Further Studies of Sequestration of Alkaloids in *Papaver somniferum* L. Latex Vacuoles

Margaret F. Roberts, B. C. Homeyer, and Thi D. T. Pham

The Department of Pharmacognosy, The School of Pharmacy, London University, 29/39 Brunswick Square, London WC 1 N 1 AX

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The uptake and sequestration of alkaloids by latex vacuoles of *P. somniferum* sedimenting at 900 × *g* and 1100 × *g* were studied; both populations take up morphine effectively. Morphine uptake by the 1100 × *g* vacuoles was stimulated by exogenous ATP and, after uptake of large amounts of alkaloid, both populations were stimulated by exogenous ATP to take up further morphine; this effect was no longer present in 900 × *g* vacuoles isolated from the latex of more mature capsules. Uptake by 1100 × *g* vacuoles was more sensitive to temperature than that of 900 × *g* vacuoles. Determination of the proton gradient across the vacuolar membrane (ΔpH), controlled dissipation of ΔpH through the use of NH_4Cl, and correlation with morphine uptake demonstrated that morphine uptake was strongly dependant on the maintenance of ΔpH. Inhibitors designed to produce loss of protons from the vacuole had, however, little effect on the system. Nitrate, as an inhibitor of tonoplast ATPase, had an inhibitory effect on morphine uptake by the 1100 × *g* vacuoles.

Uptake of meconate, [^{35}S]sulphate and L-[U^{14}C]malate, important acid constituents of the 900 × *g* vacuoles was investigated. Uptake of sulphate and malate was continuous and saturation was not reached; the rate of uptake of sulphate and malate was much lower than that of morphine and other alkaloids, and uptake of meconate by these vacuoles was not detected.

Studies on specificity of alkaloid uptake with the 900 × *g* vacuoles indicated no absolute preference for either the (+)- or the (−)-isomer of codeine. Noscapine uptake was stimulated by ATP under conditions where ATP had no effect on morphine uptake. Nicotine was not taken up, but low levels of caffeine and 1-methoxycanthin-6-one were taken up, but less effectively than morphine or noscapine.

Uptake of alkaloids by *P. somniferum* latex vacuoles is dependant on the maintenance of tonoplast ΔpH and an ATPase generates this ΔpH. Sequestration appears to involve protonation and anion-cation stabilization involving meconate and sulphate. The specificity of alkaloid uptake suggested no clear correlation with pK or lipophilicity, and some sort of channel mechanism, more related to alkaloid shape is suggested.

Introduction

In higher plants the cell vacuole has been shown in many instances to be the storage compartment for alkaloids [1–6]. Previous work on alkaloid storage in *Papaver somniferum* has shown that alkaloids are exclusively stored in vacuoles contained within the latex [7, 8] and in this respect alkaloid sequestration in *Papaver somniferum* is similar to that found in *Chelidonium majus* [9, 10]. Like other plant vacuoles, *P. somniferum* latex vacuoles have an internal pH lower than that of their cytoplasmic environment, for the latex this is normally at pH 6.2–6.8 [3]. These vacuoles also accumulate low levels of dopamine [11] and have been shown to contain most of the meconic acid found in the latex [12].

Two distinct mechanisms for alkaloid transport into vacuoles have been proposed: 1. The lipophilic alkaloids can penetrate membranes by diffusion and are trapped in the acidic vacuolar compartment by protonation and salt formation [2, 4, 13–15]. This has been referred to as the ion trap mechanism. 2. Alkaloid uptake across the vacuolar tonoplast may occur *via* catalyzed transport and some highly specific tonoplast transport systems have been reported in a variety of plants [5, 6, 16]. It has been reported that alkaloid uptake into vacuoles isolated from *Fumaria* protoplasts could be stimulated by the addition of MgATP indicating that an ATPase activity was necessary for effi-

Abbreviations: HEPES, N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; DCCD, N,N′-dicyclohexyl carbodiimide; MgATP, magnesium + adenosyltriphosphate; ΔpH, transmembrane proton gradient.

Reprint requests to Dr. M. F. Roberts.

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cient transport. A similar mechanism is required for lupanine sequestration in vacuoles isolated from *Lupinus* [6] and for senecionine-N-oxide in *Senecio vulgaris* [17]. The vacuoles of *P. somniferum* latex may be considered as specialist vacuoles due to their size, resistance to external measures designed to reduce the ΔpH and capacity to sequester millimolar amounts of alkaloid. The rapid, specific uptake of the major opium alkaloids has no absolute requirement for MgATP, does not show saturation kinetics and is largely insensitive to temperature [3].

FCCP causes some reduction in alkaloid uptake suggesting that a proton gradient across the vacuole tonoplast is essential for continued alkaloid accumulation [3] which in turn implies the existence of a proton pump. The mechanism of sequestration of alkaloids in *P. somniferum* therefore did not clearly fit either hypothesis 1 or 2. In an attempt to resolve some of the conflicting results previously obtained, further investigations of the mechanism of uptake and sequestration of morphinan alkaloids by the vacuoles of *P. somniferum* latex were undertaken.

Experimental

Chemicals

Unless otherwise stated, chemicals were purchased from Sigma, U.K. or The British Drug Houses, U.K. [^{14}C]methylamine hydrochloride (1.48–2.22 GBq mM^{-1}), $H_2{}^{35}SO_4$ (44.4–51.8 TBq mM^{-1}) and ^{14}COOH-dextran-carboxyl (18.5–74.0 MBq g^{-1}) were purchased from New England Nuclear, U.S.A.; ^3H_2O (185 MBq ml^{-1}), L-[U^{14}C]-malic acid (1.5–2.2 GBq mM^{-1}), [$^{14}CH_3$]morphine (2.07 GBq mM^{-1}) was purchased from Amersham International, U.K.; and ATP was purchased from Boehringer, F.R.G.

