Alkaloid Sequestration by *Papaver somniferum* Latex

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Z. Naturforsch. **39 c**, 876–881 (1984); received May 21/June 18, 1984

Papaver somniferum L., Alkaloid, Sequestration, Alkaloid Uptake, Organelle, Latex

The $1000 \times g$ organelles of *Papaver somniferum* latex have been shown to accumulate rapidly, large quantities of alkaloids (0.05–1 mg/mg organelle protein), particularly morphine, thebaine, codeine and papaverine and have been found to exhibit a surprising degree of specificity for the accumulation of these alkaloids. The uptake of alkaloid is independent of temperature and does not show a requirement for ATP 10^{-3} M, Mg^{2+} 10^{-3} M, or KCl 10^{-3} M. It is unaffected by known ATPase inhibitors (chlormadinone acetate 2.5×10^{-5} M, N,N-dicyclohexylcarbodiimide 5×10^{-4} M and sodium vanadate 10^{-4} M) but showed considerable increase in morphine accumulation in the presence of 4-chloromercumbenzoate 5×10^{-4} M, while N-methylmaleimide 5×10^{-4} M had no effect, and carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone 5×10^{-4} M caused a reduction (ca. 30%) of morphine uptake. An acid medium, pH 4.5–5.5, also results in decreased morphine uptake.

Introduction

Previous reports [1–6] have shown that *Papaver somniferum*, L. latex contains many of the enzymes associated with normal cellular metabolism. Although the isolated latex has been shown to carry out limited biosynthesis of papaver alkaloids [7], a survey of previous work gives no clear evidence that the latex is the primary site of alkaloid biosynthesis; however, latex and in particular the latex organelles which sediment at $1000 \times g$, has been shown to be the major site of alkaloid accumulation in the plant [7, 8]. Previous work suggests that the $1000 \times g$ sediment consists of nonhomogeneous organelles which differ in morphology, density, and enzyme as well as metabolite content [8]. The most dense population contains the bulk of the alkaloids as well as most of the catecholase and dopamine found within the latex. Monitoring plants at the approach of flowering and through to capsule formation showed rapid accumulation of alkaloids and dopamine in this organelle population [8].

Previous work on alkaloid storage in latex has investigated the uptake of sanguinarine by the latex vacuoles of *Chelidonium majus*. In these experiments, Matile and others found that sanguinarine is rapidly accumulated by a process which does not require energy [9]. A similar situation appears to exist for the accumulation of indole alkaloids into the cell vacuoles of *Catharanthus roseus* cell cultures [10]. These authors suggest the uptake of alkaloid by vacuoles may be considered in terms of the ion-trap mechanism first put forward by de Duve *et al.* [11] for the accumulation of weakly basic substances in lysosomes.

The following experiments were initiated to investigate in detail the mechanisms of alkaloid accumulation and its specificity in $1000 \times g$ organelles isolated from *P. somniferum* latex.

Abbreviations: CMA, chlormadinone acetate; CMB, 4-chloromercuribenzoate; DCCD, NN′dicyclohexylcarbodiimide; FCCP, Carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; N-MM, N-methylmaleimide.

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0341-0382/84/0900-0876 $ 01.30/0

Experimental

Plant material and isolation of 1000 × g organelles from latex

Papaver somniferum L. cult. var. Halle was grown in sequential batches in the departmental garden so that flowering occurred from June to September. Latex was collected and the $1000 \times g$ organelles isolated as previously described [8]. In experiments where additions of ATP were made, the $1000 \times g$ organelles were washed with and resuspended in buffer A at pH 7.0 twice to ensure removal of acid phosphatase and endogenous ATP [4]. Where $1000 \times g$ organelles were separated using sucrose gradients the techniques given in [8] were used.

Chemicals

[$^{14}CH_3$]morphine (56 mCi/mmol) was obtained from Amersham International, U.K., [^{3}H]codeine, [^{3}H]thebaine, [^{3}H]papaverine, and [U^{14}C]-γ-coniceine were obtained from previous biosynthetic studies with *P. somniferum* [12] and *Conium maculatum* [13]. Chemicals were of high quality, obtained from various suppliers.

