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Subcellular Localization of Alkaloids and Dopamine in Different Vacuolar Compartments of *Papaver bracteatum*[1]

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ABSTRACT

Fractionation of *Papaver bracteatum* Arya II Lindl. latex on Renografin step gradients revealed that 43% of the dopamine was compartmentalized along with α-mannosidase (40%) in vacuoles sedimenting in the 2% fraction. Twenty-two percent of the dopamine was in the supernatant, but a corresponding amount (18%) of α-mannosidase was also present suggesting vacuole breakage during isolation. By subcellular fractionation of protoplasts from cultured *P. bracteatum* cells, the 1,000*g* sedimenting organelles have been identified as the major site of accumulation of the morphinan alkaloid, thebaine (99±0.8%), and the benzophenanthridine alkaloid, sanguinarine (96±3%). Although the 1,000*g* pellet also contained 33±4% of the total alkaloid precursor, dopamine, and half of the total vacuolar marker enzyme, α-mannosidase, 62±10% of the amine was localized in the 100,000*g* supernatant. A differential distribution of the alkaloids was discovered upon resolution of the lysed protoplasts on Renografin step gradients. Over 40% of the dopamine was in the supernatant with 15% in a 2% Renografin band. The remainder was evenly distributed in denser fractions of the gradient. The 4 to 8% interface, previously found to contain the largest amount of thebaine and small amounts of sanguinarine and dopamine, has been shown to be enriched in vacuoles by electron microscopy. Using a histofluorescence method, dopamine compartmentation in vacuoles of intact cultured cells was corroborated. In summary, dopamine, sanguinarine, and thebaine occur in vacuoles of different densities. A large fraction of the total dopamine in cultured cells was found in the 100,000*g* supernatant along with 37% of the α-mannosidase suggesting that the amine may be sequestered in more fragile vacuoles than the alkaloids. The possibility that some dopamine may be cytosolic cannot be ruled out.

The catecholamine, dopamine (2-(3,4-dihydroxyphenyl)-ethylamine), is a central intermediate in the biosynthesis of isoquinoline alkaloids in higher plants (18, 19). It gives rise to both simple and 1-benzyltetrahydroisoquinolines that are ultimately transformed into a broad spectrum of alkaloids. Recently, we discovered large amounts of dopamine (>1 mg/ml) in latex of *Papaver bracteatum* and *P. somniferum* (14). Furthermore, a small fraction of this dopamine (0.1%) was localized in vacuoles of the same apparent density as those that sequester the morphinan alkaloids, thebaine, and morphine, in latex. This population of vacuoles was capable of alkaloid and dopamine transport by apparently independent mechanisms (6, 7). The storage of both primary and secondary metabolites including alkaloids in different types of vacuoles throughout the plant kingdom has been well documented (10 and references cited therein). Whether the remainder of the endogenous dopamine was present in the cytosol, other organelles, or in larger and/or more fragile vacuoles was not resolved because of our inability to account for all of the original dopamine after lengthy differential and sucrose density gradient centrifugations.

Analysis of *P. bracteatum* tissue cultures revealed large amounts of dopamine (up to 16% dry weight of the tissue) providing a system analogous to the plant latex in this respect (9). Utilizing conditions that promote cytodifferentiation to laticifer cells and thebaine accumulation (9), we were able to study the cellular localization of dopamine and thebaine (8). Initial subcellular localization studies (8) in which protoplasts were lysed and their contents resolved on Renografin step gradients revealed multiple compartments for both dopamine and the benzophenanthridine alkaloid, sanguinarine. While the largest amount of thebaine was enriched in a discrete vacuolar fraction, a percentage was always present in the supernatant fraction. A large proportion of the dopamine was also localized in the supernatant with smaller amounts occurring at the 2% Renografin interface and at the same density gradient interface as thebaine (4–8%). Multiple compartmentation of these metabolites may have been the result of vacuole lysis or of artifactual uptake into vacuoles during isolation.