Plant material

Papaver somniferum cv. Halle was cultivated at The School of Pharmacy Medicinal Plant Garden. Plants were grown in sequential batches so that flowering occurred from late June until end of October.

Isolation of latex vacuoles

Latex was collected into 700 mM mannitol to a final concentration of 50% latex. Vacuoles were sedimented by centrifuging the collected latex at 900 × *g* for 30 min and the supernatant of that centrifugation at 1100 × *g* for 30 min. The vacuoles were washed twice, and finally suspended to the original volume of buffer plus latex in a buffer of 700 mM mannitol, 100 mM HEPES adjusted to pH 6.8 with triethanolamine. This suspension was equivalent to 40 to 60 µl (900 × *g* vacuoles) and 15 to 20 µl (1100 × *g* vacuoles) intravacuolar volume for each 200 µl assay sample.

Determination of vacuolar volume

The intravacuolar volume of the 900 × *g* vacuole sample was determined by incubating a 200 µl aliquot of isolated and resuspended vacuoles with a 30 µl aliquot of tritiated water (1.11×10^3 Bq) and a 10 µl aliquot of a ^{14}COOH-dextran-carboxyl solution (3.7×10^2 Bq) for 30 min at room temperature (approximately 24.5 °C). In experiments testing the effect of substances on the transmembrane proton gradient the substances were added during this incubation. The sample was centrifuged in an Eppendorf tube at 900 × *g* for 15 min and a sample of the supernatant counted for ^{3}H and ^{14}C in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail. The pellet was suspended in 500 µl of 0.1 N HCl in methanol, digested for 30 min at room temperature, centrifuged at 2000 × *g* for 15 min and the entire supernatant counted for ^{3}H and ^{14}C. Counts were corrected for quenching, counting efficiency, and channel overlap. The concentration of tritiated water was assumed to be constant throughout the sample and the dextran restricted to the extravacuolar space. Dividing ^{3}H counts per µl of supernatant into the ^{3}H counts from the pellet provided the total sample void volume trapped in the pellet; dividing the ^{14}C counts per µl of supernatant into the ^{14}C counts from the pellet provided the trapped extravacuolar void volume; subtracting the trapped extravacuolar void volume from the total sample void volume left the intravacuolar volume [20]. This volume was very consistent for all similarly aged latex samples in a given season.

Determination of intravacuolar pH

The distribution of the membrane permeable base, methylamine, has been successfully used for

the determination of intracellular and intravacuolar pH values [19, 20]. The method is based on the assumption that uncharged compounds can easily pass across a membrane whereas charged forms are impermeant. Since the ratio of charged to uncharged compound is governed by the pH value, that value can be deduced. For most thoroughly studied situations, these assumptions have proved correct [21]. The possibility of the methylamine binding to the membrane must be considered carefully; in a vacuolar system, the possibility of differing effects of a transplasmalemma and a transtonoplast potential does not occur [22].

Intravacuolar pH was determined by incubating the vacuoles with 21 μM [^{14}C]methylamine (3.7×10^2 Bq) for 30 min and subsequent separation of supernatant and pellet and counting as described above in the determination of intravacuolar volume. The calculation was based on the equilibrium of uncharged methylamine across cell membranes and the impermeability of membranes to charged methylamine [23]. The pH gradient (pH_{in}-pH_{out}) was assumed to be equal to the $\log_{10}$ of the ratio of the proton concentration on either side of the membrane, which was equal to the $\log_{10}$ of the ratio of the probe concentrations.

Assay procedure for [$^{14}CH_3$]morphine, (+)-, (−)-[C^3H_3O]codeine, [^{35}S]sulphate and L-[$U^{14}C$]malate uptake by 900 × g and 1100 × g latex vacuoles

The method used was essentially that given in [3] except that 900 × *g* vacuoles were layered on a 20% sucrose gradient and 1100 × *g* vacuoles on a 16% sucrose gradient and centrifuged at 1000 × *g* and 1300 × *g* respectively to terminate the experiment. The resulting pellets were digested in 500 μl of .025 N HCl in methanol for 30 min centrifuged at 2000 × *g* for 15 min and 400 μl of the supernatant counted in a Packard Tri-Carb Liquid Scintillation Spectrometer using Aquasol LSC cocktail. Counts were corrected for quenching and counting efficiency. In experiments with inhibitors, the vacuole sample was preincubated with the inhibitor at 25 degrees for 20 min. In each individual experiment, duplicate samples were used and the results given are averages of at least two separate experiments.

Isolation of unlabelled alkaloids in uptake specificity experiments

1. Noscapine and nicotine

P. somniferum latex vacuole samples, 900 × *g* and 1100 × *g* were plasmolyzed in 0.025 N HCl in MeOH. After centrifugation the supernatant from each sample was evaporated to dryness and the residue taken up in a small volume of 2% sulfuric acid. The pH was adjusted to 9.5 with NH_4OH the sample placed on a column of Extrelut (Merck, Darmstadt) and the alkaloids isolated by elution with chloroform. The residue after evaporation was made up in a standard volume of MeOH for HPLC analysis.

2. Caffeine and 1-methoxycanthin-6-one

Plasmolysis of the vacuoles was carried out in a similar manner to that for the noscapine and nicotine experiments except that 0.1 N HCl was used. After centrifugation, the supernatant was extracted four times with $CHCl_3$. These extracts were combined and after evaporation the residue was made up in a standard volume of MeOH for HPLC analysis.

In all experiments with non-*Papaver* alkaloids and noscapine comparisons were made with control experiments since the vacuoles contained considerable amounts of the native alkaloids.

Quantitation of alkaloids

1. Noscapine

HPLC analysis: column, Hichrom S5W 5 μ Spherisorb (250 × 4.9 mm); mobile phase, MeOH (30 ml): $CHCl_3$ (10 ml): Et_2N (0.1 ml) of which 37.5 ml was mixed with *n*-hexane (290 ml).

