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Characterization of Alkaloid Uptake by *Catharanthus roseus* (L.) G. Don Protoplasts[1]

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ABSTRACT

The accumulation of alkaloids by protoplasts of *Catharanthus roseus* (L.) G. Don var. Little Bright Eye was studied to determine the specificity of uptake and the role of ion trapping in the storage of alkaloids. Accumulation of the indole alkaloids vindoline, ajmalicine, tabersonine, and vinblastine was found to be biphasic, with an initial burst of uptake followed by a slow, prolonged phase of accumulation. The concentration and pH dependence of the initial burst of uptake for vindoline suggested that uptake occurred by simple diffusion. Uptake of nicotine was monophasic, with a half life of 5.2 minutes. The accumulation ratio (Ci/Ce) for nicotine at steady state and for the initial burst of uptake for vindoline and ajmalicine suggested that accumulation was driven by the pH gradient between the vacuole and the external assay medium. The second, sustained phase of uptake of vindoline was sensitive to inhibition by either 20 millimolar NaN_3 or 0.5 millimolar Cu^{2+}. In azide-treated protoplasts, the uptake for vindoline conformed to the kinetics of simple diffusion, with a half life of 4 minutes. The second phase of uptake for ajmalicine, although sensitive to inhibition by Cu^{2+}, was insensitive to inhibition by NaN_3. The biphasic uptake of the indole alkaloids was not due to any significant metabolism. It is concluded that accumulation and storage of the indole alkaloids is due only partly to ion trapping of the alkaloids by the low pH of the vacuole lumen. In the case of vindoline, there appears to be a specific energy-requiring uptake that is not seen with nicotine (which is not endogenous to *Catharanthus*). Accumulation of ajmalicine appears to involve both ion trapping and an azide-insensitive component, which may be due to complexation with organic counterions and phenolics.

The vacuole of plant cells plays a central role in the storage of a wide variety of metabolites, including organic acids, sugars, and secondary metabolites (14). The vacuolar accumulation of cations such as Na^+ (2) and Ca^{2+} (21) and, in the case of CAM plants, organic acids such as malate (15) is intimately related to the pH gradient established across the tonoplast by proton pumping ATPases and pyrophosphatases. It has long been assumed that alkaloids are trapped inside the vacuole as nonpermeant salts by the low pH of the vacuole lumen. This ion trapping of the alkaloids assumes that the neutral alkaloid is able to freely diffuse through the cellular membranes in a nonmediated fashion, with the final distribution being determined primarily by the pKa of the alkaloid and the pH gradient between the extracellular media and the vacuole lumen (13, 20). This principle of the ion trapping of weak bases has been used extensively to estimate the pH gradients across membranes using [^{14}C]methylamine and other weak bases (12). Evidence in support of simple diffusion of alkaloids across cellular membranes with ion trapping in the lumen of the vacuole has been presented for the accumulation of nicotine by cultured cells of *Acer pseudoplantus* and *Nicotiana tabacum* (13) and the accumulation of tabernanthine and ajmalicine by cultured cells of *Catharanthus roseus* (20). From these studies it has been proposed that transport across the cellular membranes occurs by simple nonmediated diffusion, since nicotine and tabernanthine are not endogenous to either *A. pseudoplantus* or *C. roseus*, respectively.

The importance of ion trapping has been questioned recently by work with vacuoles isolated from *C. roseus* and *Fumaria capreolata* tissue cultures (4, 6). These transport studies have shown that there appears to be a specific transport system in the tonoplast which accumulates alkaloids endogenous to the plant in question. Based on the specificity of uptake and the effect of inhibitors it was proposed that transport of alkaloids across the tonoplast is carrier-mediated and indirectly driven by the pH gradient across the tonoplast (6). Due to the freely reversible but specific exchange of labeled alkaloid in the vacuole with externally added unlabeled alkaloid, it was proposed that retention of the alkaloids in the lumen of the vacuole is not primarily due to ion trapping of the protonated alkaloid.

In order to try and resolve some of the uncertainties concerning the transport and storage of alkaloids in plant cells, it was decided to study the uptake and storage of several different alkaloids in protoplasts prepared from *C. roseus* leaves. The indole alkaloids vindoline, ajmalicine, tabersonine, and vinblastine (see Fig. 1 for alkaloid structures), all of which are endogenous to *C. roseus*, and nicotine and morphine, which do not occur in *C. roseus*, were used to assess the specificity of uptake and the role of ion trapping. It is well known that there is a wide variation in the ability of tissue cultures to synthesize alkaloids and that the production capabilities of a particular cell strain are, in many cases, unstable with time (5). Similarly, it is reasonable to assume that if there exist specific systems involved in the intracellular transport and storage of alkaloids, these may also exhibit wide variations between different tissue culture strains. For this reason, it was decided to emphasize the use of protoplasts from leaves instead of those from tissue cultures.