In the present study, we address these possibilities in the following manner. Protoplasts were prepared from cultures exposed to conditions that induce morphinan alkaloid accumulation. Subsequent protoplast lysis was accomplished by dounce homogenization and the cellular contents were subjected to differential centrifugation. The resultant fractions were analyzed for thebaine, sanguinarine, dopamine, and the vacuolar marker enzyme, α-mannosidase. Vacuoles were further purified on Renografin step gradients and characterized by EM. Finally, in order to account for all the latex dopamine, whole latex was isolated in the presence of anti-oxidants, subjected to differential centrifugation and resolved on Renografin step gradients. Fractions were again analyzed for DA[2] and α-man.

MATERIALS AND METHODS

Plant Cell Cultures. *Papaver bracteatum* Arya II Lindl. cell culture was initiated as previously described (9).

Culture Conditions. Static cultures were maintained on Murashige and Skoog revised tobacco medium (11) supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L 6-benzylaminopurine at 24°C under 1.3 watts/m² continuous fluorescent light. To obtain hormone free suspension cultures, callus

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[2] Abbreviations: DA, dopamine; MOPS, morpholinopropanesulfonic acid; DIECA, sodium diethyldithiocarbamate; α-man, α-mannosidase; SN, supernatant; P, pellet.

tissue (approximately 1–2 g) was transferred, not later than the second week following subculture, to Erlenmeyer flasks (250 ml) containing 50 ml basal media and incubated at 24°C on a gyratory shaker (100 rpm) in the dark for 9 d.

Buffers. The following buffered solutions were used in protocols that are modifications of previously described methods (3, 16). (a) Cell digestion medium: 0.7 mM $CaCl_2$, 0.7 mM Na_2HPO_4, 0.5 mM Mes, 20 mM sodium ascorbate, 1.0 M mannitol, 2.5 mg/ml BSA, and 10 mg/ml of each of the *Aspergillus niger* hydrolases, cellulase, and hemicellulase (Sigma), and 10 mg/ml of the *Rhizopus* hydrolase, pectinase (Sigma) (pH 5.6). To this buffer was added one volume of suspension culture basal medium. (b) Vacuole buffer: 0.7 mM $CaCl_2$, 0.7 mM Na_2HPO_4, 0.5 mM MOPS, 20 mM sodium ascorbate, 1 mM EDTA, 10 mM DIECA, or 3 mM NaN_3, 0.5 M mannitol (pH 7.6). (c) Renografin gradient solutions: equimolar amounts of 3,5-diacetamido-2,4,6-triiodobenzoic acid and *N*-methyl-D-glucamine were dissolved in vacuole buffer to give a final concentration of 16% (w/v). The 2, 4, and 8% solutions were prepared by serial dilution.

Protoplast Isolation. To prepare protoplasts, the contents of 12 flasks (30 g) of hormone-free suspension culture were centrifuged at 1,000*g* for 10 min to sediment cells. the pellet was resuspended in 50 ml digestion medium and incubated at 24°C in darkness for 16 to 20 h in a gyratory shaker at 100 rpm. Subsequent steps were carried out at 4°C. The crude protoplast solution was filtered through cheesecloth, diluted 1:1 with vacuole buffer, and recentrifuged at 100*g* for 5 min. The pellet was resuspended in vacuole buffer and the 100*g* × 5 min centrifugation repeated. This wash step was repeated 2 to 3 times until the SN was colorless indicating removal of most of the hydrolytic enzymes. The P was resuspended in vacuole buffer and filtered through a 40 to 80 μm stainless steel sieve and the filtrate centrifuged at 100*g* for 5 min.

Differential Centrifugation of Vacuoles. Initially, to isolate vacuoles, the above 100*g* P was resuspended in 7 ml vacuole buffer and the protoplasts lysed with a dounce A pestle. The homogenization process was continued until protoplasts were no longer evident by light microscopy. Differential centrifugation was performed at 1,000*g* for 10 min. An aliquot of the SN was retained and the remainder was centrifuged at 100,000*g* for 1 h (Beckman SW50.1 rotor, 40,000 rpm). Each P and SN was analyzed for DA, thebaine, sanguinarine, α-man, and protein.