Buffers

A. 200 mM phosphate 500 mM mannitol, pH stated in text.
B. 20 mM phosphate O. 1% Triton X-100 pH 6.2.
C. 200 mM Tris 500 mM mannitol, pH stated in text.

Assay procedure for morphine uptake by 1000 × g organelles

In most experiments, 10 µl of [$^{14}CH_3$]morphine (1.6×10^6 dpm/ml = 0.013 µmol) was used with various additions of unlabelled morphine according to the experiment. For large additions the required morphine was dissolved in 50–100 µl of buffer A at pH 7.0. For these larger additions, alkaloids were used as their salts to ensure complete dissolution. The alkaloid solution was added to a 300 µl sample of 1000 × *g* organelles (equivalent to 150 µg protein) and incubated for 4 min at 25 °C. The sample was then layered onto 20% sucrose in buffer A at pH 7.0 and centrifuged at 2000 × *g* for 15 min to sediment the organelles. The resulting pellet was resuspended in 500 µl of buffer B for 30 min at 25 °C to plasmolyse the organelles. After centrifugation at 2000 × *g* for 15 min to remove insoluble material, 100 µl of the resulting supernatent was counted, using 10 ml Aquasol scintillation cocktail and a Packard Model 3255 Tri-Carb Liquid Scintillation Spectrometer. This method reduced contamination by material not taken up by the organelles, to less than 10% in zero time control samples and proved to be an efficient way to handle the short incubation times necessary. Where additions of other substances were made, the final volume of these plus the morphine addition was never greater than 100 µl. In experiments with inhibitors, the inhibitor was preincubated at 25 °C for 30 min with the organelle samples prior to addition of the labelled alkaloids.

In each individual experiment, duplicate samples were used and the results given are averages of at least three experiments.

Assay of alkaloids by HPLC, GLC and spectroscopy

In experiments to determine the specificity of alkaloid uptake by the 1000 × *g* organelles, uptake of nonradioactive alkaloids was monitored by HPLC [14]. Samples were brought to pH 9.5, placed on Extrelut (Merck) columns [15] and the alkaloids extracted with chloroform : isopropanol (85 : 15). After removal of the solvent the residue was dissolved in methanol (0.5 ml) and the alkaloids estimated using HPLC as described in [8, 14]. This method was suitable for separation and quantification of morphine, codeine, thebaine, reserpine, quinine and sanguinarine. The detection level was of the order of 5–10 µg. γ-coniceine was estimated by the sodium nitroprusside method [16] and also by using [U^{14}C]-γ-coniceine. Atropine and cytisine content was estimated by GLC using a 6′ column of 10% SE 30 on chromosorb WAW, 230 °C and a nitrogen flow rate of 40 ml/min; the detection level was 0.05–0.1 µg. Where radioactive alkaloids were used, the method used was as above for the assay of [$^{14}CH_3$]morphine uptake.

Determination of dopamine

The methods employed in reference [8] were used for dopamine determinations.

Light microscopy

A Leitz Orthoplan light microscope was used with oil immersion, phase contrast, magnification × 1000. The use of neutral red to determine organelle pH and of Munier's Reagent to locate alkaloids was as in [10].

Protein determinations

The method of Bradford [17], using the binding of Coomassie brilliant blue G-250 to protein was used. Samples of the 1000 × *g* organelles were pretreated for 30 min at 25 °C with an equal volume of 0.1% Triton X-100 to ensure solubilization of membrane protein.

Results

Variation in organelle size

Investigations using a light microscope with a phase contrast objective have shown the organelles

containing alkaloids to be extremely variable in size, ranging from approximately 100 to 770 nm in diameter. All the organelles within this range contained alkaloids as determined by using Munier's Reagent [10]. With this reagent an orange precipitate within the organelles could clearly be seen with the light microscope (magnification 1000 ×). It was noted that the large organelles appeared to contain more alkaloid per unit size than the small organelles. The large organelles were more fragile and were more easily plasmolysed using this technique. The larger organelles within this range are those found to sediment at 55% sucrose on continuous 30% to 65% sucrose gradients [8]. The smaller organelles were to be found trailing in the 40–50% sucrose part of the gradient. Using a discontinuous sucrose gradient it was possible to trap these lighter organelles at the 40–50% interface (Band A). The major group of organelles was, however, found at the 50–60% sucrose interphase (Band B). For this type of experiment it was essential to use latex harvested from the bud or from the flower on the day the petals opened. With latex harvested 7–21 days after petal opening the large heavy organelles were increased in size, easily aggregated and sedimented to the bottom of the gradient. The levels of alkaloid and dopamine in the two bands of organelles (A and B) were of interest. From the results in Table I, it can be seen that the lighter, smaller organelles (Band A) contained alkaloids but did not contain dopamine; whereas the denser organelles (Band B) contained both alkaloids and dopamine. Techniques to date have failed to separate the latter organelles into alkaloid and dopamine containing populations.