MATERIALS AND METHODS

Chemicals and Radiochemicals. All chemicals were of the finest grade available. Unless otherwise noted, all biochemicals were from Sigma. Ajmalicine was from Fluka Chemicals and serpentine was prepared from ajmalicine according to published procedures (1). [G-^{3}H]Vinblastine sulfate (11.5 Ci/mmol) was from Amersham. A gift of [1-^{3}H]morphine (21 Ci/mmol) from L. R. Griffing (Dept. of Biology, Texas A&M) was from Amersham. Tritiated water (1 mCi/ml) and NaB^3H_4 (0.319 Ci/mmol) were from New England Nuclear. L-[*N*-methyl-^{3}H]nicotine (15 Ci/mmol) was from American Radiolabeled Chemicals Inc. [G-^{3}H]Vindoline (5.3 Ci/mmol) was prepared by Amersham. Aquasol (New Eng-

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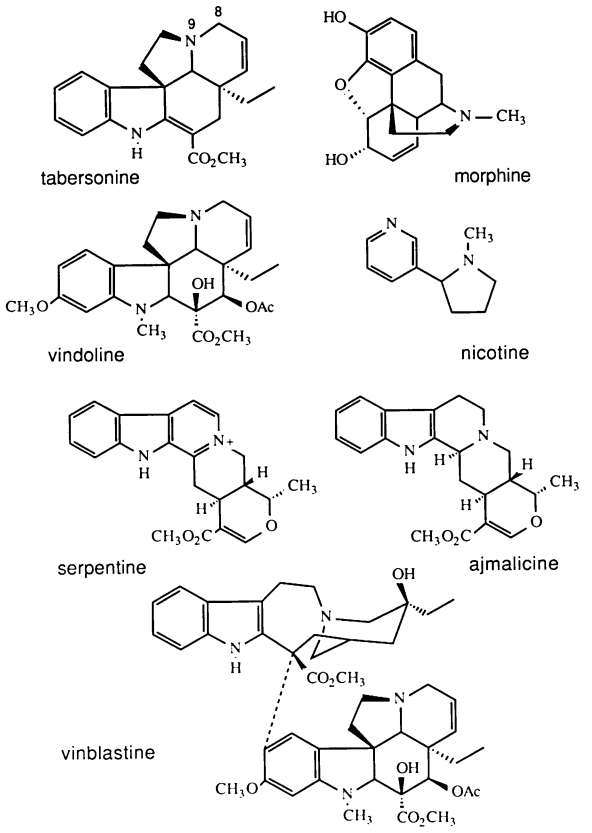

FIG. 1. Structures of alkaloids used in this study.

land Nuclear) was used for scintillation counting. Silicone oil was from Petrarch Systems Inc. (Bristol, PA). Tabersonine was from Omnium Chimique (Belgium). The apparent pKa of tabersonine was measured by titration with HCl in 50% (v/v) DMF.[3]

Plant Material and Protoplast Isolation. *Catharanthus roseus* (L.) G. Don (var. Little Bright Eye from W. Atlee Burpee Co) was grown from seed either in a greenhouse or indoors under cool-white fluorescent lights. Suspension cultures of *C. roseus* were established from surface-sterilized leaf discs of the above *C. roseus* plants cultured on solid Murashige and Skoog media (19), supplemented with 3% (w/v) sucrose, 1 μM 2,4-D, and 10 μM kinetin. After several transfers, the resulting fluffy callus was transferred to liquid media of the same composition and maintained at 120 rpm, 25°C, with subculturing once a week. The suspension cultures used in this study produced only low levels of indole alkaloids.

Protoplasts from *C. roseus* leaves were typically isolated from only the two or three youngest, fully expanded pairs of leaves from healthy, 3 month old flowering plants. Leaves (1.5–2 g) were thoroughly washed in distilled water and after removal of the midrib, were finely sliced by hand into 1–2 mm wide slices. The leaf slices were suspended in 50 ml of a digestion media of the following composition; 10 mM Mes, 0.5 M mannitol, 1 mM $CaCl_2$, 4% (w/v) Cellulase (Sigma, from *Penicillium funiculosum*), 1% (w/v) hemicellulase (Sigma, from *Aspergillus niger*), 0.2% (w/v) Macerase (Calbiochem), adjusted to pH 5.6 with NaOH. After 10 h digestion with gentle agitation under subdued light, undigested material was filtered off with a 105 μm polypropylene seive (SpectraMesh). The crude protoplasts were centrifuged at 100*g* onto a cushion of 20% (w/v) Ficoll in buffered mannitol. The protoplasts were purified by flotation twice at 100 *g* through 15% Ficoll in buffered mannitol, overlayed with buffered mannitol. The buffers used for purification of the protoplasts were as follows: buffer A, 10 mM Pipes, 1 mM $CaCl_2$, 0.5 M mannitol, adjusted to pH 6.5 with NaOH; buffer B, 20% (w/v) Ficoll in A. Intermediate concentrations of Ficoll were obtained by mixing appropriate volumes of A and B. Protoplasts from *C. roseus* suspension cultures were prepared 4 d after transfer to fresh media and were purified in a similar manner using a 6 h digestion. Unless otherwise noted, all the uptake assays were carried out using protoplasts prepared from leaves.

Synthesis of [G-^{3}H]Ajmalicine and [8-^{3}H]Tabersonine. The method of Arens *et al.* (1) was used to prepare [G-^{3}H]ajmalicine. Reduction of serpentine (8.5 μmol) with NaB^3H_4 in 0.5 ml of methanol, followed by extensive purification on TLC, afforded 1.7 μmol of [G-^{3}H]ajmalicine (20% yield, 135 mCi/mmol).