Gradient Centrifugation of Vacuoles from Protoplasts. To further resolve vacuoles, the above 100*g* P was resuspended in 2 to 4 ml of vacuole buffer and either lysed by 4 strokes of a dounce A pestle or applied directly to a Renografin step gradient (3 ml each of 2, 4, and 8% and 2 ml of 16%). After centrifugation at 100,000*g* for 2 h (Beckman SW41 Ti rotor, 32,000 rpm), gradient interfaces and intervening regions were collected and analyzed for protein and α-man and by EM.

Differential and Gradient Centrifugation of Latex. Latex was collected as previously described (14) with the following modification: the collection buffer contained 20 mM sodium ascorbate and 10 mM DIECA as anti-oxidants. Whole latex was loaded onto Renografin step gradients and centrifuged at 100,000*g* for 2 h (Beckman SW41 Ti rotor, 32,000 rpm). Fractions were collected and analyzed for DA, α-man, and protein. For differential centrifugation the whole latex was centrifuged at 1,000*g* for 10 min.

Alkaloid and Dopamine Analyses. Alkaloids and DA were quantitated by HPLC with electrochemical detection (HPLC-EC) as previously described (9, 14). Recoveries ranged from 60 to 80%.

Protein Determination. Protein was estimated by the method of Bradford (2).

α-Mannosidase Assay. A 100 μl aliquot of each gradient fraction was added to 0.1 M citric acid (pH 4.5). The reaction was initiated by addition of 0.5 ml of an aqueous solution of *p*-nitrophenyl-α-D-mannopyrannoside (3 mg/ml). The incubation (30–120 min at 25°C) was quenched by addition of 2 ml of 0.2 M boric acid (pH 9.8). Control values were obtained by adding boric acid before the addition of protein. The *A* of the *p*-nitrophenolate anion ($E = 1.8 \times 10^4$ mole/L·cm) was measured at 405 nm (1).

Electron Microscopy. The following buffered reagents were used in this protocol: Primary fixative, 2% glutaraldehyde in 0.1 M sodium cacodylate and 1 mM $CaCl_2$ (pH 8.0); cacodylate buffer, 0.1 mM sodium cacodylate, 1 mM $CaCl_2$ and 3.5% sucrose (w/v) (pH 7.25); postfixative, 1% OsO_4 in the cacodylate buffer.

Renografin gradient fractions were diluted to 12 ml with vacuole buffer and centrifuged at 100,000*g* for 1 h (Beckman SW41 Ti rotor, 32,000 rpm). Pellets were placed in primary fixative for 2 h at 4°C. Following 2 × 10 min washes in cacodylate buffer (4°C), the P were treated with the postfixative at 4°C overnight. After staining 1 h in 2.5% uranyl acetate, P were dehydrated in graded ethanol and propylene oxide and then infiltrated 2 h in 2:1 propylene oxide:Spurr, 2 h in a 1:1 mixture and overnight in a 1:2 combination. The infiltrated samples were embedded in Spurr. Grids with silver sections were stained with uranyl acetate and lead citrate and viewed on a JEOL 100 CX electron microscope.

Fluorescence Microscopy. DA was visualized in tissue by a histofluorescence method developed by De La Torre (5). Tissue sections were examined on a Leitz Dialux 20 fluorescence microscope. A BP450-490 in combination with a BG38 red suppression filter and a camera with an automatic time exposure were used.

