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Cytochemical Localization of Uricase and Catalase in Developing Root Nodules of Soybean

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Summary

Two different cytochemical methods were used to study the localization of uricase (EC 1.7.3.3) and catalase (EC 1.11.1.6) in developing root nodules of soybean (*Glycine max*) inoculated as seeds with *Bradyrhizobium japonicum*. One of the methods employs DAB (3,3'-diaminobenzidine) and detects uricase activity indirectly by coupling it to endogenous catalase activity. The other method utilizes cerium chloride to detect uricase activity directly. These methods were modified to obtain not only a strong staining reaction but also improved ultrastructural preservation. With the indirect DAB method, intense staining indicative of both uricase and catalase activity was obtained in the enlarged peroxisomes of older uninfected cells. Similar staining was observed in enlarging peroxisomes of younger uninfected cells, and in the material of associated sacs whose bounding membranes appear to arise as distensions of the ER. The observations are discussed in relation to the controversial role of the ER in peroxisome biogenesis. Although the small peroxisome-like organelles of infected cells did not give a clearly positive reaction in the indirect DAB method, they reacted positively in the cerium chloride method, and are considered to be peroxisomes.

Keywords: Cerium chloride; Diaminobenzidine (DAB); Peroxisomes; Root-nodules; Soybean; Uricase cytochemistry.

Abbreviations: DAB = 3,3'-diaminobenzidine; ER = endoplasmic reticulum.

1. Introduction

Soybean, like bean and cowpea, is an example of the ureide transporters, certain members of tropical origin in the leguminous tribe *Phaseoleae* whose root nodules produce the ureides allantoin and allantoic acid for nitrogen export in metabolic steps that go beyond those required for the synthesis of amino acids and amides by other legumes.

In soybean and many other legume root nodules, the central tissue enclosed by the cortex consists not only of cells that become greatly enlarged and heavily infected with rhizobia, but also of many smaller uninfected cells interspersed among the infected ones. In the developing soybean nodule, the peroxisomes enlarge greatly and an abundance of tubular endoplasmic reticulum appears in the uninfected ("interstitial") cells but not in the infected cells (NEWCOMB and TANDON 1981, NEWCOMB *et al.* 1985). The hypothesis that the uninfected cells play an essential role in ureide production (NEWCOMB and TANDON 1981) has been confirmed by biochemical work (HANKS *et al.* 1983).

It is now clear from the ultrastructural and biochemical data that there is a division of labor between the infected and uninfected cells, with the early steps in N_2 fixation occurring in the infected cells, and the later steps taking place in the uninfected cells. Uricase, now known to be localized in the numerous large peroxisomes of the uninfected cells, participates by converting urate to allantoin (SCHUBERT 1986). The H_2O_2 generated by uricase is destroyed by the catalase of the peroxisomes. Allantoin is then converted in part to allantoic acid by allantoinase, which is probably located in the tubular ER of the uninfected cells.

Uricase (EC 1.7.3.3) and catalase (EC 1.11.1.6) are well known components of peroxisomes generally (TOLBERT

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and ESSNER 1981), but in preparations from soybean root nodules are difficult to localize biochemically within the peroxisome owing to the extreme fragility of this organelle (HANKS *et al.* 1981). Nodule-specific uricase has, however, been localized immunocytochemically in the uninfected cell peroxisomes of soybean root nodules (NGUYEN *et al.* 1985, VANDENBOSCH and NEWCOMB 1986), while catalase has been similarly localized cytochemically (MARKS and SPRENT 1974, NEWCOMB and TANDON 1981, NEWCOMB *et al.* 1985).

Cytochemical reactions dependent on enzymatic activity provide information of a different kind than do immunocytochemical techniques based on antigenicity, and therefore may provide a means of studying other interesting questions successfully. For example, cytochemistry might be useful in determining when and where the activities of uricase and catalase appear in young developing nodules, whether the two enzymes can be demonstrated in the ER which proliferates in the uninfected cells and is closely associated with developing peroxisomes and whether activity is demonstrable in the small peroxisome-like organelles that occur in the peripheral cytoplasm of infected cells (NEWCOMB *et al.* 1985).

In seeking answers to these questions, cytochemical methods are needed which are not only sensitive but also leave the ultrastructure well preserved. One of the two methods we have used to examine these problems employs DAB and serves to localize uricase and catalase activities simultaneously (YOKOTA and NAGATA 1977). The other method utilizes $CeCl_3$ to localize the activity of uricase (VEENHUIS and WANDELAAR BONGA 1979) independently of catalase. By modifying the two methods we have been able to make improvements in both stainability and degree of ultrastructural preservation.

2. Materials and Methods

2.1. Plant Material

Soybeans (*Glycine max* (L.) Merr. cv. Evans' Certified) were treated as seeds with an inoculant containing *Bradyrhizobium japonicum* strain 3IIB110 (Nitragin Co., Milwaukee, Wis. USA). Inoculated seeds were planted in vermiculite and perlite (1 : 1) and kept under greenhouse conditions. The seedlings were fertilized twice a week with a modified Hoagland's solution minus nitrogen ($^1/_4$ strength of the solution of HOAGLAND and SNYDER (1933) but without $Ca(NO_3)_2$ or KNO_3, and with equivalent amounts of calcium and potassium added as the chloride salts). Mature nodules from plants 4–5 weeks old or developing nodules from plants 2–3 weeks old were harvested for the experiments.

