SUBCELLULAR LOCALIZATION OF A CYTOCHROME P-450-DEPENDENT MONOOXYGENASE IN VESICLES OF THE HIGHER PLANT CATHARANTHUS ROSEUS

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ABSTRACT
The intracellular location of a cytochrome P-450-dependent monoterpane hydroxylase from the higher plant, Catharanthus roseus, has been investigated. By differential and sucrose density gradient centrifugation, utilizing marker enzymes and electron microscopy, the monooxygenase was demonstrated to be associated with vesicles having a membrane thickness of 40-60 nm. The vesicles could be distinguished from endoplasmic reticulum, Golgi apparatus, mitochondria, and plasma membrane and were found in light membrane fractions containing provacuoles. Most definitive results were obtained when seedlings were ground in the presence of sand and in a medium containing sorbitol. Upon subjection of the 20,000-g pellet preparation to linear sucrose density gradient centrifugation, a threefold enrichment in hydroxylase activity was afforded in a yellow band having vesicles varying in size from 0.1 to 0.8 \( \mu m \) in diam and having a density of 1.09 to 1.10 g/cm\(^3\). Since the monooxygenase has been implicated in indole alkaloid biosynthesis in this plant, the data suggest the compartmentalization of at least a part of this pathway.

Oxygenases containing the heme protein, cytochrome P-450, are commonly embedded in cellular membranes. In animals, cytochrome P-450 systems have been found attached to the endoplasmic reticulum of liver and to mitochondrial membranes of adrenal cortex (10). Lower concentrations of P-450 heme proteins are associated with mitochondrial and microsomal fractions of other tissues as well as with hepatocyte Golgi apparatus and nuclear membranes (12). Insects (3) and Penicillium patulum (26) also possess microsomal cytochrome P-450 whereas in microorganisms such as Pseudomonas putida (9), Rhizobium japonicum (1), Candida tropicalis (5), and Streptomyces erythreus (36), cytochrome P-450-dependent monooxygenases have been recovered in a soluble form. Bacterial monooxygenases are presumed in some cases to have been originally membrane-bound although this concept is not entirely without its complications (9). A number of P-450-dependent systems from higher plants have been described as being of microsomal origin (8, 27, 30, 37). This report presents evidence that a monooxygenase (17, 24) containing a considerable fraction of the total cytochrome P-450 in the higher plant, Catharanthus roseus, G. Don (Vinca rosea) is associated with a provacuolar fraction (22). This P-450-dependent system hydroxylates
the C-10 methyl groups of the monoterpenic alcohols, geraniol and nerol, which represents one of the first steps in the biosynthesis of indole alkaloids in this plant. The results suggest at least partial compartmentalization of indole alkaloid biogenesis in vesicles of C. roseus seedlings.

MATERIALS AND METHODS

All biochemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, Mo.) except geraniol, p-chloro-N-methylaniline, and acetylacetone which were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) [1-^H]Geraniol was synthesized as previously described (24).

**Isolation of Cellular Organelles from Catharanthus roseus Seedlings**

Seed coats from 5-day-old etiolated Catharanthus roseus seedlings (germinated at 30°C over moist vermiculite) were carefully removed and the seedlings washed in distilled water. All subsequent operations were carried out at 0-4°C. Seedlings were ground gently in a cold mortar for about 2 min in 2 vol of grinding medium, and the slurry thus obtained was mixed with washed polyvinylpyrrolidone(polyclar AT) (tissue:polycar AT, 25:1, wt/wt). The grinding medium consisted of 0.4 M sucrose, 10 mM KCl, 10 mM MgCl₂, 10 mM EDTA (adjusted to pH 7.6), 1 mM dithiothreitol (DTT), and 10 mM metabisulfite dissolved in 0.1 M Tris-HCl, pH 7.6. The slurry was then squeezed through two layers of silk and the filtrate centrifuged at 3,000 g for 20 min.