Tabersonine[4] was labeled by reduction of the C_8-N_9 immonium salt of tabersonine with NaB^3H_4. Treatment of tabersonine with 1.2 equivalents of *p*-nitro-peroxybenzoic acid in methylene chloride at 4°C for 5 min gave the N_9-oxide of tabersonine (purified by TLC on silica gel plates in 9:1 [v/v] chloroform:methanol, 40% yield). Treatment of the N_9-oxide with a large excess of TFAA in methylene chloride at room temperature for 30 min generated the C_8-N_9 immonium. After evaporation of the solvent and excess TFAA under a stream of dry nitrogen, the immonium was reduced with NaB^3H_4 in methanol. Purification by TLC (chloroform and 2:7:1 [v/v/v] acetone:pet ether:diethylamine) provided 11 μmol of [8-^{3}H]tabersonine (35% yield, 77 mCi/mmol). ^{1}H and ^{13}C NMR of tabersonine labeled with NaB^2H_4 by this method indicated that the label is introduced into the C_8 position.

Assay of Alkaloid Uptake. Uptake of labeled alkaloids was assayed by silicone oil centrifugal filtration. Briefly, 1.5 ml of protoplasts were added to 2 ml of buffer A which contained the appropriate concentration of labeled alkaloid, with a final protoplast density of $\sim 2 \times 10^6$/ml. In the case where tissue culture protoplast were used, the protoplast density was $\sim 7 \times 10^5$/ml. When inhibitors were used, the protoplasts were pretreated for the indicated time before addition to the assay media, which also contained the indicated concentration of inhibitor. Unless otherwise noted, during the assay, the protoplasts were gently agitated at 25°C (pH 6.5). At the indicated time intervals, 100 μl of protoplasts were withdrawn and centrifuged through a step gradient consisting of 75 μl of buffer B, 50 μl of silicone oil (3:1 PS060.5:PS061), and 50 μl of buffer A in a 400 μl of polypropylene tube for 5 s (Beckman type E centrifuge) and subsequently frozen in liquid nitrogen until the end of the assay. The tubes were then sliced through the silicone oil with a scalpel, and the protoplast pellets were transferred to a scintillation vial for counting. Results were not normally corrected for the yield of protoplasts passing through the silicone oil since, based on protein determinations, >95% of the protoplasts routinely passed through the silicone oil (data not shown).

When making direct comparisons between uptake of different indole alkaloids, [^{3}H]vindoline and [^{3}H]vinblastine were diluted with unlabeled alkaloid to compensate for the lower specific activities of the [^{3}H]tabersonine and [^{3}H]ajmalicine. Otherwise, uptake was measured using undiluted alkaloid at the indicated concentrations.

Estimation of Protoplast Volume. Protoplast volume was estimated as the ^3H_2O accessible space measured by silicone oil centrifugal filtration. Protoplasts were equilibrated with 5 μCi of ^3H_2O for 10 min, after which they were centrifuged as described above except the upper layer of buffer A was omitted from the centrifuge tube. The ^3H_2O accessible space was typically measured over a range of 4×10^4 to 4×10^5 protoplasts and the slope of ^3H_2O accessible space versus number of protoplasts was

[3] Abbreviations: DMF, dimethyl formamide; TFAA, trifluoroacetic anhydride.

[4] The numbering system used for tabersonine is that described for aspidospermidine derivatives in Chemical Abstracts.

used as an estimate of protoplast volume.

Extraction and HPLC of Alkaloids. A combination of HPLC and scintillation counting was used to check for any metabolism of the indole alkaloids by the protoplasts. The extraction and HPLC was a modification of a previously published procedure (11). After uptake of the labeled alkaloid into the protoplasts, 15×100 μl samples of the protoplasts were centrifuged through silicone oil without the lower layer of Ficoll present. After freezing in liquid nitrogen, the protoplast pellets were cut from the bottom of the tubes and were solubilized in 500 μl of 5% (v/v) acetic acid in ethanol. After removal of the undissolved material by centrifugation, the ethanolic extract was loaded onto a silica SepPak (Waters) preequilibrated with the same solvent. After washing with ~3 ml of hexane to remove Chl and other pigments, the SepPak was flushed with ammonia gas to neutralize the acetic acid present. The alkaloids were then washed off the silica with ~5 ml of methanol. After evaporation of the solvent, an aliquot of the alkaloid extract, dissolved in methanol, was analyzed by HPLC. The total recovery of ^3H in the alkaloid fraction from this extraction procedure was typically >90% of the theoretical total.

HPLC was carried out on a C_8 column (250×4.6 mm, 5 μm particle size, Econosil, Alltech Assoc. Inc.) with UV detection at 290 nm. Elution of the column was carried out with acetonitrile and aqueous 10 mM triethylamine adjusted to pH 7.0 with formic acid using a linear gradient of 60 to 100% acetonitrile over 10 min, followed by 100% acetonitrile for 10 min at 2.0 ml/min. Fractions were collected every 0.2 min and analyzed by scintillation counting. Typically, between 95 to 105% of the total theoretical amount of ^3H injected onto the HPLC column was recovered.