2.2. Processing for Electron Microscopy

For conventional electron microscopy, the nodules were cut in halves with a razor blade and fixed in 3% glutaraldehyde in 0.05 M potassium-phosphate buffer (pH 6.8) for 2 hours at room temperature. The segments were then rinsed in 0.05 M potassium-phosphate buffer (pH 6.8) for 1.5 hours and postfixed in 2% osmium tetroxide in 0.05 M potassium-phosphate buffer (pH 6.8) for 2 hours at room temperature. They were then dehydrated in an acetone series and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome. They were mounted on copper grids and stained in aqueous 2% uranyl acetate for 10 minutes followed by lead citrate for 5 minutes. Sections were observed with a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV.

2.3. Catalase Cytochemistry

For the cytochemical localization of catalase, the nodule segments were fixed in 3% glutaraldehyde in 0.05 M potassium-phosphate buffer (pH 6.8) for 60 minutes at 0 °C and rinsed in 0.05 M potassium-phosphate buffer for 30 minutes at 0 °C. The nodule segments were then incubated in the standard 3,3'-diaminobenzidine (DAB) incubation medium (FREDERICK and NEWCOMB 1969) for 60 minutes at 37 °C in a reciprocal incubator (MagniWhirl, Blue M Electric Company) at 60 rpm. Following the incubation, the segments were rinsed in 0.05 M potassium-phosphate buffer (pH 6.8) for 20 minutes and postfixed in 2% osmium tetroxide in 0.05 M potassium-phosphate buffer (pH 6.8) for 2 hours at room temperature.

2.4. Uricase Cytochemistry Employing DAB

The method employing DAB for the cytochemical localization of uricase and catalase was modified from that of YOKOTA and NAGATA (1977). The nodule segments were fixed in 2% glutaraldehyde in 0.05 M potassium-phosphate buffer (pH 6.8) for 30 minutes at 0 °C and rinsed in 0.05 M potassium phosphate buffer (pH 6.8) for 30 minutes at 0 °C. The standard incubation medium consisted of 6 ml of 0.1 M tris-HCl buffer (pH 9.6), 4 ml of a solution of sodium urate (5 mg/ml) and 10 mg of DAB (3,3'-diaminobenzidine, Sigma Chemical Company). The segments were incubated for 90 minutes at 30 °C in a reciprocal incubator (60 rpm). In order to insure aeration, the segments were put in 30 ml breakers (diameter 3 cm) with a small amount (ca 1.5 ml) of medium so that the surface of the segments made contact with air frequently during incubation. For the control, incubation was carried out in the medium without sodium urate. Following incubation, the segments were rinsed in 0.05 M potassium-phosphate buffer (pH 6.8) for 15 minutes at room temperature and then postfixed in 2% OsO_4 in 0.05 M potassium-phosphate buffer (pH 6.8) for 1 hour at room temperature.

2.5. Uricase Cytochemistry Employing Cerium Chloride

For the cytochemical localization of uricase by the $CeCl_3$ method, the nodule segments were fixed in 3% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 6.8) for 1 hour at 0 °C, and rinsed in 0.05 M potassium phosphate buffer for 15 minutes at 0 °C. The segments were preincubated in the medium containing 10 ml of 0.1 M tris-HCl buffer (pH 9.6), 20 mg of $CeCl_3$ (cerium chloride, Sigma Chemical Company) and 42 mg of aminotriazole (3-amino-1,2,4-triazole, Sigma Chemical Company) for 30 minutes at 0 °C. The standard incubation medium consisted of 6 ml of 0.1 M tris-HCl buffer (pH 9.6) containing 20 mg of $CeCl_3$ and 42 mg aminotriazole, and 4 ml of an aqueous solution of sodium urate (Sigma Chemical

Company) (5 mg/ml). The segments were incubated in this medium for 30 minutes at 37 °C in a reciprocal incubator (60 rpm). In order to insure aeration, the segments were put in 30 ml breakers with a small amount (ca 1.5 ml) of medium so that the surface of the segments made contact with air frequently during incubation. For the control, incubations were carried out in the medium without sodium urate and also in medium containing 0.5 mM allopurinol as well as sodium urate. Following incubation, the segments were rinsed in ice-cold 0.1 M cacodylate buffer (pH 6.0) for 30 minutes to remove nonspecific precipitate, followed by 0.1 M cacodylate buffer (pH 7.2) for 10 minutes at room temperature. They were then postfixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) for 1 hour at room temperature.

Preliminary experiments were carried out to determine the optimal conditions for localizing uricase in nodules using the $CeCl_3$ method. These included different concentrations of glutaraldehyde (0, 0.1, 0.5, 1, and 3%), as well as variations in the pH of the incubation medium (pH 7.5, pH 9.6), temperature of incubation (22, 30, and 37 °C), and length of incubation (30 minutes, 1 hour, 3 hours, overnight). These parameters were tried in various combinations, the most satisfactory of which proved to be the one described in the preceding paragraph. After OsO_4 postfixation following each cytochemical procedure, the segments were processed for electron microscopy in the usual manner.

3. Results

3.1. General Features of Cells in the Infected Region of the Root Nodule

The infected region of the mature soybean root nodule is composed of two types of cells, infected and uninfected (Fig. 1). Infected cells contain numerous bacteroids enclosed in peribacteroid membranes. The plastids and mitochondria in infected cells are located at the cell periphery next to the plasma membrane. The uninfected cells, which are interspersed among the infected cells, are characterized by numerous enlarged peroxisomes and large amounts of tubular ER.

3.2. The Cytochemical Localization of Catalase with DAB

Catalase is well established as a marker enzyme for peroxisomes. In the presence of DAB and H_2O_2 in the reaction medium, this enzyme brings about the deposition in peroxisomes of amorphous, electron opaque osmium black (SELIGMAN *et al.* 1968).