2. Nicotine and 1-methoxycanthin-6-one

HPLC analysis: column, Hichrom S5W 5 μ Spherisorb (250 × 4.9 mm); mobile phase, *n*-hexane: ethyl acetate: NH_4OH (70:30:0.1).

3. Caffeine

HPLC analysis: column, Altex RP18, 5 μ, ultrasphere octyl (250 × 4.6 mm); mobile phase: 20% MeOH in H_2O.

In all cases the alkaloids were separated from the native alkaloids except for noscapine where %

uptake was determined by difference as compared with control samples.

Isolation of meconic acid

900 × *g* and 1100 × *g* vacuole samples were plasmolyzed in 0.025 N HCl in MeOH and centrifuged at 1000 × *g* for 30 min. The supernatant was placed on a cation exchange column (Dowex – 50 W H^+ form), which had been previously equilibrated with 1 N HCl and washed with distilled water. The acids were eluted with distilled water. After evaporation at reduced pressure the residue was made up in a standard volume. Meconic acid was determined by HPLC on a Hichrom RP18 10 µ (250 × 4.9 mm) column with MeOH:H_2O (17:83) containing 0.05% atropine sulphate. The column was calibrated using a standard sample of meconic acid.

Results

Separation of *P. somniferum* latex vacuoles using discontinuous sucrose gradients suggested that these vacuoles could be separated into two populations. Subsequently it was found that these two populations of vacuoles could be isolated by centrifugation at 900 × *g* and 1100 × *g* as detailed in the Experimental. This simple method of isolation yielded a higher percentage of intact vacuoles as measured using α-mannosidase as a vacuolar marker [8]. The buffer used helped to prevent aggregation of the vacuoles during their isolation and use in the experiments. This method was therefore used to produce the 900 × *g* and 1100 × *g* vacuoles used in the foregoing experiments.

Comparison of morphine uptake by the 900 × g and 1100 × g vacuoles

Both the 900 × *g* and the 1100 × *g* latex vacuoles contained the major alkaloids found in *P. somniferum:* morphine, codeine, thebaine, papaverine and noscapine. Vacuoles fed [$^{14}CH_3$]morphine showed a capacity for the sequestration of high concentrations (up to 415 mM) of alkaloid.

In the experiment shown in Fig. 1, suspending 900 × *g* and 1100 × *g* vacuoles in a bathing medium 4.5 mM with radiolabelled morphine resulted in uptake by the 900 × *g* vacuoles of over 90% of morphine offered in 2 min. It should be noted that the presence of MgATP had no effect on the rate or amount of uptake by these vacuoles; MgATP did, however, stimulate the 1100 × *g* uptake from 12% to about 17% of the morphine offered. These experiments suggested that with the 900 × *g* vacuoles, the proton gradient (ΔpH) across the membrane is sufficient to allow uptake and sequestration of all the morphine offered; however, with the 1100 × *g* vacuoles, the ΔpH requires regeneration which, from the stimulation observed with

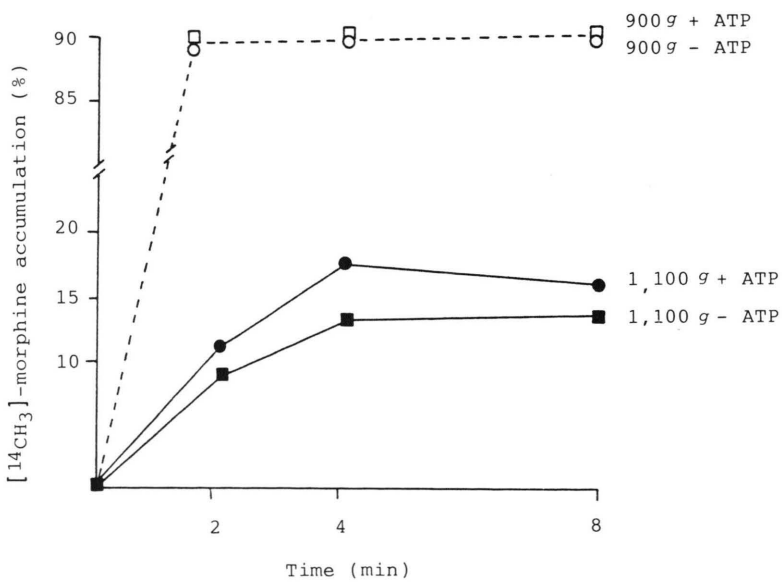

Fig. 1. The effect of MgATP on the uptake of [$^{14}CH_3$]morphine by the 900 × *g* and the 1100 × *g* vacuoles of *P. somniferum* latex. [$^{14}CH_3$]morphine (final concentration 4.5 µM, 1.0 Bq) was added to latex vacuole suspensions. Vacuolar volume: 900 × *g* = 60 µl, 1100 × *g* = 20 µl in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate, pH 6.8. MgATP: 10 mM.

MgATP, must occur through a tonoplast ATPase powered proton pump.

In the time course study shown in Fig. 2, high concentrations of morphine were fed to 900 × *g* vacuoles (2.0 mg to 60 µl vacuolar volume, ~33 mM) and 1100 × *g* vacuoles (0.2 mg to 23 µl vacuolar volume, ~3.3 mM). The uptake of morphine was rapid and complete after 8 min with the vacuole populations taking up approximately 12% (900 × *g*) and 5% (1100 × *g*) of the introduced alkaloid. When the addition of MgATP was made at 8 min, there was a distinct stimulation of morphine uptake suggesting that the high levels of morphine taken up by both vacuole populations dissipated the ΔpH across the membrane which was partially restored as a result of tonoplast ATPase activity when MgATP was added after apparent saturation.