RESULTS

Fractionation of Latex Organelles on Renografin Step Gradients. *Papaver bracteatum* latex was directly resolved on Renografin step gradients and the resultant fractions analyzed for DA, α-man, and protein. The results (Fig. 1) indicate that DA and α-man are compartmentalized in a vacuolar fraction sedimenting to the 2% Renografin layer. DA and α-man were also present at the top of the gradient (SN). Throughout the gradient the distribution of DA closely paralled that of α-man suggesting that DA is primarily contained in vacuoles. These results are in agreement with those obtained from differential centrifugation of *P. bracteatum* latex in which 39±1.4% DA and 12±0.7% α-man were detected in the 1000*g* SN, with the remainder in the 1000*g* P. Similar results were obtained with latex from *P. orientale.*

Subcellular Fractionation of Organelles from Protoplasts by

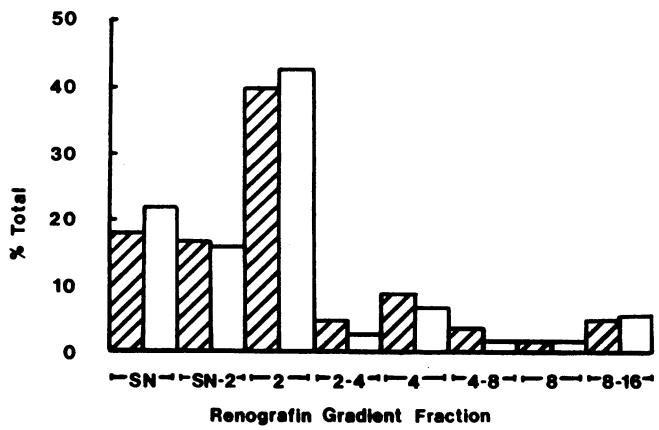

FIG. 1. Distribution of *P. bracteatum* latex DA and α-man on Renografin step gradients. Open bars represent DA and the shaded α-man. Data represents results obtained from two independent experiments. See "Materials and Methods" for experimental details.

Differential Centrifugation. *P. bractectum* protoplasts were prepared from cell suspension cultures by cell wall digestion with hydrolytic enzymes and their cell membranes were gently disrupted by dounce homogenization. Protoplast formation and complete disruption was carefully monitored by light microscopy. Subsequent to protoplast lysis, cellular contents were fractionated by differential centrifugation and analyzed for DA, thebaine, sanguinarine, and α-man. The results (Table I) clearly indicate that two-thirds of the DA was localized in the 1,000*g* SN whereas thebaine and sanguinarine were predominantly localized within the 1,000*g* pellet. Centrifugation of the 1,000*g* SN at 100,000*g* for 1 h demonstrated that DA is not compartmentalized in microsomal vesicles. The equal distribution of α-man activity, in 1,000*g* sedimenting and SN fractions suggests vacuole breakage even during isolation under these gentle conditions of cell lysis.

Subcellular Fractionation of Vacuoles from Protoplasts by Gradient Centrifugation. Originally (8) protoplasts were often fractionated without dounce homogenization thereby a more gentle lysis was achieved during ultracentrifugation. This also permitted recovery of intact protoplasts and cells at the 8 to 16% interface. To better characterize the vacuolar fractions by EM, protoplasts were lysed completely by dounce homogenization prior to ultracentrifugation. The cellular contents were layered onto a Renografin step gradient. After centrifugation, DA analyses of the collected fractions revealed >40% of the catecholamine was recovered from the gradient with the greatest proportion in the SN band (Fig. 2). Consistent with earlier studies (8) recovery of a percentage of the α-man activity in the SN fraction suggested some disruption of vacuoles during their isolation.

The 2% band contained 15% of the DA and comparable amounts of α-man whereas the remainder of the amine was distributed uniformly in more dense fractions of the gradient. With dounce homogenization, a discrete band of vacuoles (Fig. 3) enriched (over the original protoplasts [Fig. 4]) in thebaine was localized in the 4 to 8% interface. Without dounce homogenization, intact segments of protoplasts were frequently encountered. As seen in earlier Renografin profiles (8), some DA was also present in the 4 to 8% band. In conclusion, dounce homogenization of the protoplasts did not change the distribution of DA but did provide better enrichment of vacuoles at the 4 to 8% Renografin interface.