To obtain preliminary data on the subcellular localization of the hydroxylase, the following differential centrifugations were carried out. The 3,000 g supernate was centrifuged at 10,000 g and 15,000 g for 15 min each. Pellets were gently washed with small amounts of medium A (0.4 M sucrose, 1 mM EDTA, 1 mM DTT in 0.1 M Tris-HCl, pH 7.8) and resedimented. The 10,000 g and 15,000 g pellets were then resuspended in medium B (0.1 M Tris-HCl, pH 7.8 containing 1 mM DTT and 0.2 M sucrose). The 15,000 g supernate was further centrifuged at 100,000 g for 75 min to obtain the particulate fraction, designated as microsomes. The microsomal pellet was washed with small amounts of wash medium A and resedimented.

In experiments in which sucrose density gradient centrifugations were carried out, the 3,000 g supernate was subjected to centrifugation at 20,000 g for 20 min. This pellet, referred to as a vacuolar fraction, was gently resuspended in medium B, and 1.0 ml was carefully layered on the top of a stepped gradient composed of (a) 10 ml of 40% (wt/vol) sucrose, (b) 10 ml of 20% (wt/vol) sucrose and (c) 9 ml of 15% (wt/vol) sucrose. All sucrose solutions were prepared in 0.05 M Tris-HCl, pH 7.6 containing 1 mM EDTA and 1 mM DTT. The gradients were centrifuged at 20,000 rpm for 8 h in a Beckman Model L2-65 ultracentrifuge with a SW 25.1 rotor at 4°C (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). After centrifugation, the gradient was fractionated with an ISCO density gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebr.). Fractions of 1.1 ml were collected, and the sucrose concentration in each was determined on a Bausch & Lamb Abbe refractometer (Bausch & Lomb Inc., Analytical Systems Div., Rochester, N. Y.). Each fraction was tested for hydroxylase as well as other enzyme activities.

When sucrose gradient centrifugation was carried out to determine the effect of Mg++ on sedimentation behavior, 3 mM or 20 mM MgCl₂ was included in the same gradient as described above to prevent the dissociation of membrane-bound ribosomes (15). In some experiments, homogenates were prepared in a medium containing 20 mM EDTA, 30 mM MgCl₂, 1 mM DTT, and 0.5 M sucrose in 0.1 M phosphate buffer, pH 7.4 (38). The cell-free extract was centrifuged at 3,000 g, and the supernate was layered onto a discontinuous gradient of 0.8, 1.0, 1.2, and 1.4 M sucrose containing 20 mM EDTA, 30 mM MgCl₂, 1 mM DTT, and 0.1 M phosphate buffer, pH 7.4. The gradient was centrifuged at 100,000 g for 1 h in a SW 41 rotor (Beckman Instruments, Spinco Div.).

**Isolation of Vacuoles**

**METHOD I:** The method entailed maceration of C. roseus cotyledons with pectinase (34) followed by gentle crushing. The cotyledons have been found to be the site of the bulk of the hydroxylase activity in seedlings (G. Fiskum, unpublished observations). Cotyledons (2 g) were sliced into 1-mm sections and incubated for 105 min with 0.5% pectinase in 0.5 M mannitol at 20°C. The cotyledons were then transferred into a homogenizing tube, and 5 ml of 0.7 M mannitol was added. They were then gently crushed with a loose-fitting Teflon pestle and filtered through cotton gauze. The filtrate was centrifuged at 1,000 g for 5 min to give a vacuolar pellet. The pellet was washed three times with 0.1 mM CaCl₂ in 0.7 M mannitol. Finally, the pellet was suspended in 5% (wt/vol) Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) in 0.7 M mannitol and resedimented. The Ficoll centrifugation was repeated two more times, and the final pellet was used to assay for hydroxylase activity.

The integrity of the vacuoles was monitored by light microscopy with a neutral red stain.

**METHOD II:** Large amounts of intact vacuoles were isolated from the 5-day-old C. roseus seedlings after the method of Matile and Wiemken (23). The crude vacuolar pellet (20,000 g) was prepared by gently grinding the seedlings in the presence of sand and in a sorbitol medium. Thereupon, it was further purified on a discontinuous sucrose gradient (40%, 20%, and 15% wt/vol), or a linear gradient (15-50% wt/vol sucrose, centrifuging in a SW 41 rotor (2 h, 125,000 g).