Despite differences in the amount of alkaloid fed to the two vacuole populations and the differences in vacuolar volume used, the 900 × *g* vacuoles proved to have superior capacity to sequester alkaloids (Fig. 2).

Efflux of alkaloids from latex vacuoles

Previous experiments [3] have shown that [$^{14}CH_3$]morphine is retained in the vacuoles after uptake even if the pH of the buffer suspending the vacuoles is lowered to 5.5–5.6. Vacuole populations, 900 × *g* and 1100 × *g*, fed ^{14}C-labelled morphine prior to isolation were washed twice to remove contaminating [$^{14}CH_3$]morphine. These vacuoles were suspended in buffer and fed with further large amounts of unlabelled morphine (2.0 mg to a 200 µl sample of the 900 × *g* vacuoles with a 60 µl vacuolar volume and 0.2 mg to a 200 µl sample of the 1100 × *g* vacuoles with a 23 µl vacuolar volume). Vacuole samples were incubated at room temperature and harvested after 30, 60, and 120 min. There was no significant movement of ^{14}C-labelled morphine out of the vacuoles into the buffer. The alkaloid appeared to be tightly retained within the vacuole and was only released on plasmolysis of the vacuoles. In this respect there was apparently no difference between the 900 × *g* and the 1100 × *g* vacuoles.

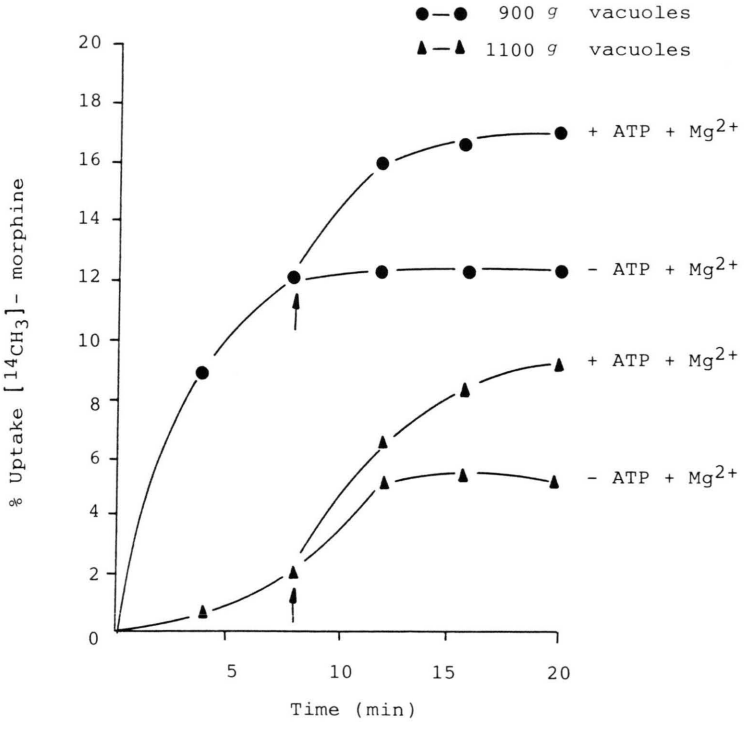

Fig. 2. The accumulation of high concentrations of [$^{14}CH_3$]morphine in 900 × *g* and 1100 × *g* *P. somniferum* latex vacuoles in the presence and absence of MgATP. High concentrations of [$^{14}CH_3$]morphine (10 mg ml^{-1} for 900 × *g* vacuoles, 1.0 mg ml^{-1} for 1100 × *g* vacuoles) were used to preload isolated latex vacuoles. Vacuolar volume: 900 × *g* = 60 µl, 1100 × *g* = 23 µl in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate, pH 6.8. MgATP (10 mM) was added to some samples 8 min after the start of incubation with morphine.

Studies using ATPase inhibitors and ionophores

A number of inhibitors designed to affect the proton gradient across the vacuole membrane or inhibit tonoplast or plasmalemma ATPases were used. At the concentrations given in Table I, most of the ionophores and ATPase inhibitors used had little effect on reducing the uptake of [$^{14}CH_3$]-morphine by either the 900 × *g* or the 1100 × *g* vacuoles, suggesting a stable environment in which the proton gradient was not readily perturbed by the conditions used. However, the 1100 × *g* vacuoles showed reduced uptake (83% of control) with nitrate, an inhibitor of tonoplast ATPase, and in the presence of MgATP, a slight stimulation with FCCP and DCCD. In the absence of MgATP these latter two reagents resulted in a slight inhibition of uptake.

The effects of temperature on the 900 × g and 1100 × g vacuoles

Although previous experiments [3] carried out on vacuoles which sedimented at 1000 × *g*, and were therefore rich in the 900 × *g* vacuoles of the present experiments, had suggested that morphine uptake was largely independent of temperature, close inspection suggested that differences in uptake of [$^{14}CH_3$]morphine were observable if uptake into the vacuoles was measured after 4 min and these results are given in Fig. 3. It was observed that temperature more radically affected the uptake of morphine into the 1100 × *g* vacuoles, where an increase in temperature from 0 °C to 30 °C resulted in an 8-fold increase; uptake of morphine by 900 × *g* vacuoles over the same temperature range increased by only 2-fold. The calculated activation energy (E_a) of morphine uptake for the 900 × *g* vacuoles is 22.18 kJ · mol^{-1}; that for the 1100 × *g* vacuoles is 58.08 kJ · mol^{-1}. The value for the 900 × *g* vacuoles lies at the upper limit of the accepted values for passive diffusion, while that for the 1100 × *g* vacuoles is well into the range associated with facilitated uptake [6].

