

Nile red fluorescence demonstrates lipid in the envelope of vesicles from N₂-fixing cultures of *Frankia*

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Using Nile red as a fluorescent stain for lipid, we investigated the composition of the envelope of vesicles of *Frankia* strain HFPCc13 from cultures induced in N-free medium at 1 and 20 kPa O₂. Vesicles and nitrogenase activity appeared in the cultures at both pO₂; on average, vesicles viewed by dark-field microscopy were larger and had thicker envelopes at 20 kPa O₂ than at 1 kPa O₂. Envelopes of Nile red-stained vesicles fluoresced red under incident green light. When samples of Cc13 were extracted through a lipid-solvent series and then stained, vesicles still fluoresced red but lacked a distinct peripheral fluorescent ring. These results are consistent with the view that the envelope of *Frankia* vesicles consists largely of lipid and serves as a barrier to diffusion of O₂.

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Une investigation portant sur l'enveloppe des vésicules de la souche HFPCc13 de *Frankia* a été entreprise à l'aide du rouge de Nil, un colorant fluorescent des lipides, et ce, à partir de cultures induites sur un milieu libre d'azote mais en présence d'O₂ à raison de 1 et de 20 kPa. Les vésicules ainsi que l'activité nitrogénase sont apparues dans les cultures sous les deux pressions d'O₂. Les vésicules soumises à la microscopie en fond noir ont présenté des enveloppes plus larges et plus épaisses sous 20 kPa d'O₂. Les enveloppes des vésicules colorées au rouge de Nil ont produit une fluorescence rouge sous une lumière incidente verte. Lorsque des échantillons de Cc13 furent retirés d'une série de solvants des lipides et colorés, les vésicules ont encore produit une fluorescence rouge, mais il leur manquait un anneau périphérique distinct fluorescent. Ces résultats concordent avec le point de vue que l'enveloppe des vésicules de *Frankia* est largement constituée de lipides qui servent de barrière à la diffusion de l'O₂.

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Introduction

Extreme sensitivity of nitrogenase to O₂ seems to be a law of nature for N₂-fixing bacteria (Postgate 1982). *Frankia*, the N₂-fixing actinomycete symbiont of root nodules of numerous woody plants, has evolved swollen multicellular hyphal ends termed vesicles wherein nitrogenase is protected by a multilaminar envelope that impedes diffusion of O₂ (Torrey and Callahan 1982; Murry *et al.* 1984, 1985; Noridge and Benson 1986; Parsons *et al.* 1987). In many strains of *Frankia* grown *in vitro*, vesicles develop when aerated cultures with an adequate C supply are deprived of utilizable combined N (Tjepkema *et al.* 1980, 1981; Fontaine *et al.* 1984). In *Frankia* strain HFPCc13 the thickness and the number of laminae of the vesicle envelope increase as N₂-fixing cultures adapt to increases of ambient pO₂ (Parsons *et al.* 1987).

Torrey and Callahan (1982) discussed the similarities between the multilaminar envelope of *Frankia* vesicles and that of heterocysts of certain N₂-fixing cyanobacteria (Haselkorn 1978). They cited the "strong evidence that the laminated layers in heterocysts of cyanobacteria are made of glycolipids and that these glycolipid layers serve to limit gaseous exchange into and out of the heterocyst, site of the enzyme nitrogenase" (Torrey and Callahan 1982). Since, however, the multilaminar envelope of *Frankia* vesicles has not been chemically analyzed, its lipid nature remains likely rather than certain. We tested for the presence of lipid in *Frankia* vesicles by using Nile red, a fluorescent, hydrophobic

benzophenoxazinone dye whose spectrofluorometric and staining properties were recently described (Fowler and Greenspan 1985; Greenspan and Fowler 1985; Greenspan *et al.* 1985). Greenspan *et al.* (1985) showed that Nile red selectively dissolves and fluoresces in intracellular lipid droplets of cultured animal cells. We hoped that the dye would selectively stain the vesicle envelope in N₂-fixing cultures of *Frankia* strain HFPCc13, and thereby confirm the presence of lipid.

