily exchanged with excess vindoline in the medium. The recently proposed ion-trap mechanism for alkaloid accumulation in *C. roseus* cells (Neumann et al. 1983) is thus at variance with our results.

A typical property of specific transport systems in general is the selectivity for the respective substrate. This observation has been extended for vacuolar transport systems with hexose analogues (Guy et al. 1979) and amino acids (Ohsumi and Anraku 1981) and is fully confirmed by the results presented in Table 6. Vacuoles took up only those alkaloids in substantial amounts which are indigenous to the plant from which the vacuoles had been isolated. Thus, vacuoles from *C. roseus* and *Rauwolfia serpentina* excluded benzylisoquinoline and tropane alkaloids, and preferably accumulated the indole alkaloids. Although ajmaline is one of the main alkaloids of *Rauwolfia serpentina*, the extent to which this alkaloid accumulated inside isolated vacuoles of this plant species was only small compared with the alkaloid accumulation in vacuoles of *C. roseus*.

It may be possible that not the product, ajmaline itself, but a precursor is preferably taken up by the vacuoles and is then metabolized to ajmaline, perhaps during the passage across the tonoplast membrane. Surprisingly, serpentine, an indole alkaloid which is typical for *C. roseus* as well

as for *Rauwolfia serpentina* and which is beyond doubt located inside the vacuole of *C. roseus*, was never taken up from vacuoles of *C. roseus* or *Rauwolfia serpentina*. These results let us presume that not serpentine itself but the precursor ajmalicine is taken up by the vacuoles. As it passes the tonoplast membrane, serpentine may be produced from ajmalicine by dehydration. The specificity of alkaloid uptake was further demonstrated with *Daucus carota* vacuoles which did not accumulate any of the alkaloids offered. The results of Renaudin (1981), who reported an unspecific accumulation of tabernanthin by whole cells from cell suspension cultures of *C. roseus* and *Acer pseudoplatanus* and proposed a diffusive uptake of this alkaloid, do not contradict our results with isolated vacuoles. The transport processes across the plasmalemma may be quite different from those at the tonoplast membrane; therefore, results obtained with whole cells are not necessarily comparable with those found for transport processes at the level of isolated vacuoles.

Summarizing our results, we can conclude that alkaloid transport at the tonoplast is an active, energy-requiring process which is highly specific for the respective substrate. A specific transport mechanism at the tonoplast membrane is responsible for the selective uptake of alkaloids into the vacuoles; this mechanism mediates the accumulation of natural compounds within the cell and may, therefore, also influence the productivity of a plant cell.

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