The effect of changes in the membrane proton gradient (ΔpH) on alkaloid uptake by latex 900 × g vacuoles

The measurement of ΔpH

A time course of uptake of methylamine by *P. somniferum* latex vacuoles indicated a very rapid penetration by the probe into the intravacuolar space followed by a constant ratio of methylamine inside/outside the vacuoles for at least 30 min, Fig. 4. The successful use of the reagent depends on the lack of binding to macromolecules within the intravacuolar space or to the membrane itself. Osmotic rupture of the vacuoles accomplished liberation of over 95% of the methylamine in every instance. Most investigations were carried out at the natural pH of the latex, pH 6.8, and the ratio of the concentration of intravacuolar methylamine to that outside remained constant over a range of 100 to 750 μM.

Table I. The effect of inhibitors on [$^{14}CH_3$]morphine accumulation by the 900 × *g* and 1100 × *g* vacuoles of *P. somniferum* latex in the presence of 5 mM MgATP. Buffer: 100 mM HEPES, 700 mM mannitol, pH 6.2 – 200 μl. Vacuoles: 900 × *g*, 60 μl vacuolar volume; 1100 × *g*, 20 μl vacuolar volume. [$^{14}CH_3$]morphine: 4.5 μmolar (1.0 Bq per assay) final concentration. Uptake was measured after 4 min after preincubation with inhibitors for 20 min.

Inhibitor	Conc. [M × 10^{-4}]	[$^{14}CH_3$]morphine uptake % Control		
		900 × *g* ±MgATP	1100 × *g* +MgATP	1100 × *g* −MgATP
Control		100	100	100
FCCP	5	97	122	86
DCCD	5	97	102	79
KNO_3	50	96	83	99
Orthovanadate	50	97	99	99

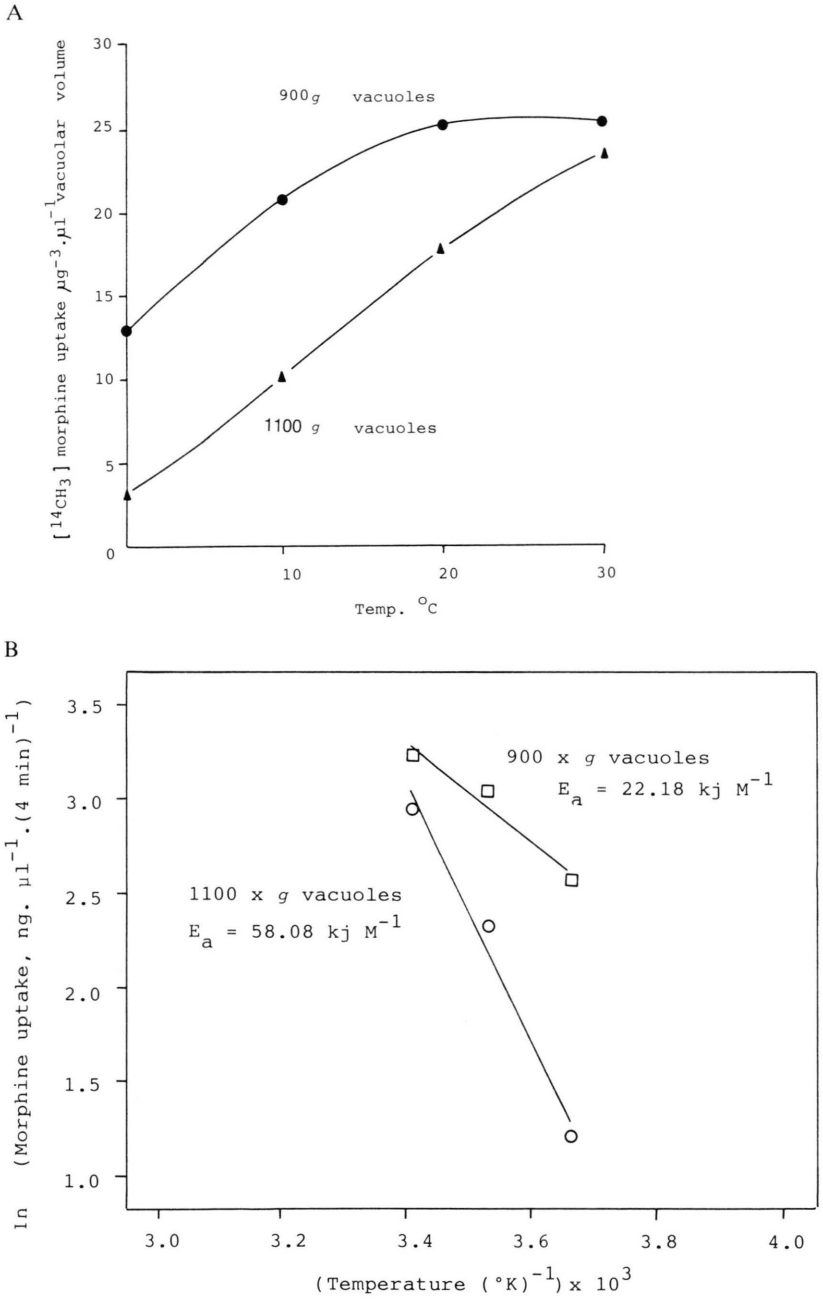

Fig. 3. The effect of temperature on alkaloid uptake by 900 × *g* and 1100 × *g* vacuoles from *P. somniferum* latex. [$^{14}CH_3$]morphine, final concentration 85 µM, 3 Bq, was incubated with latex vacuole suspensions for 4 min. Vacuolar volume: 900 × *g* = 60 µl, 1100 × *g* = 20 µl in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate buffer, pH 6.8. Fig. A: orthographic plot; Fig. B: Arrhenius plot.

Using similar techniques it was possible to calculate that at the natural pH of the latex which seasonally varies between 6.2 and 6.8 the internal pH of the vacuoles was always approximately one pH unit lower than the external pH.