When soybean root nodule segments were incubated with DAB and H_2O_2, regardless of the age sampled between 2 and 5 weeks, the peroxisomes were heavily stained, indicating catalase activity (not illustrated; *cf.*, NEWCOMB *et al.* 1985). A deposit of electron opaque material was also seen in the cell wall, especially in the middle lamella. This is ascribable to peroxidase activity (SEXTON and HALL 1978).

3.3. Indirect Localization of Uricase Using Urate and DAB

3.3.1. Uricase Activity in Peroxisomes of Uninfected Cells

Incubation of nodule segments in the medium containing DAB and urate resulted in intense staining of the peroxisomes in uninfected cells (Figs. 2 and 3). In this case a positive reactions depends on the activity of catalase on the H_2O_2 produced endogenously by uricase; staining thus results from the combined activity of the two enzymes. Paracrystalline regions in peroxisomes of uninfected cells also contained reaction product (Fig. 2, arrow).

No deposit was seen in the peroxisomes of control tissue incubated in the medium without urate (Fig. 4). However, a deposit of electron opaque material was frequently seen in the mitochondrial cristae (Fig. 4).

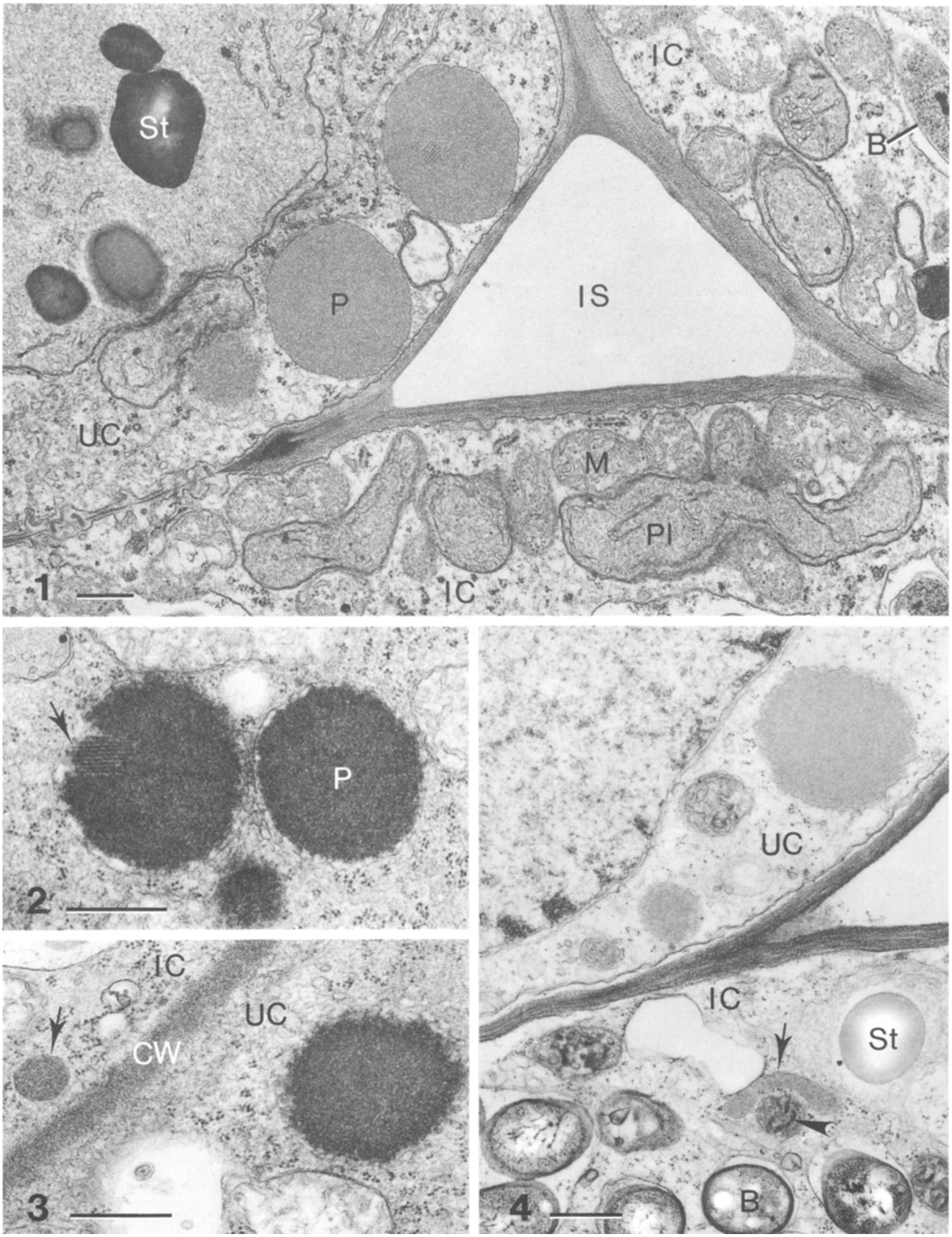
3.3.2. Response of the Peroxisome-like Organelles in Infected Cells

Mature cells infected with bacteroids contain occasional small, single-membrane bounded organelles previously interpreted as undeveloped or abortive peroxisomes (NEWCOMB *et al.* 1985). When tissue was incubated with DAB and urate, these bodies showed a weakly positive reaction (Fig. 3, arrow). In a direct test for catalase activity (*i.e.*, DAB plus H_2O_2), they gave an ambiguous result interpretable as either a negative or a weakly positive response (not illustrated).