Histofluorescence Studies of Cultured *P. bracteatum* Cells. In contrast to its vacuolar localization in latex, two-thirds of the DA in *P. bracteatum* cell cultures appeared to be in SN fractions. Nevertheless, the co-occurrence of α-man in these same fractions made a cytosolic designation equivocal. Therefore, it was of interest to resolve the question of the compartmentation of this catecholamine in cultured cells by examining intact tissue. Using a histofluorescence method developed to track dopaminergic neurons in brain (5), it was ascertained that a considerable amount of DA is localized in vacuoles in intact cells. As seen in Figure 5B green fluorescent product by reaction of DA plus glyoxylate appeared to delineate spherical intercellular organelles that we believe to be DA containing vacuoles. Similar compartmentation of DA was discovered in intact plant tissue as well (data not shown). First, since the principal of the method is based on the Pictet-Spengler condensation of the amine with glyoxylic acid to yield a fluorescent isoquinoline derivative, water-treated tissue controls were found to be devoid of green fluorescence (Fig. 5). Some autofluorescence may be seen in cell walls of control. Bright yellow fluorescent organelles, Taylor image of yellow, and orange may also be observed occasionally in cells. In addition, rat striatal tissue which is rich in DA gave a comparable color. Finally, cell cultures of *Thalictrum foliolosum* which contains trace amounts of DA (9), were also examined and found to contain an endogenous fluorescent metabolite which differed in color from the DA adduct.

Table I. *Fractionation of DA, Thebaine, Sanguinarine, and α-Man from Cultured P. bracteatum Cells by Differential Centrifugation*

See Material and Methods for details. Data represents the average of three experiments performed in duplicate.

Fraction	Dopamine[a]	Thebaine	Sanguinarine	α-Man
		%		
1,000*g* SN	67 ± 4	0	3 ± 3	53 ± 7
1,000*g* P	33 ± 4	99 ± 0.8	96 ± 3	47 ± 7
100,000*g* SN	62 ± 10	0	3 ± 4	37 ± 8
100,000*g* P	2 ± 0.1	1 ± 0.8	1 ± 0.7	6 ± 0.4

[a] An average of 82% of the total DA in protoplasts was recovered in the 1000*g* SN and P.

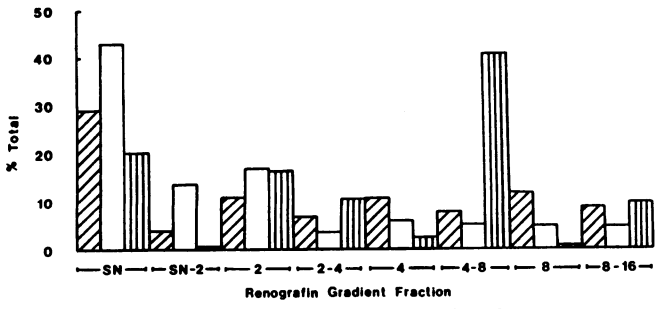

FIG. 2. Renografin gradient centrifugation of *P. bracteatum* protoplast contents. Protoplasts were lysed by dounce homogenization prior to ultracentrifugation. Fractions were analyzed for α-man (angle bars), DA (open bars), and thebaine (vertical bars). Data represents the means from two independent experiments. See "Materials and Methods" for experimental details.

DISCUSSION

The data reported here indicate a co-localization of α-man and DA with a distribution that is distinctly different from that of thebaine. In latex, a vacuolar fraction at the 2% Renografin interface contains 40% of the α-man and DA (Fig. 1). The use of latex provided the mildest conditions of organelle isolation. However, in lysed and dounced protoplasts, a less gentle method of isolation, we could not obtain conclusive evidence for the localization of α-man and DA in subcellular fractions. Over 40% of each was also in the SN.

Two interpretations are possible. If we assume that α-man is a vacuolar marker, as shown in other tissues (1) as well as in lysed, but undounced *Papaver bracteatum* protoplasts (8), then DA could be localized in vacuoles. However, the possibility that all or some DA is in the cytosol cannot be excluded on the basis of the subcellular fractionation results. More conclusive evidence that DA is in vacuoles is provided by fluorescence microscopy (Fig. 5). At least in certain cells, DA is localized in small round organelles measuring ≤3.2 μm in diameter which we believe to be vacuoles.