The effect of changes in ΔpH on morphine uptake

The effect of varying ΔpH was investigated using ammonium chloride as described in [24]. Ammonia, as ammonium chloride (1–50 mM), added to the highly buffered 900 × *g* vacuole sus-

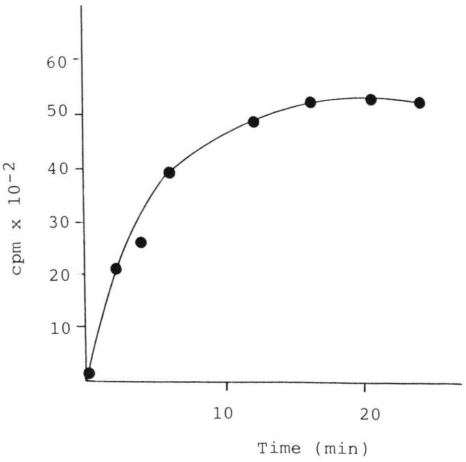

Fig. 4. Time course for [^{14}C]methylamine uptake by *P. somniferum* latex 900 × *g* vacuoles. The 900 × *g* vacuoles (vacuolar volume 43 µl) in 200 µl 100 mM HEPES, 700 mM mannitol, 100 µM molybdate buffer, pH 6.8 with 21 µM [^{14}C]methylamine (7.4×10^2 Bq). The ratio of [^{14}C]methylamine inside/outside the vacuole was linear within a range of 0–200 µM.

pensions, showed a dose dependant decrease in the ΔpH. Addition of morphine at the concentrations used in this series of experiments (4.5 µM) had no significant effect on ΔpH measurements. A reduction in the uptake of [$^{14}CH_3$]morphine paralleled in the reduction in ΔpH (Fig. 5).

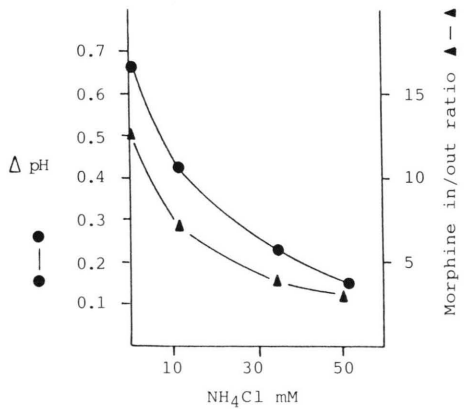

Fig. 5. The effect on morphine uptake by 900 × *g* vacuoles of perturbation of ΔpH using ammonium ions. Vacuolar volume: 43 µl in 200 µl 200 mM HEPES, 500 mM mannitol, pH 6.8. Probes: For ΔpH, 21 µM [^{14}C]methylamine (7.4×10^2 Bq); for [$^{14}CH_3$]morphine uptake, a concentration of 270 µM, 3.7×10^2 Bq was used.

Specificity of alkaloid uptake

The latex vacuoles of *P. somniferum* sequester alkaloids of three basic structural types: the morphinanes: morphine (pK_1 7.8, pK_2 9.85), codeine (pK 7.95), and thebaine (pK 7.95); phthalideisoquinolines: noscapine (pK 6.18); and benzylisoquinolines: papaverine (pK 5.90).

Previous investigation indicated that latex vacuoles show a remarkable degree of specificity for these alkaloids [3]. To extend this study a further group of alkaloids was investigated. In Table II a comparison of the uptake of [$^{14}CH_3$]morphine with (+)-[C^3H_3O]codeine and (−)-[C^3H_3O]codeine at 25 °C by 900 × *g* vacuoles confirms the efficiency of uptake of morphine and indicates that little distinction is made between the natural, (−), and the unnatural, (+), isomers of codeine except that uptake of the (−)-isomer in all experiments was marginally better than the (+)-isomer.

Surprisingly, while uptake of noscapine was marginally less efficient than uptake of morphine, noscapine uptake in the presence of MgATP was enhanced. This enhancement with MgATP is not observed with morphine in the 900 × *g* vacuoles under similar conditions. Nicotine at pH 6.8 was not taken up by the 900 × *g* vacuoles, whereas caffeine and 1-methoxycanthin-6-one were taken up to a small extent (18% and 4% respectively). Uptake of these alkaloids was less efficient than that of [^{14}C]methylamine (24%) or morphine (98%).

Table II. Specificity of alkaloid uptake by the 900 × *g* latex vacuoles. Buffer: 100 mM HEPES, 700 mM mannitol, pH 6.8 (200 µl). Vacuoles: 900 × *g*, 63 µl vacuolar volume. Incubation was for 20 min at 25 °C followed by vacuole isolation and processing as given in the Experimental.

Alkaloid	µmol per 200 µl latex Input	Uptake	[%] Uptake
[$^{14}CH_3$]morphine	0.25	0.245	98
	32.2	29.0	90
(−)-[C^3H_3O]codeine	0.5	0.49	98
(+)-[C^3H_3O]codeine	0.5	0.45	90
Noscapine (−MgATP)	10.2	6.3	62
(+MgATP)	10.2	8.3	82
Nicotine	200	0	0
Caffeine	7.7	1.4	18
1-Methoxycanthin-6-one	10.0	0.4	4
[^{14}C]methylamine	0.2	0.048	24

The uptake of meconic acid, sulphate and malate by 900 × g vacuoles

In experiments in which unlabelled meconic acid was introduced to 900 × *g* vacuoles, no detection of uptake by the vacuoles was observed. However with the high levels of meconate already within the vacuole it was thought that the HPLC method used did not have the sensitivity to detect small changes in meconic acid against this high background and therefore radiolabelled meconic acid would be required to determine any uptake into the vacuoles. Preliminary investigations shown in Fig. 6 have demonstrated that both radiolabelled sulphate and malate are taken up by the 900 × *g* vacuoles. The uptake of malate in the presence of MgATP was stimulated, however, similar use of MgATP during sulphate uptake gave equivocal results. The rate and level of uptake of both acids is low compared with the uptake of morphine. This is probably related to ionization in the bathing medium which is such (pH 6.2–6.8) that, for example, only about 2% of the malate would not be ionized and consequently available for uptake in the molecular form.