3.3.3. Positive Reactions for Uricase in ER Derivatives and Enlarging Peroxisomes of the Uninfected Cells

Ultrastructural changes in the uninfected cells are especially pronounced during the period extending from about 12 days to 4 weeks after inoculation. By the start of this stage the infected and uninfected cells have already become differentiated from one another; as differentiation continues the peroxisomes in the uninfected cells enlarge and the ER proliferates into a rich meshwork that surrounds and frequently envelopes them (*cf.*, Figs. 3–5 in NEWCOMB *et al.* 1985). During this period the peroxisomes possess diverse shapes and frequently appear to be indistinctly demarcated from the ER. Accumulations of moderately electron dense material in sacs that appear to be distensions of the ER are common.

When the indirect DAB methodology to localize uricase is applied, the material in closely associated sacs is



Figs. 1–4

stained along with the matrix in the central part of the peroxisomes. Various examples are shown in Figs. 5–16. The figures are arranged in a developmental sequence corresponding approximately to the stage of peroxisome maturity as indicated by age of the nodule and by content, size and contour of the organelle. Inclusions of reaction product similar to those in the figures are common during this stage of nodule development.

A later but still not fully mature stage in the development of an uninfected cell is seen in Fig. 17. The peroxisomes are now spherical and the matrix fills the organelle except for a thin peripheral region next to the bounding membrane.

3.4. The Cytochemical Localization of Uricase with $CeCl_3$

3.4.1. Modifications of the $CeCl_3$ Method

In applying the $CeCl_3$ method for the localization of uricase in nodule cells, we tried various fixative concentrations and incubation conditions. Good ultrastructural preservation is needed to distinguish peroxisomes from lipid bodies, which often become electron dense during the procedure, and to distinguish the peroxisome-like bodies of infected cells from small profiles of plastids. We obtained the best results by prefixing the nodule slices in 3% glutaraldehyde for 1 hour at 0 °C and then incubating them in the reaction medium for 30 minutes at 37 °C and a pH of 9.6.

3.4.2. Reactivity of Peroxisomes in Uninfected Cells to $CeCl_3$

Incubation with $CeCl_3$ and urate resulted in the deposition of electron opaque material in the peroxisomes of uninfected cells (Fig. 18). Paracrystalline bodies in the peroxisomes are also strongly labeled (not illustrated). The deposit is believed to be a precipitate of cerium perhydroxide produced by the reaction between $CeCl_3$ and the H_2O_2 generated by uricase; thus it serves to localize this enzyme. The electron dense material produced by this method is finer textured than that obtained with DAB.

Two different control procedures were used to assess the validity of this method for localizing uricase activity, one in which urate was omitted and one in which the competitive inhibitor allopurinol was added. Without urate the peroxisomes stained no more intensely than other organelles, *e.g.*, plastids (Fig. 19). Addition of allopurinol to the incubation medium containing urate also reduced deposition in the peroxisomes (Fig. 20 compared to Fig. 18).

3.4.3. Reactivity of Peroxisome-like Bodies in Infected Cells to $CeCl_3$

Large peroxisomes are never observed in the infected cells. The occasional small, frequently elongated peroxisome-like organelles in these cells gave a strongly positive response indicative of uricase activity when nodule tissue was incubated with $CeCl_3$ and urate (Fig. 21, arrows). This contrasts with a weakly positive response of these bodies to tests for catalase activity. The deposit was absent from these organelles in the tissue incubated in control media (Figs. 19 and 20, arrows).

4. Discussion

Localization of uricase during development of soybean root nodules was examined by two different cytochemical methods. One of these depends on the addition of both urate and DAB. The DAB is polymerized by catalase acting on the H_2O_2 produced endogenously by uricase (YOKOTA and NAGATA 1977). An electron dense

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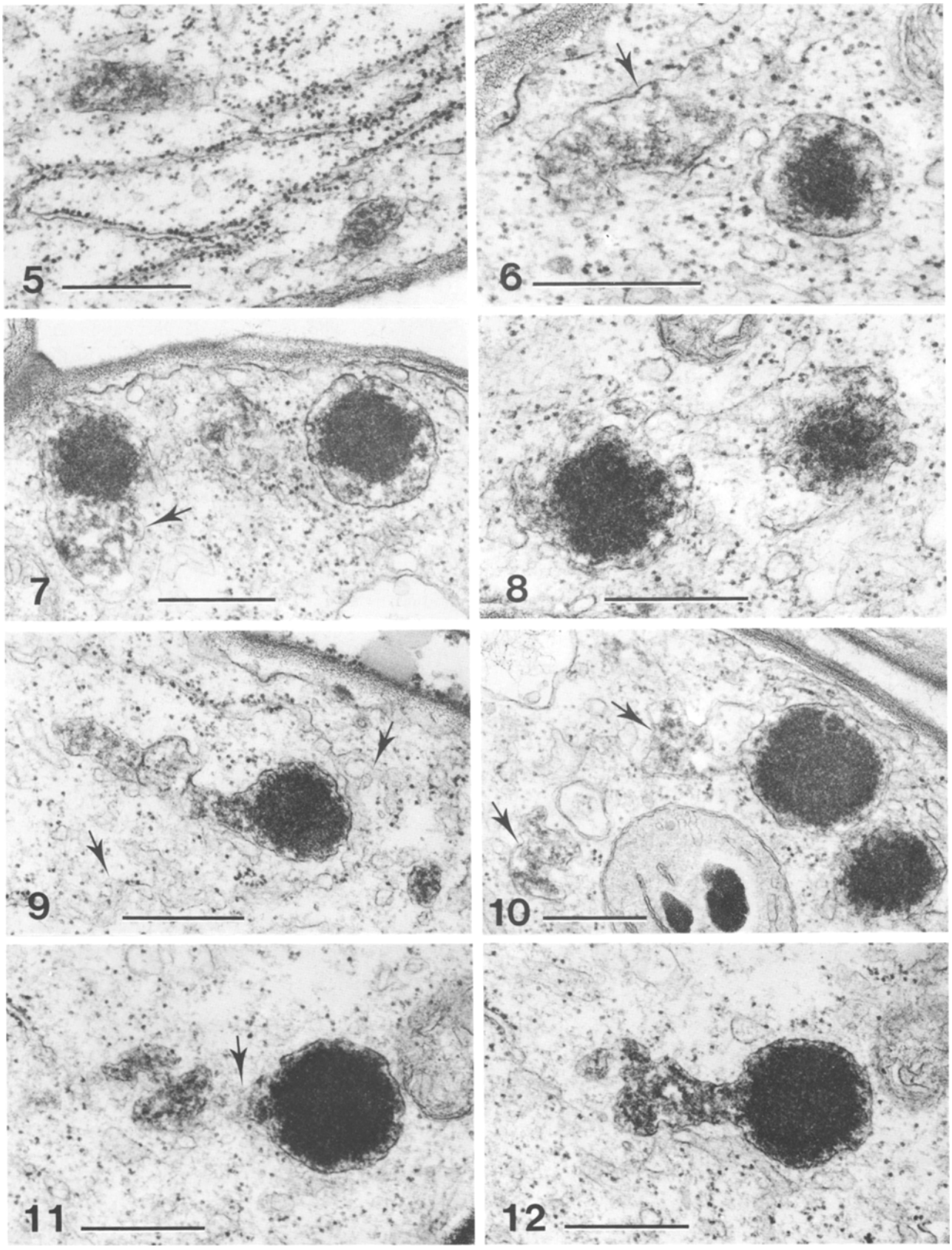
Figs. 1–4. Scale bars = 0.5 μm