Thebaine-storing organelles were identified as vacuoles by EM (Fig. 3) and marker enzyme analysis after resolution on Renografin gradients. The 4 to 8% Renografin interface, from which vacuoles were collected, had previously (8) been identified as 10-fold enriched in thebaine, but had not been shown to be the exclusive site of accumulation. Originally, it proved to contain 40% of the total thebaine recovered from the gradient with a large variable amount (10–30%) in the SN. In a series of experiments the fluctuation of α-man recoveries in the SN paralleled that of thebaine. It now appears, in light of nearly complete recovery of alkaloids in the 1,000*g* pellet, that vacuoles undergo disruption during gradient ultracentrifugation.

The differential localization of DA and alkaloids in vacuoles

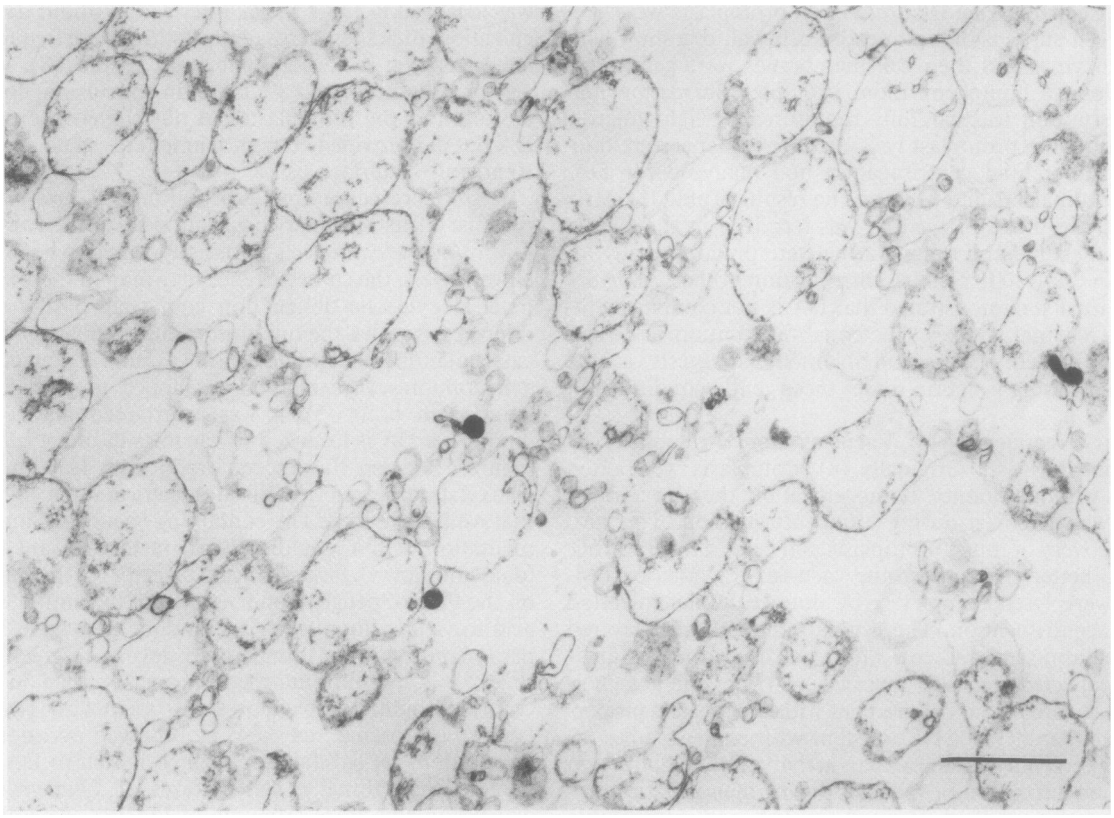

FIG. 3. Electron micrograph of vacuoles enriched at the 4 to 8% Renografin interface. This fraction is 10-fold enriched in thebaine (8). See "Materials and Methods" for experimental details on sample preparation. Vacuoles varied in size with an average diameter of 1 to 1.5 μm. Some vacuoles contain internalized membranes as seen in intact protoplasts. Bar = 1 μm (×15,000).