RESULTS

Specificity of Alkaloid Accumulation. Figure 2 shows that uptake by mesophyll protoplasts of *Catharanthus roseus* of vindoline, ajmalicine, tabersonine, and vinblastine, all of which are endogenous to *C. roseus*, is biphasic. In each case, uptake is characterized by an initial, rapid burst of uptake, followed by a slower, linear phase of uptake. The relative magnitudes of the initial burst of uptake and the rate of the slower second phase appear to depend on the specific alkaloid in question. In the case of vindoline, the second phase of uptake continues linearly for greater than 3 h (data not shown). In contrast, uptake of morphine and nicotine do not show this biphasic nature in *C. roseus* protoplasts. Nicotine shows a monophasic uptake which reaches a steady state by 25 min, while morphine shows only a very slow, linear uptake. With the exception of morphine, uptake of each alkaloid into protoplasts prepared from suspension cultures of *C. roseus* was monophasic without the second sustained phase seen with the leaf protoplasts.

Concentration and pH Dependence of Uptake. Vindoline was the only *Catharanthus* alkaloid for which the initial velocity of uptake could be accurately estimated. The initial burst of uptake for both ajmalicine and tabersonine was too rapid for accurate measurement and that of vinblastine was both too short and small. The initial rate of uptake of [^3H]vindoline, diluted with unlabeled vindoline to give the appropriate concentration, was estimated from the first 3 min of uptake and found to be linear from 100 nM up to >100 μM. At concentrations higher than 100 μM, the initial velocity increased more rapidly, possibly due to nonspecific disruption of the membranes by the hydrophobic alkaloid.

The pH dependence for uptake of vindoline was measured by adding the protoplasts, in unbuffered mannitol, to the labeled vindoline, buffered at the appropriate pH with a combination of 10 mM Mes, Pipes, or Tris. Uptake was measured for the first 4 min to obtain a linear estimate. Initial uptake reached a maximum at pH 7.5, with uptake above this pH dropping off due to protoplast lysis. At pH 5.5, uptake was ~50% that of the maximum. Subsequent assays were carried out at pH 6.5 since this gave the best compromise between rapid uptake and protoplast stability.

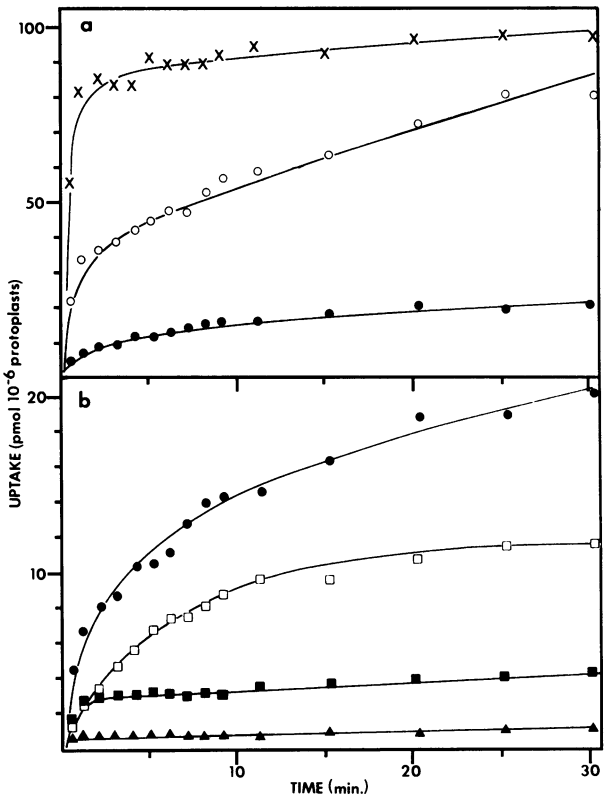

FIG. 2. Time course of alkaloid uptake of labeled alkaloids by *C. roseus* leaf mesophyll protoplasts. Protoplasts at ~2×10^6/ml were incubated with; 0.21 μM [^3H]vindoline (●), 0.24 μM [^3H]ajmalicine (○), 0.22 μM [^3H]tabersonine (×), 0.23 μM [^3H]vinblastine (■), 0.13 μM [^3H]nicotine (□), or 0.13 μM [^3H]morphine (▲). b is an expanded vertical scale of a.