Fig. 1. Representative profile of a portion of an uninfected cell (upper left) adjacent to two infected cells in the infected region of a mature soybean root nodule. The tissue was processed in the usual manner and was not subjected to cytochemical treatment for localization of uricase or catalase. *B* bacteroid, *IC* infected cell, *IS* intercellular space, *M* mitochondrion, *P* peroxisome, *Pl* plastid, *St* starch, *UC* uninfected cell. × 20,000

Fig. 2. Intense staining of peroxisomes (*P*) of an uninfected cell in soybean root nodule tissue treated with DAB plus urate. Note staining of paracrystalline body (arrow). × 36,000

Fig. 3. Nodule tissue treated with DAB plus urate. Staining of the peroxisome in the uninfected cell (*UC*) is heavy. Whether the small peroxisome (arrow) in the infected cell (*IC*) has been stained is not clear. *CW* cell wall. × 36,000

Fig. 4. Incubation of nodule tissue with DAB in the absence of urate. No electron dense deposit is seen either in the peroxisomes of the uninfected cell (*UC*) or in the small peroxisome (arrow) in the infected cell (*IC*). An electron opaque deposit is seen in the mitochondrial cristae (arrowhead). *B* bacteroid, *St* starch. × 26,000



Figs. 5–12

deposit (osmium black) demonstrates uricase activity indirectly by coupling it to endogenous catalase activity. The other method utilizes $CeCl_3$ to demonstrate uricase activity directly without depending on catalase activity. We have modified both methods so as to obtain strong staining and improved ultrastructural preservation.

In applying the indirect DAB method, we obtained an especially intense staining in the peroxisomes of uninfected cells. Compared to the conventional method for the localization of catalase, in which tissues are incubated with DAB and H_2O_2, the staining is enhanced and the ultrastructure is better preserved because the H_2O_2, which commonly disrupts the cytoplasm when added externally, is produced within the peroxisomes, where it is immediately broken down by catalase.

If the results of the indirect DAB method are negative, then both the conventional DAB method for catalase and the $CeCl_3$ method for uricase must be used to determine whether either enzyme is present. By using the two procedures in combination, we were able to demonstrate activity of both enzymes at early stages in the development of peroxisomes of uninfected cells and in the ER or ER derivatives closely associated with enlarging peroxisomes. In contrast, we found high uricase but low catalase activity in the small peroxisome-like organelles present in infected cells. Demonstration of peroxisomal uricase activity in the uninfected cells by two different cytochemical methods confirms the earlier work on the localization of nodule-specific uricase in this organelle by immunocytochemistry (NGUYEN *et al.* 1985, VANDENBOSCH and NEWCOMB 1986), and indicates that uninfected cell peroxisomes are major sites of allantoin production in soybean root nodules.

The staining of crystalloids in the peroxisomes by the indirect DAB method is not necessarily indicative of the presence of uricase, since the positive reaction could result from catalase activity. H_2O_2 for the catalase could diffuse into the crystal after being produced by the action of uricase on urate in the peroxisomal matrix. However, staining of the crystalloids also by the cerium chloride method suggests that they do contain uricase activity. When immunogold labeling was used to detect a nodule-specific form of uricase, similar inclusions remained unlabeled under conditions in which the matrix was richly labeled with gold particles (VANDENBOSCH and NEWCOMB 1986). One possible explanation for the lack of agreement is that the crystalloids do indeed contain uricase, but in the paracrystalline state the antigenic sites of the protein are blocked and are unable to bind with the antibodies even though the protein retains enzymatic activity.