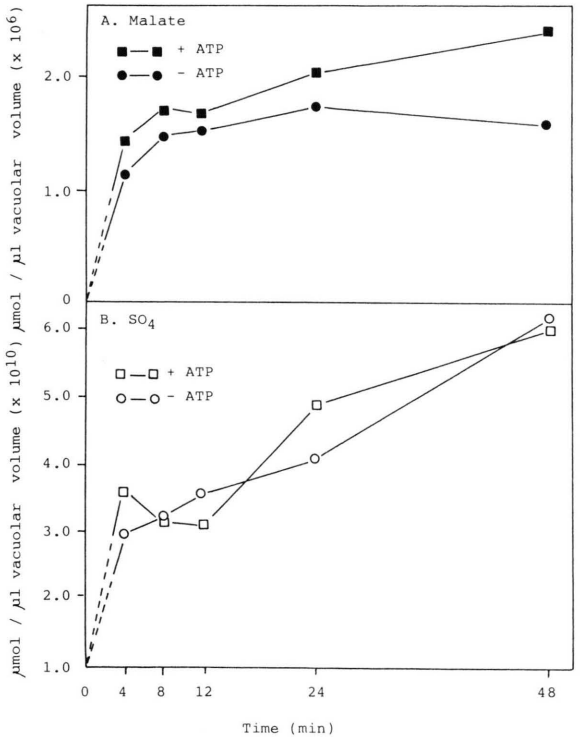

Fig. 6. The uptake of L-[U^{14}C]malate and [^{35}S]sulphate by the 900 × *g* latex vacuoles of *P. somniferum*. A. L-[U^{14}C]-malic acid at a final concentration of 16.4 μM, 3.6 Bq was added to a vacuolar suspension sample, vacuolar volume (900 × *g* vacuoles) 29 μl, in 200 μl 100 mM HEPES, 700 mM mannitol, 100 μM molybdate, pH 6.8. MgATP additions were made for a final concentration of 10 mM. B. [^{35}S]H_2SO_4 at a final concentration of 16 μM (10.0 Bq) was added to an identical vacuolar sample, as in 6 A.

Discussion

In the foregoing experiments and those detailed in [3], and in [18], an attempt has been made to determine whether the alkaloids penetrate the latex vacuole tonoplast by diffusion with entrapment in the acidic vacuolar compartment by protonation and formation of non-diffusable salts [2, 3, 13–15] or whether alkaloid uptake across the vacuolar membrane results from catalyzed transport [3, 5, 6, 16]. In considering these possibilities, it is important to distinguish between two processes, the passage of molecules through the tonoplast (diffusion or by transport protein) and the driving force for alkaloid accumulation in the vacuole against a concentration gradient (membrane energization or ion trap mechanism).

Time course studies of the uptake of [$^{14}CH_3$]-morphine by latex 900 × *g* and 1100 × *g* vacuoles (Fig. 1) suggest that both groups of vacuoles take up morphine rapidly *via* a diffusion process with equilibrium attained in 2–4 min.

Previous results with a vacuole population sedimented at 1000 × *g* [3] have shown that saturation kinetics were not attainable and that there was no absolute requirement for MgATP. Furthermore, uptake of morphine was not particularly sensitive to temperature.

All these conditions would support a theory postulating simple diffusion, however, in the present experiments, while the 900 × *g* vacuoles showed absolutely no stimulation of uptake in the presence of MgATP, the 1100 × *g* vacuoles showed very slight stimulation and these 1100 × *g* vacuoles were also much more sensitive to temperature (Fig. 3). The 1100 × *g* vacuoles exhibited an activation energy (E_a) for morphine uptake of 58.08 kJ · M^{-1} (Fig. 3 B), while the 900 × *g* vacuoles had an E_a = 22.18 kJ · M^{-1}. The 900 × *g* vacuoles are at the upper limit of activation energies associated with diffusion and the 1100 × *g* vacuoles are well

above the minimum activation energy level associated with facilitated uptake as discussed by Mende and Wink [6]. Closer inspection of the two vacuole populations [8, 18] showed that the 900 × *g* vacuoles contained most of the meconic acid, sulphate, malate and phenolic acids of the latex with only a very small amount present in the 1100 × *g* vacuoles. Investigation of these acids in the vacuoles during capsule maturation [18] revealed a strong correlation between acid and alkaloid content throughout maturation of the capsule for the 900 × *g* vacuoles which was not observable for the 1100 × *g* vacuoles, although these vacuoles still contained significant amounts of alkaloid [18]. In the 900 × *g* vacuoles therefore the acid content of the vacuole plays a major role in the maintenance of the acidity of the vacuole, a factor which is less apparent for the 1100 × *g* vacuoles.

In considering the theory of simple diffusion, the hydrogen ion concentration of the external medium would be expected to influence uptake. Measurements of morphine uptake were low at a pH similar to that of the vacuole internal pH (5.2–5.8) rising to a maximum at pH 8.0 with a 20% fall off at more alkaline pH [3]. The present investigation of vacuole internal pH (pH_{in}) using [^{14}C]methylamine as probe showed that a difference of $\simeq$ one pH unit was maintained when the external pH (pH_{out}) was that of the latex (*i.e.,* between 6.2 and 6.8).