Table I. Levels of morphinan alkaloids in bands A and B of organelles obtained with discontinuous gradients.

Alkaloid	µg/mg Protein	
	Band A	Band B
Narcotine	49.9	52.6
Papaverine	51.7	100.0
Codeine	96.5	118.3
Thebaine	89.6	316.9
Morphine	600.0	1326.8
dopamine	nil	178.6

The uptake of morphine by the 1000 × g organelles

The effect of time and temperature

Initial experiments showed that [$^{14}CH_3$]morphine was rapidly taken up by the 1000 × *g* organelles. Using a 10 µl addition of [$^{14}CH_3$]morphine (16070 dpm equivalent to 0.00013 µmole) to 300 µl of 1000 × *g* organelles, the uptake of morphine had been completed within 6 min and the rate of uptake was essentially the same whether the experiments were carried out at 0, 25, or 30 °C. Uptake of morphine, therefore, appeared to be independent of temperature.

In a further series of experiments, unlabelled morphine additions of 39 µg and 320 µg with 10 µl of [$^{14}CH_3$]morphine-HCl (1.6×10^6 dpm/ml ≅ 0.013 µmol) were made and estimates of morphine uptake made at intervals over a period of 4 min. In each case more than 73% of the added morphine was taken up by the 300 µl sample of 1000 × *g* organelles after 4 min.

In a third series of experiments, five additions of 530 µg of morphine each were made over a period of time with each addition made 4 min after the previous one. A total of 2650 µg of morphine was added and of this 1279 µg (48%) was taken up in 20 min.

The effect of pH

In these experiments 500 µl samples of the 1000 × *g* organelles were used with 10 µl of a morphine [$^{14}CH_3$]morphine HCl, 2.0×10^6 dpm = 0.016 µmol + 2.5 mg cold morphine-HCl all in 1 ml) solution. The incubation time was 4 min. The results given in Fig. 2 show that pH has a marked effect on the

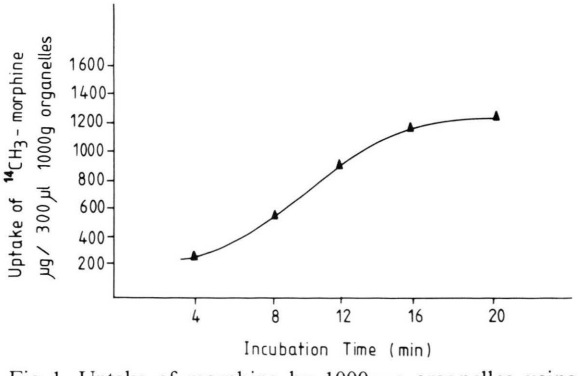

Fig. 1. Uptake of morphine by 1000 × *g* organelles using sequential additions of morphine.

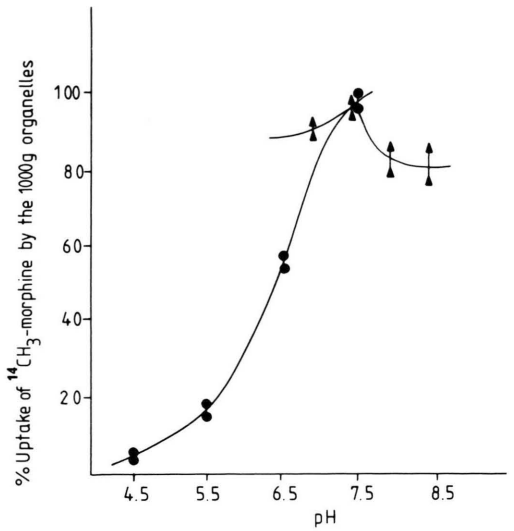

Fig. 2. The effect of pH on the uptake of morphine by the $1000 \times g$ organelles. ●—● buffer A; ▲—▲ buffer C.