Our cytochemical observations on the ER are of particular interest because the role of the ER in peroxisome biogenesis is controversial. The suggestion that peroxisomes originate from endoplasmic reticulum was based on ultrastructural observations of a close association between these organelles and the ER in both animal and plant cells (HRUBAN and RECHCIGL 1969, LAZAROW and FUJIKI 1985). Biochemical work on the ER and the membrane component of glyoxysomes (*i.e.*, the peroxisomes of germinating fatty seedlings) led to formulation of the concept that the glyoxysomal membrane is derived directly by a process of vesiculation and excision from a specific region of the ER (BEEVERS 1979). However, GOLDMAN and BLOBEL (1978) showed that uricase and catalase in rat liver cells are synthesized on free polysomes and proposed that these cytoplasmically synthesized matrix enzymes are

Figs. 5–12. Scale bars = 0.5 μm. Developing peroxisomes in uninfected cells in the infected region of soybean root nodule tissues stained with DAB plus urate. The figures are arranged corresponding approximately to stage of peroxisome development

Fig. 5. An early stage in a young uninfected cell showing two peroxisomes stained for uricase activity. Rough ER cisternae are prominent. × 46,000

Fig. 6. Sac (arrow) containing material stained for uricase activity. The nearby peroxisome is only partly filled by heavily stained matrix. × 60,000

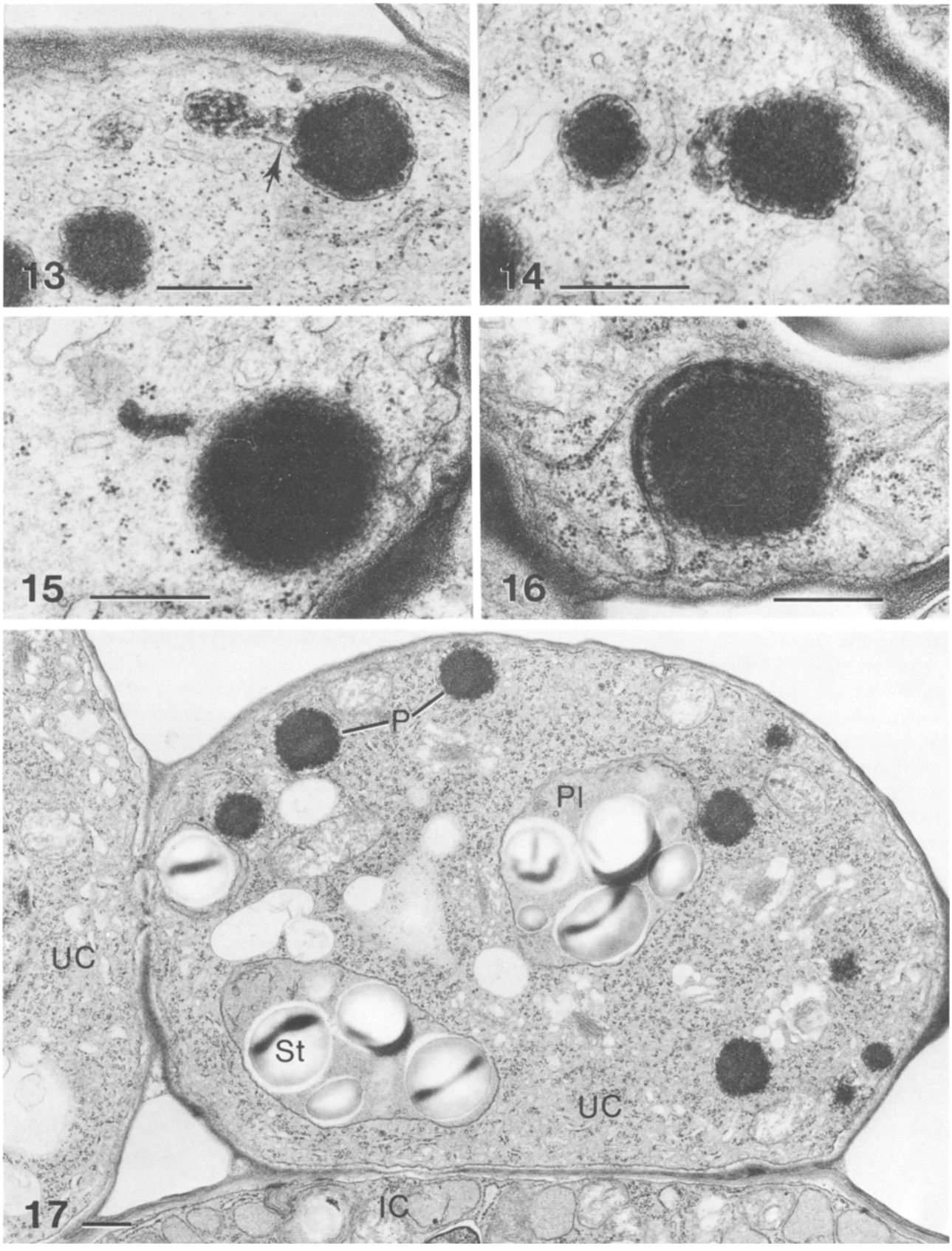
Fig. 7. The sac (arrow) of flocculent material on the left is clearly connected to the peroxisome. The material has been stained for uricase activity. × 42,000

Fig. 8. Clumps of material stained for uricase activity are in tubules and cisternae closely associated with the two peroxisomes. × 51,000

Fig. 9. An elongate sac containing stained material is clearly continuous with the main body of the peroxisome. Note the plexus of tubular ER in the vicinity (arrows). × 43,000

Fig. 10. Sacs (arrows) interpreted as derivatives of the ER in the vicinity of two peroxisomes. Material in the sacs has reacted positively in the test for uricase activity. × 37,000

Figs. 11 and 12. Closely spaced sections through the same peroxisome. The indistinct neck (arrow) between the sac of stained material in Fig. 11 is clearly seen to be connected to the peroxisome in Fig. 12. × 44,000



Figs. 13–17

imported into "nascent" peroxisomes which are in the process of budding from the ER. A similar model is supported by recent studies on glyoxysome formation (TRELEASE 1984). Also NGUYEN *et al.* (1985) recently showed that the nodule-specific uricase of soybean is preferentially synthesized on free polysomes and that the peptide lacks a signal sequence, implying that it is not processed cotranslationally into the ER lumen.