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Enzyme Assays

Cytochrome oxidase: the oxidation rate of the reduced cytochrome c was determined by recording the absorbance change at 550 nm after the procedure of Mann and Gest (18). NADPH-cytochrome c reductase and NADH-cytochrome c reductase were assayed according to the method of Ernst et al. (6). N-demethylase activity was determined by a modified Nash method (4) with p-chloro-N-methylamphetamine as substrate. Malate dehydrogenase was assayed as described by Ochoa (28), using oxaloacetate as substrate. Esterase activity was determined according to the method of Matile (19), using p-nitrophenyl acetate as the substrate. Glucose-6-Pase activity was assayed essentially after the procedure described by Barrett (2), using glycero-2-phosphate (Method I) and p-nitrophenyl phosphate (Method II) as substrates. Geraniol hydroxylase was carried out as previously described (24). Cytochrome P-450 was estimated according to the method of Omura and Sato (29), using either a Cary Model 14 (Cary Instruments, Fairfield, N.J.) or an Aminco-Chance DW-2 (American Instrument Co., Inc., Silver Springs, Md.) split beam spectrophotometer. Particulate fractions were resuspended in 0.1 M phosphate buffer, pH 7.4, containing 1.15% KCl and 1 mM DTT. After the suspension was reduced, with a few crystals of dithiobisnitrobenzene, CO was bubbled into the sample cuvette for 30 s. Protein determinations were made according to the method of Lowry et al. (16) and, in some cases, a modified Lowry method (31) which corrects for phenolic contaminants.

Electron Microscopy

Vacuolar pellets were resuspended in 2.5% glutaraldehyde in phosphate buffer and fixed for 1 h at 4°C. The cell pellet was rinsed through subsequent changes of phosphate buffer. Postfixation was done in 2% buffered osmium tetroxide and dehydration in increasing percentages of ethanol solutions. Cell pellets were embedded in Spur plastic (Polysciences Inc., Warrington, Pa.), and sections were cut on an LKB-Huxley ultramicrotomes (LKB Instruments, Inc., Rockville, Md.). Sections were stained with lead citrate and uranyl acetate and examined with a Philips EM300 or a Hitachi HU-11E electron microscope.

RESULTS

Initial studies on the geraniol hydroxylase (24) were performed utilizing a microsomal pellet derived by centrifugation of a crude cell-free extract, first at 10,000 g for 20 min and then the resulting supernate at 100,000 g for 1 h. This afforded a 10,000 g pellet which exhibited no hydroxylase activity, while the 100,000 g pellet contained virtually all detectable activity. In an attempt to improve the overall yield of the geraniol hydroxylase, the isolation procedure was modified to include a 3,000 g centrifugation, thereby facilitating removal of cell debris including the seed coat which is difficult to separate manually from 5-day-old seedlings. It was observed that, upon centrifugation at 3,000 g initially, the subsequent 10,000 g pellet exhibited considerable hydroxylase activity which was associated with a yellow particulate fraction (Table I). Presumably, a black polymeric constituent of the seed coat had previously inhibited enzyme activity in this 10,000 g pellet. Furthermore, if a 15,000 g pellet was obtained after a 3,000 g spin, its geraniol hydroxylase activity was 5 times greater than that of the microsomal pellet originally studied (24). Indeed, careful differential centrifugation under conditions designed to prevent aggregation of endoplasmic reticulum repeatedly revealed that less than 10% geraniol hydroxylase activity was associated with the 100,000 g microsomal fraction (Table I). If the 20,000 g and 100,000 g pellets were recombined, an increase in activity was observed commensurate with the activity of the fraction added. But when the 20,000 g pellet was extracted with chloroform-methanol (2:1) and the lipid added to the microsomes, no stimulation of geraniol hydroxylase activity was observed.