Calculation of Accumulation Ratios. The ratio of alkaloid accumulated inside the protoplasts *versus* the external concentration of alkaloid (Ci/Ce) requires an estimate of the protoplast volume. The ^3H_2O accessible space in the protoplasts was used as an estimate of the protoplast volume. A plot of ^3H_2O accessible space *versus* number of leaf mesophyll protoplasts was linear, with a slope of 12 μl/10^6 protoplasts or 12 pl/protoplast (see Table I for volume estimates). This corresponds to an average diameter of 30 μm, which closely agrees with that estimated microscopically. An estimate of the pH of the vacuole lumen can be calculated from the accumulation ratio using the previously described (22) formula:

$$Ci/Ce = \frac{1 + 10^{ppKa-pHi}}{1 + 10^{pKa-pHe}}$$

In order to calculate the vacuolar pH (pHi) from the above equation, the distribution of the alkaloid must be determined primarily by diffusion of the neutral form of the alkaloid and must be at a steady-state level. The Ci/Ce for nicotine used to calculate pHi was estimated after 40 min incubation. Clearly, due to the biphasic nature of the uptake of ajmalicine, vindoline, and tabersonine by *C. roseus* mesophyll protoplasts, the accumulation ratio used to calculate pHi for these alkaloids must be estimated from the region in which the initial burst of uptake

Table I. *Estimation of Vacuolar pH (pHi) from Uptake of Alkaloids by Protoplasts*

The values of Ci/Ce for vindoline, ajmalicine, and tabersonine were estimated from the magnitude of the initial burst of uptake observed for each alkaloid. Alkaloid uptake was measured at the same concentrations as in Figure 2. The estimate of Ci/Ce for vindoline uptake into azide-treated protoplasts and for nicotine uptake was obtained after 40 min incubation at an assay of pH 6.5. The estimates of protoplast volume and vacuolar pH were calculated as described in the text.

	pK_a	Mesophyll Protoplasts (12 pl/proto-plast)		Tissue Culure Protoplasts (14 pl/proto-plast)	
		Ci/Ce	pHi	Ci/Ce	pHi
Vindoline					
$-NaN_3$	5.5	3.4	5.1	4.8	4.9
$+NaN_3$		4.3	4.9		
Ajmalicine	6.3	14	5.0	27	4.7
Tabersonine	5.9	40	4.2	47	4.1
Nicotine	8.02	12	5.4	14	5.3

turns into the second, sustained phase. Table I shows the accumulation ratios and the calculated pHi values obtained. The pK_a value listed for tabersonine was estimated from titration in 50% (v/v) DMF, the others are literature values (9, 17). Close agreement was obtained for the pHi calculated from vindoline, ajmalicine, and nicotine uptake into both leaf and cultured cell protoplasts. Simple ion trapping according to the above equation is not, however, able to explain either the magnitude of the initial burst for tabersonine or the second phase of uptake for all four of the *Catharanthus* alkaloids into leaf protoplasts. Because of the slow rate of accumulation, estimates of pHi were not calculated from the uptake of morphine or vinblastine.

Effect of Inhibitors on Uptake. In the case of vindoline transport, pretreatment of protoplasts with 20 mM NaN_3 for 15 min results in a monophasic uptake, without the sustained phase of transport seen in the control assay (Fig. 3a). A replot of vindoline uptake by azide-treated protoplasts as $\ln[1 - C_t/C_\infty]$ *versus* time is linear, indicating that uptake conforms to the kinetics of simple diffusion, with a half-life of approximately 4 min (data not shown). Under conditions of equilibrium exchange (8), where the azide-treated protoplasts are equilibrated with different concentrations of unlabeled vindoline before addition of a low concentration of ^{3}H-vindoline, this half-life was found to be constant, again suggesting that the first component of uptake is due to simple diffusion and not to carrier-mediated transport (data not shown). Treatment with NaN_3 had no effect on uptake of either morphine or ajmalicine by *C. roseus* protoplasts (Fig. 3b).

The second phase of uptake of vindoline was also found to be sensitive to inhibition by Cu^{2+}. Pretreatment for 1 min with 0.5 mM Cu^{2+} completely inhibited the second phase of uptake, without significantly inhibiting the initial uptake (Fig. 3a), while 0.1 mM Cu^{2+} had no effect on uptake. Similarly, the second phase of uptake of ajmalicine was also found to be sensitive to 0.5 mM Cu^{2+} (Fig. 3b). Although the Cu^{2+} treatment caused some aggregation of the protoplasts, no visible damage was apparent.

Efflux of accumulated vindoline could be rapidly induced by treatment of the protoplasts with 20 mM NH_4Cl. On treatment with NH_4Cl, the vindoline content rapidly decreased to the same level seen when vindoline was accumulated by protoplasts pretreated with 20 mM NH_4Cl (Fig. 4a). As with azide-treated protoplasts, the kinetics of influx and efflux under the influence of NH_4Cl were consistent with simple diffusion. If the protoplasts were treated with NH_4Cl up to 40 min after the start of the assay, the intracellular levels of ^{3}H always relaxed to the same level