A different model proposes that new peroxisomes are formed by the fission of preexisting peroxisomes rather than by budding from the ER (LAZAROW and FUJIKI 1985). According to this model, the protrusions ("tails") frequently exhibited by peroxisomes are interpreted as parts of a peroxisomal reticulum rather than as derivatives of the ER, since a role for the ER in peroxisome biogenesis was not supported by their biochemical or ultrastructural work (LAZAROW *et al.* 1982). The ER is still believed to be the site of phospholipid synthesis for the peroxisomal membrane, however (LAZAROW and FUJIKI 1985).

Our observations strongly support a role for the ER in peroxisome biogenesis. In electron micrographs of developing soybean root nodules, the endoplasmic reticulum is closely associated physically with peroxisomes (NEWCOMB *et al.* 1985). Moderately electron dense material is commonly seen in structures that appear to be tubules or distended sacs of the ER in the vicinity of enlarging peroxisomes (NEWCOMB *et al.* 1985). Material in similar sacs reacts positively for uricase and catalase, as shown in the present illustrations (Figs. 5–16).

These micrographs are representative of the appearance of the cytoplasm in the vicinity of peroxisomes in the uninfected cells during a 2- to 3-week period when these organelles are enlarging and becoming filled with matrix. The clumps of membrane-bounded material that react positively for uricase and catalase are not seen at either younger or older stages of nodule development. Their close association with the peroxisomes and the reciprocal relationship noted between their abundance and the progressive filling out and rounding up of the peroxisomes in late stages of enlargement, taken together, argue for their contributory role.

Our observations can be reconciled with recent evidence on other systems (LAZAROW and FUJIKI 1985) by assuming that while the membranes of the sacs arise as distensions of ER, the accumulations within them consist of uricase, catalase and other proteins that have been taken up posttranslationally following their synthesis on free polysomes. We suggest that these sacs must then either fuse with preexisting peroxisomes or develop into new ones.

Our use of $CeCl_3$ to detect uricase activity largely confirms the results obtained with DAB, but also contributes some new information. The $CeCl_3$ method was introduced by BRIGGS *et al.* (1975) in localizing NADH oxidase in human polymorphonuclear leukocytes. VAUGHN *et al.* (1982) used $CeCl_3$ to localize uricase in sesbania, soybean and alfalfa root nodules, while KAUSCH and HORNER (1985) employed the same method to demonstrate uricase activity in *Yucca* root peroxisomes. VAUGHN *et al.* (1982) interpreted their results on soybean root nodules to indicate that enlarged peroxisomes occur not only in the uninfected cells but also occasionally in the infected cells, and that these large peroxisomes react positively to $CeCl_3$ in both cell types. However, in their figures it is difficult to distinguish peroxisomes from lipid bodies, which often become highly electron dense during the procedure and come to resemble peroxisomes closely. Our observations extending over a several year period do not substantiate their claim that enlarged peroxisomes occur in the infected cells of soybean nodules.

The enzymatic properties of the small peroxisome-like bodies of infected cells are of special interest because it is important to know whether the ability to convert uric acid to allantoin is confined solely to the uninfected cells. Although nodule-specific uricase was not detectable in these bodies by means of immunogold labeling (VANDENBOSCH and NEWCOMB 1986), the intense staining obtained with cerium chloride clearly