The findings prompted an examination of the subcellular location of the hydroxylase by differential and sucrose density gradient centrifugation, monitoring by marker enzymes and electron microscopy. As seen in Table I, marker enzymes for mitochondria (cytochrome oxidase) and microsomes (N-demethylase) were observed to occur predominantly in the expected fractions. Other enzyme activities such as phosphatases and esterases were found to be distributed generally. Approx. 60% of the total geraniol hydroxylase activity was associated with the 10,000 g pellet, whereas another 25-30% of the total activity was detected by CO difference spectra was in the same fraction (Table I). Presumably, a black polymeric constituent of the seed coat had previously inhibited enzyme activity in this 10,000 g pellet. Furthermore, if a 15,000 g pellet was obtained after a 3,000 g spin, its geraniol hydroxylase activity was 5 times greater than that of the microsomal pellet originally studied (24). Indeed, careful differential centrifugation under conditions designed to prevent aggregation of endoplasmic reticulum repeatedly revealed that less than 10% geraniol hydroxylase activity was associated with the 100,000 g microsomal fraction (Table I). If the 20,000 g and 100,000 g pellets were recombined, an increase in activity was observed commensurate with the activity of the fraction added. But when the 20,000 g pellet was extracted with chloroform-methanol (2:1) and the lipid added to the microsomes, no stimulation of geraniol hydroxylase activity was observed.

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Most of the Glu-6-P activity was found in post 20,000 g fractions, but this enzyme is a questionable marker for plant endoplasmic reticulum (15). To secure a better microsomal marker enzyme and also to define the role of the remaining 58% of cytochrome P-450 associated with the 100,000 g pellet in C. roseus, its N-demethylase activity was investigated. Cytochrome P-450-dependent
TABLE I
Enzyme Activities (nmol/min/mg protein) of Fractions Obtained by Differential Centrifugation

| Enzyme Activity                        | Pellet          | Cell-free extract | 10,000 g | 15,000 g | 100,000 g | 100,000 g |
|----------------------------------------|-----------------|-------------------|----------|----------|-----------|-----------|
| Total protein (mg)                     | 840             | 42.9              | 9.3      | 20.6     | 732       |           |
| P-450 concn (pmol/mg protein)          | 0.088           | 0.45              | 0.86     | 0.364    | 0         |           |
| Geraniol hydroxylase                   | 11.3            | 0.14              | 0.33     | 1.24     |           | 0         |
| N-Demethylase                          | 113             | 8.5               | 17.0     | 15.9     | 0         |           |
| NADPH-cytochrome c reductase           | 124             | 1,960             | 430      | 137.3    | 69.5      |           |
| Cytochrome oxidase                     | 30              | 0                 | 0        | 70       | 30        |           |
| Malate dehydrogenase                   | 30              | 30                | 32       | 10       | 50        |           |
| Acid phosphatase                       | 230             | 80                | 60       | 200      | 253       |           |
| p-Nitrophenyl-acetate esterase         | 64              | 33                | 38       | 52       | 91        |           |

* The CO-difference spectrum of the dithionite-reduced protein (2 mg/ml) was taken with a 20,000 g pellet in this experiment.
† 90% of this activity found in the 20,000 g pellet.

N-demethylases have been demonstrated to be localized in microsomes of castor bean endosperm on the basis of isopycnic density and marker enzyme studies (37). Table I reveals that N-demethylase was found predominantly (70%) in the 100,000 g pellet of C. roseus.

To identify the subcellular locus of the hydroxylase, succrose gradient centrifugations and marker enzyme studies were utilized. After a preliminary 3,000 g spin, a 20,000 g pellet was obtained and separated on a discontinuous 15-40% sucrose gradient. As seen in Fig. 1, the hydroxylase was observed to occur in a single band with a density centered at 1.13 g/cm³. Included in this yellow band were both NADH- and NADPH-cytochrome c-reductase and acid phosphatase activities (Fig. 2). A mitochondrial enzyme marker (succinate dehydrogenase) was found exclusively at a density of 1.16 g/cm³. N-Demethylase was not detectable in succrose gradient fractions of 20,000 g or 100,000 g pellets. Its absence may be attributable to the instability of the enzyme during the 8 h centrifugation and/or the lack of sensitivity of the formaldehyde assay at the low protein concentrations obtained.