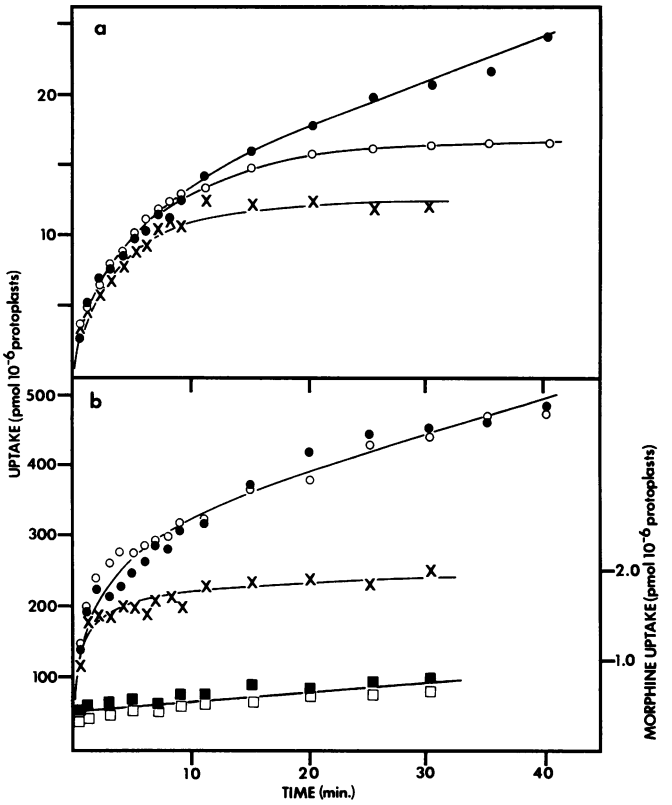

FIG. 3. Effect of inhibitors on the uptake of alkaloids by *C. roseus* leaf mesophyll protoplasts. Protoplasts incubated with either 0.22 μM [^{3}H]vindoline (a) or 1.5 μM [^{3}H]ajmalicine (b) were pretreated with either 20 mM NaCl for 15 min (●), 20 mM NaN_3 for 15 min (○), or 0.5 mM $CuCl_2$ for 1 min (×). Protoplasts incubated with 0.10 μM [^{3}H]morphine (b) were pretreated with either 20 mM NaCl for 15 min (■) or 20 mM NaN_3 for 15 min (□).

seen when the protoplasts were pretreated with NH_4Cl before the start of the assay. Efflux of ajmalicine could also be rapidly induced by NH_4Cl, although, in this case, a short rapid efflux of ^{3}H was followed by a long, slow efflux that only slowly approached the intracellular level of ^{3}H of protoplasts pretreated with NH_4Cl (Fig. 4b). As uptake continued, this slow component of the efflux came to account for a progressively larger proportion of the NH_4Cl induced efflux.

Metabolism of Alkaloids. Protoplasts incubated with each labeled indole alkaloid were centrifuged through silicone oil, extracted, and assayed for any metabolism by HPLC/scintillation counting as previously described. Of the indole alkaloids examined, only ajmalicine showed any observable metabolism over the short assay period used (Fig. 5). However, even in the case of ajmalicine, the amount of metabolism observed was relatively insignificant, with less than 2% of the total ^{3}H appearing in other compounds.

DISCUSSION

Protoplasts from leaves of *Catharanthus roseus* are able to accumulate all the alkaloids tested. Uptake of all four *Catharanthus* alkaloids was biphasic, characterized by an initial, rapid burst of uptake followed by a long, slow phase of linear uptake. The magnitude of the initial burst of uptake and the rate of the second phase of uptake depended on the specific alkaloid. In contrast, uptake of morphine was slow and linear, and uptake of nicotine was monophasic, reaching a steady state after 25 min (Fig. 2).

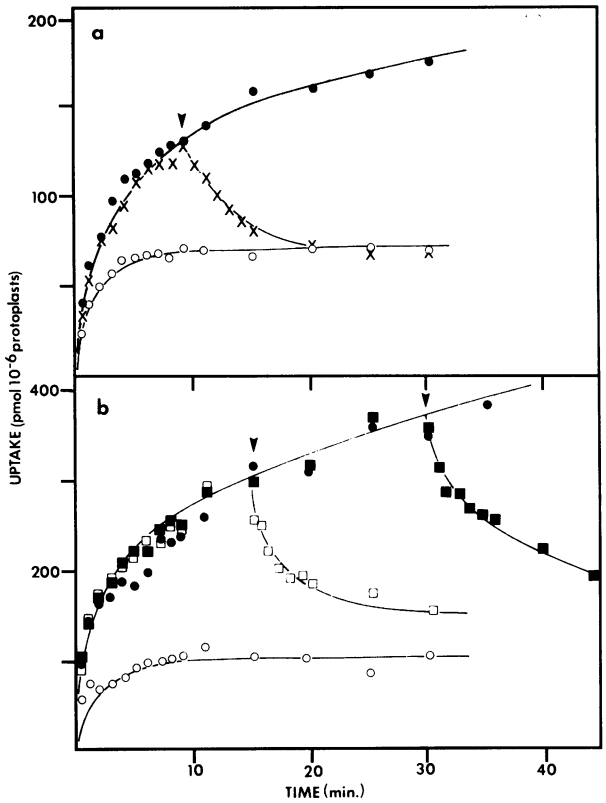

FIG. 4. Effect of NH_4Cl on uptake of alkaloids by *C. roseus* leaf mesophyll protoplasts. Protoplasts incubated with either 2.3 μM [^{3}H]vindoline (a) or 1.1 μM [^{3}H]ajmalicine (b) were treated with NH_4Cl at a final concentration of 20 mM at the indicated times. For the control assays (○), the protoplasts were pretreated with 20 mM NH_4Cl for 2 min before the start of the assay.