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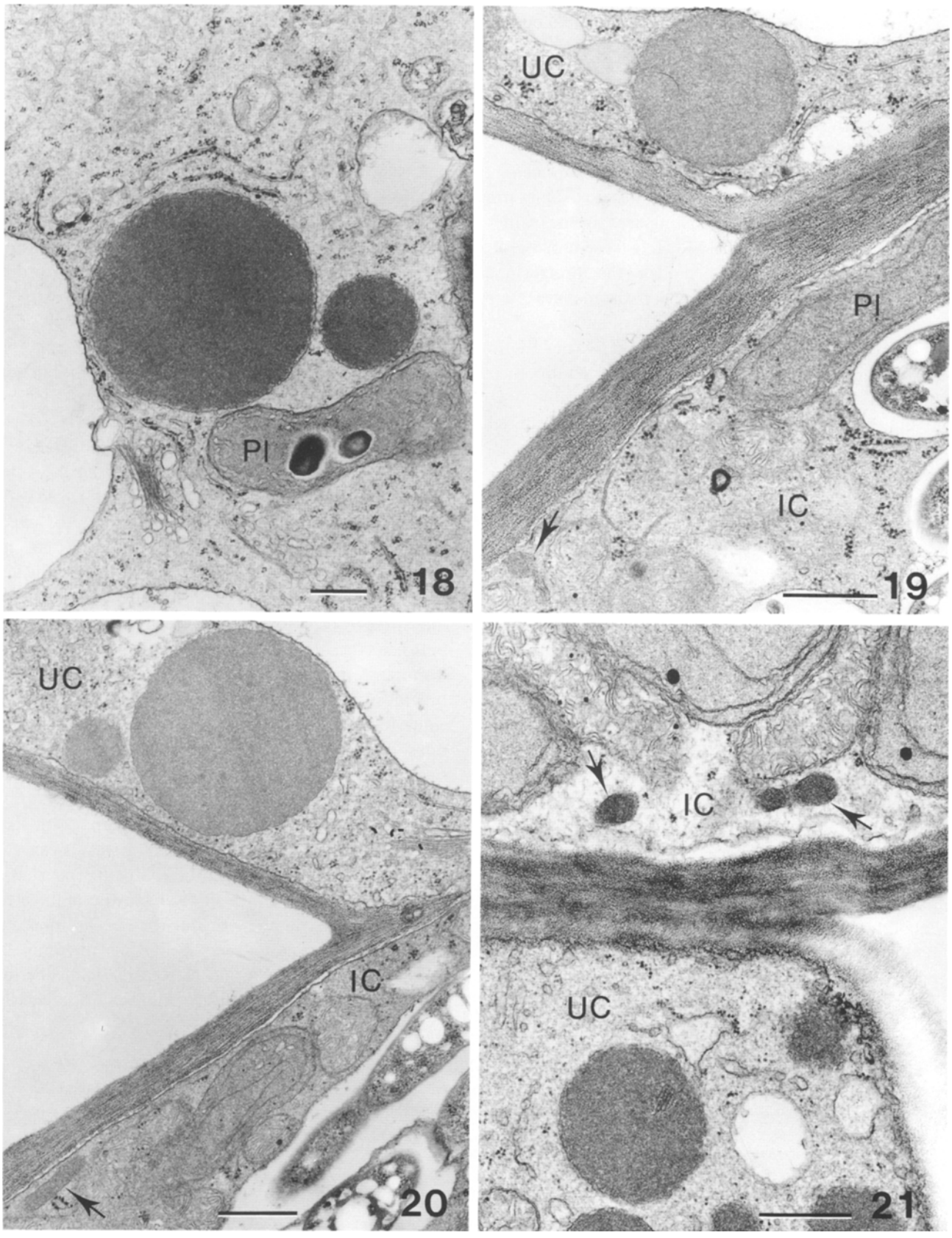
Figs. 13–17. Scale bars = 0.5 μm. Continuation of series illustrating peroxisome development in uninfected cells in the infected region of soybean root nodules. Nodule segments treated with DAB plus urate. The matrix heavily stained for uricase activity now nearly fills the peroxisomes. Sacs and tubules associated with the peroxisomes are now more compact and the contents are more condensed and more intensely stained than in Figs. 6–12.

The sac in Fig. 13 is connected (arrow) to the peroxisome. Fig. 16 is unusual in containing stained material in a flattened ER cisterna rather than in an irregularly shaped sac.

Fig. 13 × 34,000. Fig. 14. × 46,000. Fig. 15. × 43,000. Fig. 16. × 39,000

Fig. 17. Late stage in the development of an uninfected cell in the infected region of a soybean root nodule. The nodule sections were incubated with DAB plus urate. The matrix of the enlarged and now spherical peroxisomes has reacted strongly in the test for uricase activity. *IC* infected cell, *P* peroxisome, *Pl* plastid, *St* starch, *UC* uninfected cell. × 17,000



Figs. 18–21

indicates the presence of uricase. Lack of labeling in the immunogold procedure may simply reflect the comparatively low sensitivity of this approach under the conditions employed. When DAB is used to demonstrate the presence of catalase in the organelle, the results are equivocal, and indicate only low activity at best. However, we conclude, on balance, that the bodies should be considered peroxisomes.

The cytochemical methods we employed are dependent on enzymatic reactions to bring about staining on the ultrastructural level, and provide information of a different sort than do immunocytochemical techniques, whose specific immunological reactions are dependent on the recognition of particular antigenic sites (WACHSMUTH 1980). Furthermore, tissue treatment preceding the tests for enzymatic activity is much less drastic than the dehydration and embedment in plastic that usually precedes testing for antigenicity. Whereas immunogold labeling, utilizing antiserum against a purified subunit of nodule-specific uricase, detected antigenicity only in the matrix of peroxisomes in uninfected cells (VANDENBOSCH and NEWCOMB 1985), both cytochemical methods detected uricase activity also in sacs associated with enlarging peroxisomes of uninfected cells, and the $CeCl_3$ method detected uricase in the small peroxisomes of infected cells. Thus the cytochemical methods appear in these cases to have been considerably more sensitive in indicating the presence of uricase.

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Figs. 18–21. Scale bars = 0.5 μm

Fig. 18. Portion of uninfected cell of soybean root nodule tissue incubated with $CeCl_3$ and urate. The peroxisomes have been stained positively for uricase activity. *Pl* plastid. × 20,000

Fig. 19. Control: incubation of soybean root nodule tissue with $CeCl_3$ in the absence of urate. No electron opaque deposition has taken place in the large peroxisome of the uninfected cell (*UC*) or the small peroxisome (arrow) in the infected cell (*IC*). *Pl* plastid. × 34,000

Fig. 20. Control: incubation of soybean root nodule tissue with $CeCl_3$ and urate in the presence of allopurinol. Deposition of electron dense reaction product has been greatly reduced, both in the peroxisomes of the uninfected cell (*UC*) and in the small peroxisome (arrow) of the infected cell (*IC*). × 28,000

Fig. 21. Portions of an infected cell (*IC*) and an uninfected cell (*UC*) in soybean root nodule tissue incubated with $CeCl_3$ and urate. The small peroxisomes (arrows) in the infected cell have been stained, indicating uricase activity, as have the peroxisomes in the uninfected cell. × 33,000

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