$1000 \times g$ organelle ability to take up morphine. Maximal uptake was observed in the range pH 7.3 to 7.5 with both buffers. In preliminary experiments in which organelles were allowed to accumulate [$^{14}CH_3$]morphine at pH 7.0 and then were reisolated and suspended in buffers of differing pH, the lack of loss of morphine into the supernatant suggested that organelles remained essentially intact over the range tested. This was borne out by observing the organelles at different pH using the light microscope with a phase contrast objective. These experiments also demonstrate that there was no significant movement of morphine through the organelle membrane into the surrounding medium during the period of the experiment.

The specificity of the 1000 × g organelles for the uptake of morphinan alkaloids

From previous research [8] it was known that essentially all the alkaloids in the latex were found in the $1000 \times g$ organelles. The fact that the present experiments have investigated morphine is due in part to the ready availability of [$^{14}CH_3$]morphine and also because it is the major alkaloid accumulated in *P. somniferum*. Since codeine and thebaine are similar in structure to morphine, the uptake of these alkaloids was investigated as was the uptake of papaverine, sanguinarine, and a series of non-papaver alkaloids (Table II). Alkaloid uptake by the $1000 \times g$ organelles was measured by HPLC, GLC, or assay of radioactive label according to the methods given in the experimental. The results in Table II show that the morphinan alkaloids morphine, codeine, and thebaine are readily taken up by the $1000 \times g$ organelles as is the benzylisoquinoline alkaloid papaverine. Neither the quaternary alkaloid, sanguinarine, nor the non-papaver alkaloids are taken up by the organelles.

In a second series of experiments [$^{14}CH_3$]morphine (1 mg) was added to the $1000 \times g$ organelles with 1 mg of either codeine or thebaine. After a 4 min incubation at room temperature, samples were processed and [$^{14}CH_3$]morphine uptake found to be very little different from that in samples to which the other alkaloids had not been added.

Investigation of the requirement for the accumulation of protons by an active pump mechanism and its role in morphine accumulation

It has been suggested that alkaloids could be accumulated by an ion trap mechanism in acidic vacuoles [10]. Using neutral red according to Strugger [19] showed that most of the alkaloid containing vesicles of the $1000 \times g$ organelle fraction took up this stain readily and have an estimated pH of about 3.0. In a series of experiments in which additions of ATP and Mg^{2+} were made to samples of $1000 \times g$ organelles, neither ATP nor Mg^{2+} showed any enhancement of morphine uptake by the organelles. Organelle samples which had been pretreated with inhibitors of ATPase still took up neutral red and therefore samples were also pre-

Table II. Uptake of alkaloids by the $1000 \times g$ organelles of *P. somniferum*. All alkaloids were dissolved in standard buffer as their hydrochloride salts to ensure dissolution and 1 mg in 100 µl buffer was added to 300 µl of $1000 \times g$ organelles and incubated for 20 min at room temperature (24.5 °C).

Alkaloid	% Uptake of alkaloid administered
Morphine	68
Codeine	63
Thebaine	64
Papaverine	50
γ-coniceine	nil
Reserpine	nil
Atropine	nil
Quinine	nil
Cytisine	nil
Sanguinarine	nil

Table III. Effect of ATP, Mg^{2+}, and ATPase inhibitors on morphine uptake by $1000 \times g$ organelles.