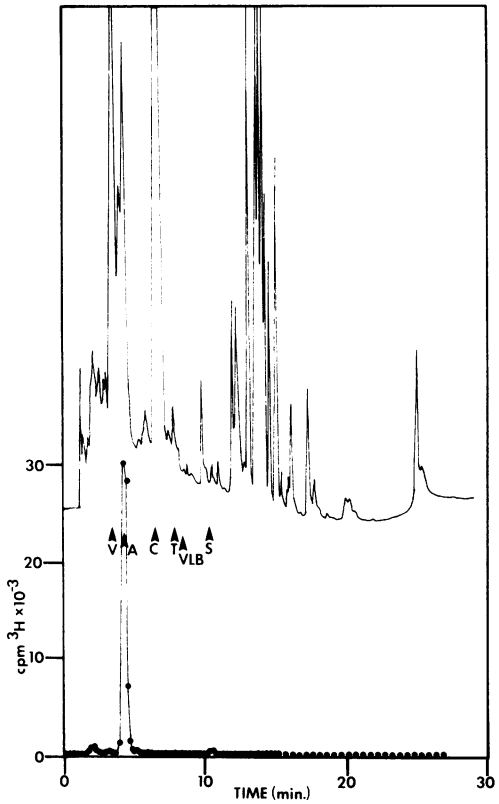

FIG. 5. Assessment of metabolism of labeled alkaloids by leaf mesophyll protoplasts. After 60 min incubation with 1.4 μM [^{3}H]ajmalicine, the protoplasts were extracted, and the alkaloid fraction was analyzed by HPLC/scintillation counting as described in "Materials and Methods." The retention times of vindoline (V), ajmalicine (A), catharanthine (C), tabersonine (T), vinblastine (VLB), and serpentine (S) are indicated.

Evidence for simple diffusion of the alkaloids into the protoplasts was provided by the kinetics of uptake for nicotine and the concentration and pH dependence of the initial rate of uptake for vindoline. The initial velocity of uptake of vindoline was linear over a 1000-fold concentration range between 100 nM and 100 μM. In addition, the initial rate of uptake increased between pH 5.5 to 7.5, at which point damage to the protoplasts began to take place. At pH 5.5, the initial rate of uptake was about 50% of the maximum observed at pH 7.5, which would be expected if only the neutral form of vindoline, with a pK_a of 5.5, was being transported. This agrees with previous results which indicated that only the neutral forms of ajmalicine (21) and nicotine (13) were involved in diffusion into cultured cells.

The consistency of the calculated pHi values obtained from the uptake of nicotine, and the magnitude of the initial burst of uptake for both ajmalicine and vindoline (Table I) using both leaf and cultured cell protoplasts, suggested the involvement of ion trapping in an acidic compartment inside the protoplast, presumably the vacuole. Since nicotine is not endogenous to *Catharanthus*, it is unlikely that there is any transport system present which could directly affect its distribution. The accumulation ratios are also clearly too large to be accounted for by diffusion only into a small cytoplasmic volume. For these reasons, it is unlikely that the initial burst of uptake for the *Catharanthus* alkaloids represents a simple filling of the cytoplasm, followed by slower accumulation into the vacuole. Tabersonine, however, was accumulated to an extent much greater than predicted from this model. This could be due either to a significant diffusion of the protonated form of tabersonine or to a large amount of intracellular binding to membranes (12). Due to the low rate of uptake for both vinblastine and morphine, pHi values were not estimated from these alkaloids.

Simple diffusion of the alkaloids with ion trapping is not able to account for the second, sustained phase of uptake seen for the *Catharanthus* alkaloids (Fig. 2). The second phase of uptake for both vindoline and ajmalicine was found to be sensitive to the effects of metabolic inhibitors, while the second phase of uptake for tabersonine and vinblastine was not active enough to study reliably in this way. The second phase of uptake for vindoline and ajmalicine showed different sensitivities to both NaN_3 and NH_4Cl. After pretreatment of protoplasts with 20 mM NaN_3 for 15 min, vindoline appears to diffuse into the protoplasts according to the kinetics of simple diffusion, whereas ajmalicine uptake is insensitive to NaN_3 (Fig. 3). Elimination of the second phase of uptake of vindoline is probably due to lowered ATP levels induced by the NaN_3. The calculated accumulation ratio of vindoline in protoplasts pretreated with 20 mM NaN_3 was 4.3 (Table I), suggesting that the vindoline can be accumulated in the vacuole due to the preexisting pH gradient across the tonoplast in an energy independent manner.

Treatment of the protoplasts with 20 mM NH_4Cl induces a rapid efflux of accumulated vindoline (Fig. 4a). Regardless of the time at which the NH_4Cl was added, vindoline levels always relaxed to the level seen when protoplasts were pretreated with NH_4Cl. After treatment with 20 mM NH_4Cl, the accumulation ratio was ~1.8, indicating that under these conditions vindoline is almost evenly distributed between the medium and the protoplasts, due to dissipation of transmembrane pH gradients throughout the protoplast. Although treatment of protoplasts with NH_4Cl caused an efflux of accumulated ajmalicine, there

was a slow component to the efflux that was not observed with vindoline (Fig. 4b). As the incubation time increased before the addition of NH_4Cl, the slow component came to account for a larger proportion of the efflux, while the rapid portion of the efflux remained roughly constant. HPLC/scintillation counting of protoplasts extracted after 60 min of incubation with [^{3}H]ajmalicine indicated that this was not due to metabolism of the ajmalicine to a less readily diffusible form (Fig. 5).