The transtonoplast pH gradient (ΔpH) has been shown to be important in the regulation of alkaloid uptake into the vacuole, since reductions in ΔpH by NH_4Cl (Fig. 5) brought about a reduction in morphine uptake by the 900 × *g* vacuoles. The data in Fig. 2 shows that the uptake of large amounts of morphine by both the 900 × *g* and the 1100 × *g* vacuoles resulted in a reduced rate of uptake, this reduced rate could be improved by the addition of MgATP. This phenomenon was observable with both vacuole populations. The direct correlation between ΔpH and morphine uptake and the effect of MgATP on a saturated system seems to demonstrate that the function of an ATPase is to power a proton pump which restores the ΔpH. The stimulation of alkaloid uptake by MgATP in the 900 × *g* vacuoles was only observable with latex collected from young capsules (day 2–3 after petal opening) collected early in the season; presumably, the older vacuoles contained enough anions (sulphate, meconate and malate) [18] to stabilize a sufficient quantity of protons to maintain a ΔpH adequate for uptake and sequestration. Inhibitors (Table I) designed either to dissipate ΔpH or inhibit tonoplast ATPase were relatively unsuccessful in perturbing the system, presumably because of the high levels of anions and native alkaloids already present which stabilize the protons present within the vacuoles. Previous work [18] showed that during capsule maturation the vacuolar concentration of these anions is increasing at a rate which allows for the maintenance of a tonoplast ΔpH in addition to establishing a correlation between dibasic acids and alkaloids which would support protonation and complex salt formation (ion trap) as a method of permanent sequestration. Results in [18] suggested that the 1100 × *g* vacuoles are probably a younger form of the 900 × *g* vacuoles and since in these vacuoles a correlation between acid and alkaloid was not apparent, it was assumed that at this stage in vacuole development the tonoplast ATPase plays a more important role in the maintenance of ΔpH than it does in the 900 × *g* vacuoles. The distinctly different activation energies of the two vacuolar populations, discussed above, lends support to this hypothesis. The alkaloids, however, are as tightly held within the 1100 × *g* vacuoles as they are in the 900 × *g* and no alkaloid efflux was observed under the conditions used for these experiments. This result was not expected because of the apparent lack of correlation between alkaloid content and acid content, however, it is possible that other anions, *i.e.,* chloride, are important counterbalance ions [18]. In *Chelidonium majus* latex, vacuolar levels of chelidonic acid and phenolic compounds far exceed the levels of alkaloids and these, too, appear to be important components of an ion trap mechanism [25]. In *P. somniferum* latex vacuoles, similar significant amounts of phenolic material, other than morphine, are also found [8, 18] and must also play a role in the ion trap mechanism along with the anions L-malate, meconate and sulphate.

Important to an ion exchange mechanism of accumulation is the uptake of these materials into the vacuole or their synthesis as is suggested for the phenolic material present in *Chelidonium majus* [25]. Matile [25] failed to show uptake of chelidonic acid into the vacuoles of *C. majus* latex and similar problems were found in our experiments with

meconic acid. However, experiments with [^{35}S]sulphate and L-[U^{14}C]malate showed that uptake into the *P. somniferum* vacuoles was relatively slow and only marginally stimulated by the addition of MgATP (Fig. 5). The levels of sulphate taken up in 30–60 min would not readily be detected without the use of labelled material and the same could well be true for meconic acid, since the vacuoles contain large concentrations against which small changes due to uptake were being measured. The biosynthetic pathway and the site of biosynthesis of this unusual acid (pK_2 2.3) remains to be elucidated and so the question of its origins in the vacuoles remains open.

One problem remains which is not totally resolved by the present experiments. Previous results [3] suggested that the opium alkaloids were specifically taken up by the latex vacuoles. If movement across the vacuolar membrane is the result of simple diffusion as has been assumed for methylamine (pK 10.6) then low specificity and selectivity for the passage of alkaloids across the vacuolar membrane might be expected. While morphine (pK_1 6.13, pK_2 9.85), codeine (pK 6.05), thebaine (pK 6.05), papaverine (pK 8.07) and noscapine (pK 6.18) are readily taken up by the latex vacuoles, other alkaloids such as reserpine (pK 6.6), atropine (pK 4.35), quinine (pK_1 5.07, pK_2 9.7), cytisine (pK_1 6.11, pK_2 13.08) [3] and nicotine (pK_1 6.16, pK_2 10.96) do not pass through the latex vacuolar membrane. Present experiments, Table II, reveal however that caffeine (pK 14.0) and 1-methoxycanthin-6-one are taken up to a small extent. It has been suggested that "ion trapping" may be limited to alkaloids with relatively low pK values with the more basic alkaloids, *e.g.*, lupinine (pK 9.1), requiring active transport across the membrane [6]. Other groups [26–28] have suggested that the accumulation of bases is dependant on pK; the more alkaline the pK the higher the accumulation ratio. The present results and those given in [3], however, indicate that there is no clear correlation between pK and accumulation values, nor does there appear to be a correlation with lipophilicity since morphine, one of the least lipophilic of the group is taken up by the latex vacuoles with maximum efficiency. The results seem to suggest that molecular shape is important, although no distinction is made between (+)-codeine and (−)-codeine.

A recent paper by Hauser and Wink [29] reporting on the latex of *Chelidonium majus,* reports low selectivity for the latex vacuoles and a decrease in uptake of alkaloid with exogenous ATP. We, on the other hand, have found specificity of uptake with *P. somniferum* latex vacuoles (Table I), and have demonstrated that ATP does have an effect on morphine uptake by the $1100 \times g$ vacuoles, although it does not increase uptake in mature, $900 \times g$, vacuoles unless they have been exposed to an exceedingly high level of morphine (Fig. 2).

None of the evidence presented based on our present experiments or in [3] would support a requirement for a carrier protein to effect transport across the membrane and the high degree of specificity observed for the uptake of a limited range of alkaloid as opposed to others of similar pK and lipophilicity would not support the simple diffusion theory. The idea of specific channels involving the protein and/or the lipid component of the membrane has to be a consideration.

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