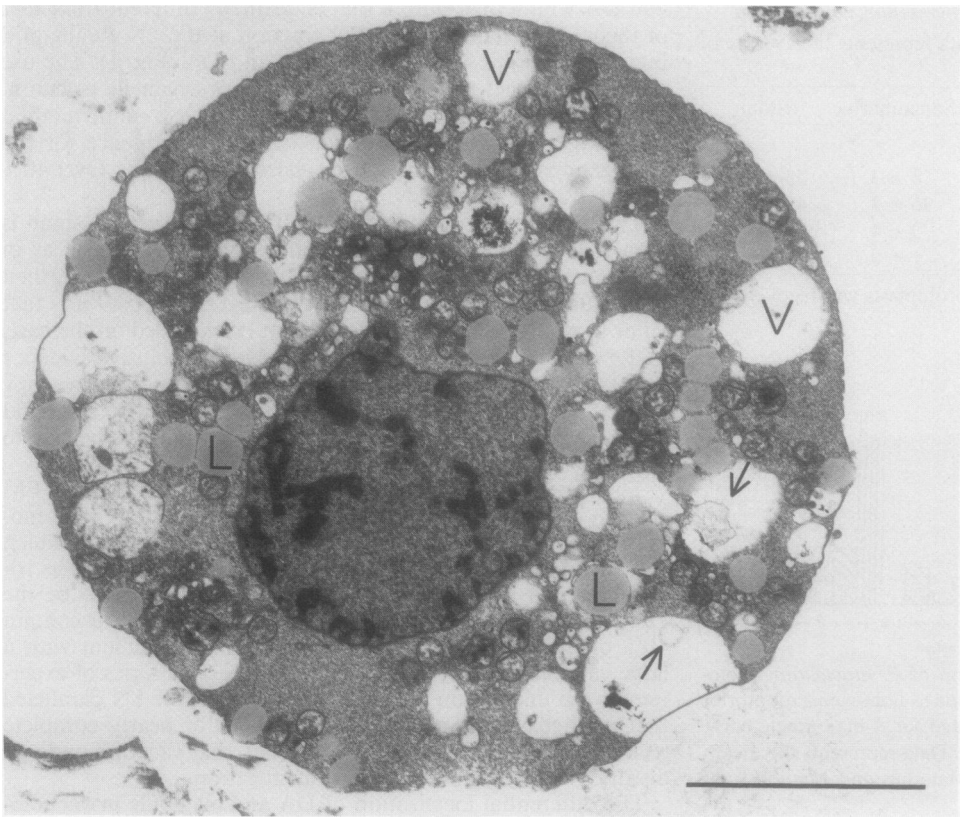

Fig. 4. Electron micrograph of a typical protoplast prior to ultracentrifugation. Vacuoles (V) vary in both size and electron dense contents. Note the preponderance of lipid droplets (L) and membranes internalized in vacuoles (arrow). Bar = 5 μm (×6250).