Pretreatment with 500 μM Cu^{2+} for only one min was found to inhibit the second phase of uptake for both vindoline and ajmalicine (Fig. 3). Using isolated vacuoles of *C. roseus*, it was shown that the uptake of vindoline could be inhibited by similar concentrations of Cu^{2+} (4). It has been reported that low concentrations of Cu^{2+} inhibit the tonoplast H^+-ATPase of yeast (10) although the tonoplast H^+-ATPase of plants is not inhibited by Cu^{2+} (3). Thus, it is not clear from these results if this inhibition is due to a direct interaction of Cu^{2+} with a tonoplast proton pump or an inhibition of an unrelated alkaloid transport system.

The sensitivity of the second phase of uptake of vindoline to both NaN_3 and Cu^{2+} suggests that the sustained uptake is due to an energy-dependent process that is specific for vindoline. It is unlikely that this simply represents a basal level of proton pumping across the tonoplast, which would pull increasing amounts of alkaloid across, since nicotine, an alkaloid not found in *Catharanthus*, did not show this biphasic uptake. Previous work with suspension cultured cells of *A. pseudoplantus* and *C. roseus* also did not show this type of sustained uptake (13, 20). In addition, none of the alkaloids tested showed biphasic uptake into protoplasts prepared from *Catharanthus* tissue cultures (data not shown). As is the case with many of the biosynthetic enzymes for the indole alkaloids, it is possible that many of the tissue cultures examined may lack specific transport systems for alkaloids that are found in the plant.

Although small, the initial burst of uptake for vinblastine was consistently observed in *C. roseus* mesophyll protoplasts, whereas morphine always showed a very slow linear uptake without an initial burst. It has previously been shown using a different transport assay (18) that uptake of morphine by *C. roseus* mesophyll protoplasts is linear for >180 min before reaching a steady state. Differences in the assays and the presentation of results make it difficult to compare the results presented here with that study. It was considered possible that the low rate of net morphine uptake was due to a balance between diffusion into the protoplast with an energy-dependent excretion against a concentration gradient. The lack of effect of NaN_3 on morphine uptake indicated this was not the case (Fig. 3b). The reason for the biphasic uptake of vinblastine is not known at this time. Uptake of vinblastine and morphine is probably limited by an intrinsically low permeability of membranes for these two alkaloids.

It has been suggested that binding of alkaloids to organic counterions and phenolics could assist in the ion trapping of alkaloids inside the vacuole (20). The slow formation of these nondiffusible alkaloid complexes would be expected to produce biphasic uptake kinetics (16). In the case of ajmalicine, the second phase of uptake may have been caused by this type of slow complexation with counterions and phenolics. This would explain the insensitivity of ajmalicine uptake to NaN_3 and the slow phase of NH_4Cl-induced efflux. This type of trapping, however, is unlikely to be responsible for the biphasic kinetics seen for vindoline uptake because of the azide sensitivity observed. The effect of Cu^{2+} on the second phase of uptake of both vindoline and ajmalicine may be due to an inhibition of a specific transport system for both these indole alkaloids, as suggested previously with isolated vacuoles (4).

It is interesting to note that of the alkaloids tested only vindoline, one of the major alkaloids which accumulates in *Catharanthus* leaves (23), was clearly shown to have an energy dependent accumulation. The other three indole alkaloids tested, although endogenous to *Catharanthus*, occur only at very low levels in the leaves. Ajmalicine, although produced at high levels in the roots and in tissue cultures (1, 5), is virtually absent from the leaves. Tabersonine, a precursor of vindoline, is present at significant levels only in etiolated seedlings. During the greening of etiolated seedlings, tabersonine levels drop sharply with a concomitant increase in the levels of vindoline found in the cotyledons (7). Tabersonine was undetectable by either HPLC or TLC in mature leaves of *Catharanthus* (Fig. 5). This suggests that an efficient channeling of intermediates from tabersonine to vindoline occurs normally in the leaf. If this channelling takes place, it is questionable whether a significant amount of storage of these biosynthetically active alkaloids occurs in the vacuole under normal conditions. It is possible that only the major alkaloids of the leaves such as vindoline are accumulated in the vacuole by a specific energy-requiring system, while any vacuolar storage of the minor, biosynthetically active alkaloids such as tabersonine occurs by nonmediated diffusion across the tonoplast.

Although the low rates of uptake for vinblastine and morphine suggest that certain alkaloids have an intrinsically low permeability even when in the uncharged form, this was clearly not the case with the other alkaloids tested. From the results obtained with vindoline uptake, it appears that ion trapping of the alkaloid by low pH plays an important role in the retention of the alkaloid in the vacuole. The behavior of ajmalicine suggests that complexation of certain alkaloids with counterions or phenolics may also play a role in the retention of alkaloids in the vacuole. As indicated by the energy dependence for vindoline uptake, it is probable that there exist specific, energy dependent transport systems responsible for the transport and storage of the major alkaloids stored in the cell.

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