Fractions 19-21 contained all of the geraniol hydroxylase activity of the 20,000 g pellet and also gave the characteristic CO difference spectrum of reduced cytochrome P-450 and an oxidized vs. reduced spectrum characteristic of the cytochrome b₅ type. The density (1.13 g/cm³) of fractions 19-21 is similar to that observed for a vacuolar fraction (provacuoles) obtained by Matile (19), Matile et al. (20), and Matile and Moor (22) from corn and tobacco seedlings (1.138 g/cm³). Marker enzyme studies for esterase and acid phosphatases (Fig. 2) are in agreement with his findings. On the other hand, Semadeni (32) has interpreted fluorochrome staining patterns to indicate that fragments of ER occur at a density of 1.07 g/cm³. Glu-6-P activity was found in this study, and by Semadeni (32) at the same density of 1.07 g/cm³. One of the NADPH-cytochrome c reductase activity bands (Fractions 9 and 10, Fig. 1) also possessed this density. Although Lord et al. (15) have reported a band of Glu-6-P activity and NADPH-cytochrome c reductase activity at 1.07 g/cm³, it was proposed that the bulk of the endoplasmic reticulum of castor bean endosperm is associated with a light membrane fraction having a density of 1.12 g/cm³. This assignment was supported by isopycnic centrifugation in the presence and absence of Mg++. In the presence of Mg++, ribosomes are retained on the ER membrane, thereby affording a fraction with a density of 1.16 g/cm³. The experiments described above (15) were repeated and, although shifts in RNA and protein profiles were observed as reported, geraniol hydroxylase activity remained in the same fractions with the same density in both the presence and absence of 3 and 20 mM Mg++. Furthermore, the 100,000 g microsomal pellet was found...
FIGURE 1 Fractionation of enzymes in the 20,000 g pellet by discontinuous sucrose gradient centrifugation. Multiple enzyme analyses were carried out on each fraction. Activity is expressed in nmoles/hour/milligram protein for the geraniol hydroxylase and ΔA_{340}/min/mg protein for NADH- and NADPH-cytochrome c reductases.

FIGURE 2 Fractionation of enzymes in the 20,000 g pellet by discontinuous sucrose gradient centrifugation. Multiple analyses were carried out on each fraction. Activity is expressed in nmoles/minute/milligram protein for β-glycerophosphate phosphatase and p-nitrophenol phosphate phosphatase. Hydroxylase activity is expressed as in Fig. 1.

to have an isopycnic density of 1.12 g/cm³ in agreement with Beever's findings. Thus, the geraniol hydroxylase was associated neither with the rough endoplasmic reticulum membranes nor with the bands at 1.07 g/cm³.

Finally, a 3,000 g supernate of a cell-free homogenate was fractionated on a discontinuous gradient of 0.8, 1.0, 1.2, and 1.4 M sucrose in the presence of 20 mM EDTA and 30 mM Mg²⁺ as described by Williamson et al. (38). In this manner, four discrete bands were obtained of which the first, at the 0.8 M sucrose interface, contained 90% of the total hydroxylase activity. The specific activity of this yellow band was 0.48 nmol/min/mg protein. The remainder of the hydroxylase activity (0.05 nmol/min/mg protein) was detected at the
interface between 0.8 and 1.0 M sucrose. Of the bands resolved, only the hydroxylase-containing fraction was yellow initially. The others changed from a buff-color to yellow when subsequently pelleted out.

In another approach which affords a pellet of intact vacuoles, cotyledons of 5-day-old etiolated C. roseus seedlings were subjected to maceration with pectinase (34), followed by gentle crushing and centrifugation in 5% Ficoll at 1,000 g for 5 min. This pellet exhibited geraniol hydroxylase activity having, in some experiments, specific activities 6 times greater than those of the 1,000 g supernate. In such preparations as compared to the usual method (cf. Table I), hydroxylation rates were relatively low (0.1-0.2 nmole/min/mg protein). The loss of enzyme activity may be due to the lengthy procedure or the introduction of proteases which are contaminants of commercial pectinases.