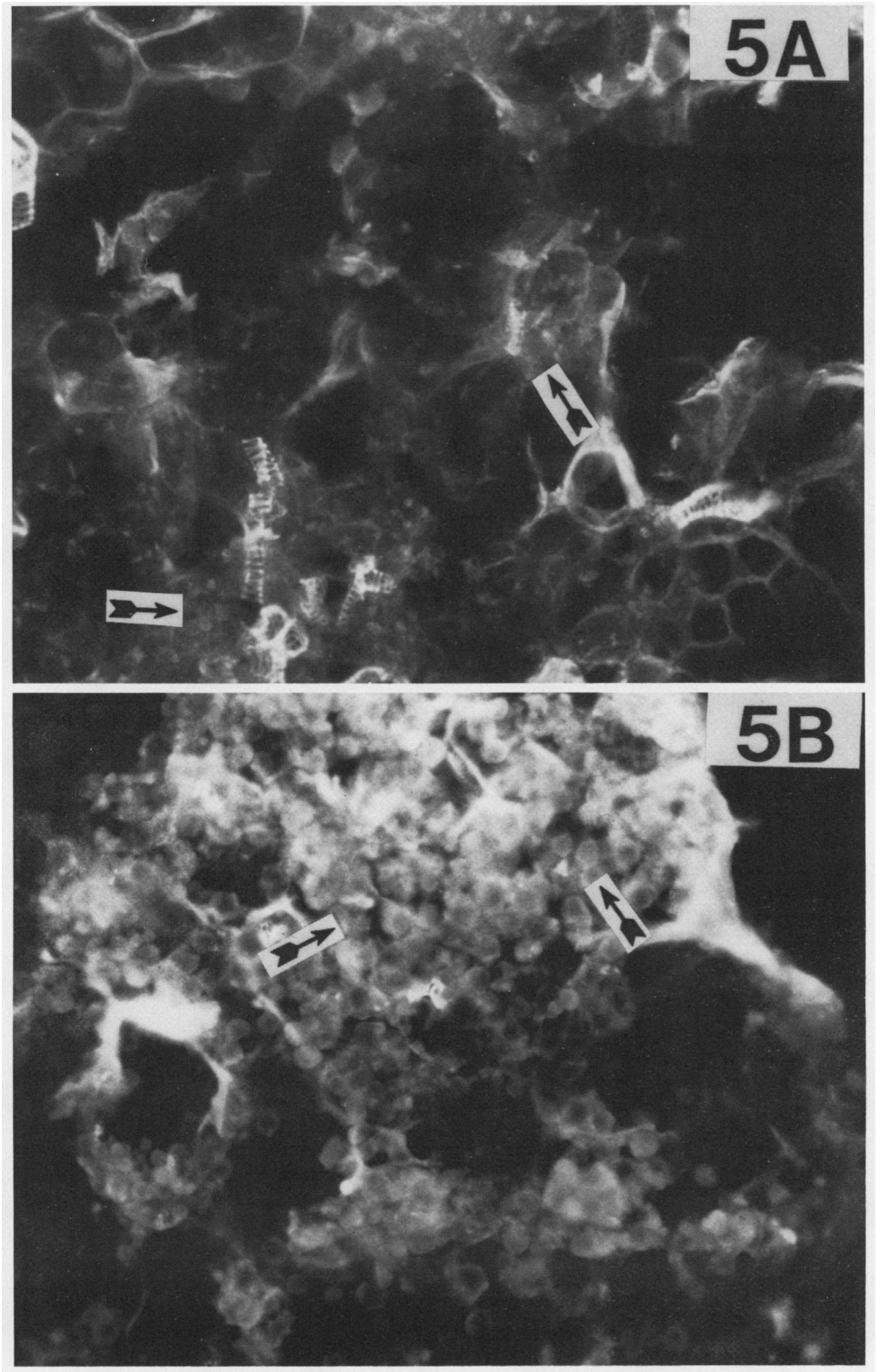

FIG. 5. Histofluorescence of cultured *P. bracteatum* cells. A, Water treated control (×200); B, glyoxylate treated section (×312). Vacuoles (arrow) fluoresce bright green as not seen in controls.

of different densities appears, at first, to present logistical problems for alkaloid biosynthesis. Dopa decarboxylase (13) and *S*-norlaudanosoline synthase (15, 17), two enzymes involved in DA synthesis and transformation, are generally thought to be cytosolic, but the possibility that they are located in the same fragile vacuoles which contain DA cannot be ruled out. Earlier studies (6–8, 10, 16) suggest that different plants contain different types of vacuoles. Taken together, the previous data and those reported here are consistent with the concept of the existence of heterogeneous populations of vacuoles within the same plant that have specific roles with respect to alkaloid synthesis and storage. This is consistent with the differences of pH optima observed for the different enzymes of alkaloid synthesis (12–15, 17) as well as the differences in both the subcellular localization and uptake of thebaine and sanguinarine into vacuoles (6–8).

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