Additions, final concentration	Morphine accumulation % of control
None	100
ATP 10^{-3} M	100
Mg^{2+}, 10^{-3} M	100
ATP + Mg^{2+}, 10^{-3} M	100
Ouabain, 10^{-4} M	82
Chlormadinone acetate (CMA), 2.5×10^{-5} M	87
Sodium vanadate, 10^{-4} M	82
NH_4Cl, 1 and 2×10^{-3} M	91
KCl, 1.5, and 10×10^{-3} M	103
CMB, 1, 2.5 and 5×10^{-4} M	218
DCCD, 1 and 5×10^{-4} M	100
N-MM, 1 and 5×10^{-4} M	91
FCCP, 1 and 5×10^{-4} M	66

treated with cold morphine (1 mg per 300 µl sample) for a further 16 min in an attempt to dissipate the proton gradient prior to the commencement of the experiment. Even after this pretreatment, the inhibitors had little inhibitory effect on morphine uptake. No effect was observed with either NH_4Cl or KCl, while neither DCCD nor N-MM showed inhibition, CMB showed considerable stimulation and FCCP demonstrated a 30% reduction of morphine uptake by the $1000 \times g$ organelles (Table III).

Discussion

In the foregoing experiments an attempt has been made to further investigate the mechanism of accumulation of morphinan alkaloids in *P. somniferum* latex. Accumulation occurs rapidly in the $1000 \times g$ organelles, which vary in size from 100 nm to 770 nm. The larger organelles contain large amounts of alkaloid. The overall size and fragility of these organelles would suggest that the concentration of bases, in this case alkaloids, was such that water has entered osmotically to swell the organelle and so produce the large organelles observed. However these organelles remain acidic [19] and as experiments show continue to be capable of accumulating further alkaloid material. This group of organelles also contains most of the latex dopamine and experiments to date with continuous and discontinuous sucrose gradients have failed to show separate storage organelles for alkaloids and for dopamine in this more dense organelle population. The smaller organelles of Band A do not accumulate dopamine but this may be a feature of development rather than an inherent difference from the larger organelles of Band B. Both alkaloids and dopamine give black precipitates with OsO_4 and therefore the fact that these organelles have been previously shown to contain different levels of dense material with the electron microscope [8] would be no indication as to their alkaloid or catecholamine contents.

The uptake of morphine is apparently insensitive to temperature and morphine moves rapidly across the membrane into the organelle with maximal uptake of mg amounts by a 300 µl sample of the organelles after 6 min. Much larger amounts of alkaloid were taken up by the organelles if morphine is added sequentially to the organelle sample and it appears that the capacity of the organelles for morphine storage is far in excess of normal natural requirements. Since codeine, thebaine and papaverine are accumulated with similar ease this lends further support to the idea that these alkaloids are essentially formed outside of and stored within the $1000 \times g$ organelles. The rapidity of uptake of morphine by the organelles and the lack of temperature dependence suggest a channel protein mechanism rather than a protein carrier mechanism for passage through the organelle membrane. The channel protein mechanism in general does not involve coupling to an energy source and permits much larger fluxes across the membrane than do carrier protein mechanisms [18].

For the concentration of morphine and other alkaloids to build up within the organelle some trapping mechanism must be involved. Matile *et al.* [9] suggest that, in the case of sanguinarine uptake by organelles in *Chelidonium majus* latex there was an exchange with other alkaloids moving out of the organelle into the vacuolar sap. Neumann *et al.* [10] suggest that alkaloids could accumulate in the vacuole by an ion trap mechanism in acidic vacuoles. Further it is suggested that alkaloids are trapped as a result of protonation, the organellar membrane being only slightly permeable to the protonated form [20]. Salt formation or binding to organic substances may also occur [21]. The pH differences across the organellar membrane may be caused by a H^+ ion pump in that membrane. A proton gradient was present between organelle and supernatant in papaver latex as was proven by the neutral red estimation of pH and from the effect of pH change

on morphine uptake by the organelles. At pH 4.5 morphine accumulation was negligible but increased rapidly to a plateau at pH 7.5–8.5. In many instances [22] proton gradients result from the activity of membrane ATPase; however, the investigation of the involvement of ATP and Mg^{2+} in morphine accumulation proved negative as did the experiments with CMA, vanadate, and ouabain, all known to be inhibitors of ATPase. Providing that the inhibitors reached the site of action it must be assumed that membrane ATPase was not primarily responsible for the observed proton gradient. Treatment of the $1000 \times g$ organelles with NH_4Cl at 2 mM, used to promote leakiness of a membrane with respect to protons, did little to change the organellar uptake of either the neutral red dye or of morphine. Some success was observed with the proton gradient dissipator FCCP which did show some inhibition of morphine uptake at 10^{-4} M, but at twice that concentration produced plasmolysis of the organelles. All other methods used, except for change in pH of the suspending medium, failed to significantly reduce the proton gradient across the organellar membrane and similarly failed to significantly reduce morphine uptake. The entire problem of the development of the acidity of the $1000 \times g$ organelles and its relation to morphine accumulation appears, therefore, unresolved.