Thus, another method (23) was adopted to secure a vacuolar population, and this proved to be most definitive and most suitable for routine work. The procedure entailed grinding seedlings in the presence of sand and in 0.5 M sorbitol, 50 mM Tris-HCl buffer, pH 7.6, 1 mM EDTA, a medium in which meristematic vacuoles were found to be very stable (20). Upon centrifugation on a discontinuous sucrose gradient for 2 h at 125,000 g, the hydroxylase was again obtained in a discrete yellow band at the interface between 15% and 40% sucrose and exhibited maximal activities (5.2 nmol/min/mg protein). The same homogenization procedure has been repeated, but the 20,000 g pellet was loaded onto a linear sucrose gradient (15-50%). As predicted by Matile and Wiemken (23), the yellow vacuolar band became more diffuse on such a gradient due to the heterogeneity in density of the vacuolar population. The monoterpene hydroxylase and cytochrome c reductase activities appeared as a broad band in a density range of 1.09 to 1.10 g/cm³, again coincident with the yellow color (Fig. 3). In addition, a threefold increase in the specific activity of the hydroxylase of these fractions over the 20,000 g pellet was observed, thereby affording the purest membrane preparation that we have obtained. Furthermore, recovery of the hydroxylase activity applied to the gradient was 95% as compared to 25-30% in the earlier procedure (Figs. 1 and 2). In addition to the selective advantages of the utilization of a linear gradient, at least three other factors are apparent which may contribute to the observed enrichment and recoveries. These are the use of sand for homogenization, the sorbitol medium, and the shorter gradient centrifugation time (2 h).

When the pelleted yellow band from fractions 19-21 (Figs. 1 and 2) was examined by electron microscopy, it was found to be composed of nu-
merous small vesicles of varying diameter (0.1-0.3 μm) (Fig. 4). The thickness of the membranes varied from 40 to 90 nm, with the majority of the vesicular membranes measuring 40-60 nm. Profiles of presumed cisternae of the Golgi apparatus were also present. No mitochondria were detected in this yellow band, but they were present in subsequent fractions (Fig. 5). The mitochondria were morphologically similar to those of the intact tissue. Examination of the band obtained by sucrose gradient centrifugation (15) of the microsomal (100,000 g) pellet showed both vesicles of varying size and membrane profiles (Fig. 6). The membranes comprising this fraction measured 40-90 nm. The thicker, pleomorphic membranes which were in greatest abundance in this fraction appeared to be derived from the plasmalemma. In contrast, electron micrographs (Fig. 7) of the yellow hydroxylase-containing band obtained by the Morré method (38) revealed vesicles of 0.2-2 μm diameter and only 40-60 nm membrane thickness.

Electron micrographs of the hydroxylase fraction of highest specific activity obtained from linear sucrose density gradients (Fig. 3) revealed a dense vacuolar population ranging in size from 0.1 to 0.8 μm (Fig. 10). Some distribution of size with density could be observed, the larger vacuoles occurring at a lighter density than the smaller vacuoles which were found at the lower portion of the yellow band. The enrichment in hydroxylase specific activity paralleled the purification of the vacuolar fraction as evidenced by electron microscopy (Fig. 10). Vesicles that contained a yellow pigment and were similar in size to the larger vacuoles found at 1.09 g/cm³ were observed in free-hand sections of cotyledons by light microscopy.

Examination by electron microscopy of fixed tissue of 5-day-old etiolated cotyledons shows a large central vacuole with electron-dense inclusions (Fig. 8). In Fig. 9, it can be seen that the large central vacuole is derived by coalescence of provacuoles which resemble, with respect to membrane thickness and size range, the vesicles found in the yellow hydroxylase-containing band obtained by the Morré method (see Fig. 7).

An examination by scanning electron microscopy of the 5-day-old etiolated C. roseus seedlings show the presence of two large cotyledons and the shoot apex which consisted of the apical meristem surrounded by 3-4 minute leaf primordia. Cryo-fractured sections of the cotyledons revealed the following layers: single-celled layers of epidermic and palisade parenchyma cells, 4-5 layers of mesophyll parenchyma cells, and a single layer of hypodermis.