The fact that CMB caused stimulation of morphine uptake by the $1000 \times g$ organelles is of some curiosity since no such stimulation was observed with N-MM. One can only suggest that CMB may in some way be affecting a regulatory protein associated with morphine accumulation. This however requires further investigation.

Finally we concerned ourselves with the specificity of uptake of alkaloids by the $1000 \times g$ latex vacuoles. As expected from the results in Table II, in which accumulation of other alkaloids was compared with that of morphine, the uptake of codeine, thebaine, and papaverine was approximately that of morphine. Codeine and thebaine, however, had no apparent effect on morphine accumulation within the organelles and it is, therefore, possible that each alkaloid has its own specific channel protein. Somewhat surprisingly, we were unable to detect any uptake of the papaver alkaloid, sanguinarine, which, although it is not normally found in *P. somniferum* plants is commonly found in tissue cultures derived from this plant where it does occur in vacuoles [23]. This failure to pass through the organellar membrane may relate to the charge on the nitrogen and requires further investigation. Other, non-papaver, alkaloids did not accumulate in the $1000 \times g$ latex organelles of *P. somniferum* and it is proposed that the accumulation of alkaloids by the $1000 \times g$ organelles is a process requiring a highly specialized mechanism in which both shape and charge on the molecule are important.

[1] M. F. Roberts, Phytochemistry **10,** 3021 (1971).
[2] M. F. Roberts, Phytochemistry **13,** 119 (1974).
[3] M. D. Antoun and M. F. Roberts, Phytochemistry **14,** 909 (1975).
[4] M. D. Antoun and M. F. Roberts, Phytochemsitry **14,** 1275 (1975).
[5] M. D. Antoun and M. F. Roberts, Planta Med. **28,** 6 (1975).
[6] M. F. Roberts and M. D. Antoun, Phytochemsitry **17,** 1083 (1978).
[7] J. W. Fairbairn and M. J. Steele, Phytochemistry **20,** 1031 (1981).
[8] M. F. Roberts, D. McCarthy, T. M. Kutchan, and C. C. Coscia, Arch. Biochem. Biophys. **222,** 599 (1983).
[9] Ph. Matile, B. Jans, and R. Rickenbacher, Biochem. Physiol. Pflanzen **161,** 447 (1970).
[10] D. Neumann, G. Krauss, M. Hieke, and D. Groger, Planta Med. **48,** 20 (1983).
[11] D. de Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof, Biochem. Pharmacol. **23,** 2495 (1974).
[12] T. M. Kutchan, M. F. Roberts, and C. C. Coscia, to be published.
[13] M. F. Roberts, Phytochemistry **14,** 2393 (1975).
[14] United Nations Document ST/SOA/SerH, Bi, 33.
[15] M. Wink and T. Hartmann, Z. Pflanzenphysiol. **102,** 337 (1981).
[16] M. F. Roberts, Phytochemistry **17,** 107 (1978).
[17] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).
[18] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson, Chapter 6, Molecular Biology of the Cell, Garland, New York 1983.
[19] S. Strugger, Praktikum der Zell- und Gewebephysiologie der Pflanze. Springer, Berlin, Göttingen, Heidelberg 1969.
[20] R. F. Dawson, Plant Physiol. **21,** 115 (1946).
[21] B. P. Jans, Ber. Schw. Bot. Ges. **83,** 306 (1973).
[22] S. Schuldiner, H. Fishkes, and B. I. Kanner, Proc. Natl. Acad. Sci. USA **75,** 3713 (1978).
[23] T. M. Kutchan, S. Ayabe, and C. J. Coscia, in: The Chemistry and Biology of the Isoquinoline Alkaloids. Eds.: J. D. Phillipson, M. F. Roberts, and M. H. Zenk. Springer-Verlag Berlin, Heidelberg, New York, Tokyo, in press.