DISCUSSION
The main body of evidence described herein established clearly that the geraniol hydroxylase is not associated either with the bulk of the microsomal membranes or with mitochondrial membranes. Thus, by differential centrifugation, 70-80% of the hydroxylase can be sedimented at 20,000 g whereas most of the microsomal fraction of this plant occurs in the 100,000 g pellet as established by the microsomal marker enzyme, N-demethylase. Furthermore, in sucrose gradients with high Mg²⁺ concentrations, rough ER was pelleted whereas the hydroxylase was not. Finally, electron microscope examination of the hydroxylase-containing yellow band from sucrose gradients (Figs. 7 and 10) clearly excluded the presence of either mitochondria or microsomes. Mitochondria and microsomes were detected in other fractions having the expected densities in all cases.

The yellow color of the hydroxylase-associated membranes suggests contamination with plastids or plastid fragments. However, plastids are more dense, and their membrane fragments often distribute among several bands including mitochondrial and more dense fractions. The distribution of the hydroxylase as a discrete band in all of our gradients obviates a plastid origin. In fact, the yellow pigment associated with the hydroxylase can be removed with chloroform:methanol (2:1), and the extract exhibits a visible spectrum similar to that of the carotenoid, lutein (17). Lembi and Morré (14) have found lutein in vacuoles of corn tissue. Examination by light microscopy of free hand sections of C. roseus cotyledons revealed the presence of vacuoles containing yellow pigment. The presence of a high lipid content in intact vacuoles (Figs. 7 and 10) would contribute to their low isopycnic density.

That the cytochrome P-450-dependent hydroxylase is associated with vacuolar membranes is supported by evidence from several experimental observations. Included are the occurrence of the hydroxylase in the vacuolar pellet, obtained after gentle disruption of protoplasts, and the 1.15 g/cm³ density of the hydroxylase-containing band in sucrose gradients prepared according to the methods described in references 19-22. In the latter gradient the hydrolytic enzyme distribution profile
FIGURE 4 Electron micrograph of the pelleted yellow band obtained by sucrose gradient centrifugation after the procedure of Matile (19) and Matile et al. (20). Note the presence of numerous membrane profiles and vesicles measuring 40-90 nm in thickness. × 23,500.

FIGURE 5 Electron micrograph of pooled fractions 23 and 24 from the above sucrose gradient centrifugation. Note the presence of intact mitochondria (arrows). × 23,500.
FiGurE 10  Electron micrograph of vesicular fractions having highest hydroxylase specific activities obtained by linear sucrose gradient centrifugation after homogenization in sorbitol (23). × 28,000.

resembles that observed for corn and tobacco seedling extracts, and the hydroxylase appears in a fraction that has been assigned as containing pro-vacuoles on the basis of enzyme marker studies and electron microscopy (19–22, 32). By discontinuous sucrose density centrifugation utilizing the Morré method (38), and by homogenization in sorbitol, followed by centrifugation on linear gradients (Fig. 3), the hydroxylase is enriched in dense vacuolar fractions essentially homogeneous within the size range of 0.1 to 0.8 μm (Figs. 7 and 10). The sorbitol method affords excellent recoveries of hydroxylase activity. The threefold enrichment in hydroxylase specific activity in the

FiGurE 6  Electron micrograph of the major protein band obtained by sucrose gradient centrifugation of the microsomal pellet after the procedure of Lord et al. (15). Note the presence of membrane profiles and vesicles measuring 50 nm in thickness. × 66,000.

FiGurE 7  Electron micrograph of the pelleted yellow band obtained by sucrose gradient centrifugation after the procedure of Williamson et al. (38). The vesicles which contained 90% of the total hydroxylase activity ranged in size from 0.2 to 2 μm in diam and 40–60 nm in membrane thickness. × 26,000.

FiGurE 8  Electron micrograph of intact mesophyll cell from 5-day-old etiolated cotyledons. Note the large central vacuole (V) with electron-dense inclusions. × 17,000.

FiGurE 9  Electron micrograph of intact mesophyll cell from 5-day-old etiolated cotyledons. Note the presence of pro-vacuoles (PV) undergoing fusion with each other and coalescing with the central vacuole (V) (arrow). × 17,000.
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