Materials and methods

The *Frankia* strain used was HFPCc13 (Catalog no. HFP020203, referred to here as Cc13) isolated from root nodules of *Casuarina cunningghamiana* Miq. (Zhang *et al.* 1984). The isolate was maintained at 27°C in 50 mL of NH₄⁺-containing culture medium (BAP medium of Murry *et al.* 1984, with 5 mM sodium pyruvate as C source) in 250-mL flasks on a gyratory shaker operating at 90 rpm. Induction of vesicles was initiated by harvesting 14-day-old BAP-grown mycelium by centrifugation, washing twice with N-free medium, and finally resuspending in N-free medium (B medium of Murry *et al.* 1984, with 10 mM sodium pyruvate as C source) and incubating as before.

Growth of 100-mL N-free cultures of varying pO₂ was conducted in 1-L canning (preserving) jars with gas-tight lids containing a septum seal. Gas exchange was effected by purging each jar for 10 min with the approximate gas mixture filtered through two in-line sterile 0.22-μm membrane filters. The pO₂ of each jar was measured daily by taking a 0.1-mL sample in a sterile syringe and analyzing it by gas chromatography.

Acetylene reduction assays were used to determine nitrogenase activity. At each assay time, duplicate 3.5-mL septum-capped tubes were flushed with the appropriate gas mixture and acetylene was added as substrate to provide 10 kPa. A 1-mL aliquot of culture was taken with a sterile syringe and injected into an assay vial; after 30 min 0.1 mL of gas was drawn for analysis of ethylene. The gas sample

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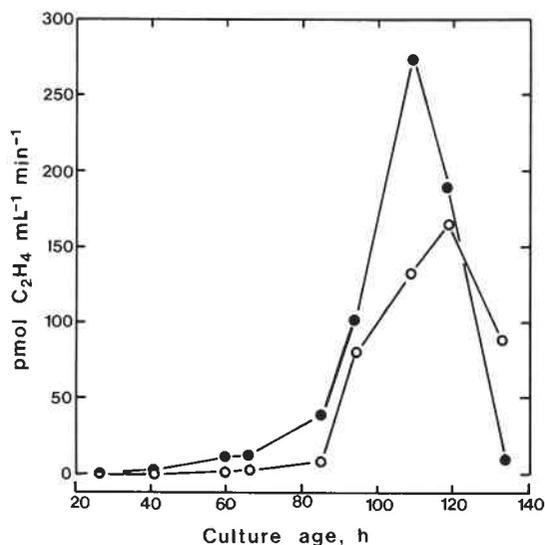


FIG. 1. Time dependence of acetylene reduction rate (nitrogenase activity) of *Frankia* strain HFPCc13 following transfer from NH_4^+ -containing medium to N-free medium at 1 kPa O_2 (●) or at 20 kPa O_2 (○).

was injected into a Carle 9500 gas chromatograph equipped with a 1.0-m Porapak T column and a flame ionization detector. All assay results are expressed as $\text{pmol C}_2\text{H}_4 \text{ mL}^{-1} \text{ min}^{-1}$.

Culture samples were fixed, following centrifugation, by adding to the pellet 1 mL of 3% glutaraldehyde (from 50% glutaraldehyde, Fisher Scientific) in 25 mM potassium phosphate, pH 6.9. Fixation times varied from 2 h to 10 weeks; after 24 h, fixing vials were transferred from room temperature to 4°C. For observation or further processing the fixed mycelium was washed twice with distilled water.

Nile red (Eastman Kodak Co., Rochester, NY) was dissolved in acetone at 1 mg mL^{-1} to make a stock solution that could be refrigerated or frozen. The staining solution proper contained Nile red, 5 $\mu\text{g mL}^{-1}$, in 50% aqueous glycerol. Following centrifugation, a pellet of fixed *Frankia* was resuspended in 1 mL of Nile red staining solution. After 1 h the suspension was concentrated to about 0.1 mL by centrifugation, and a drop of the concentrate, still in staining solution, was mounted between alcohol-cleaned slide and cover slip. Photomicrography was done no less than 12 h later to permit substantial evaporation of water from the mounting solution.

Half of the 118-h sample from 20 kPa O_2 was extracted with lipid solvents before being stained. The solvent sequence was 50% isopropanol (aqueous), 100% isopropanol (1 h), hexane/isopropanol::3/2 (v/v, 1 h), 100% isopropanol, 50% isopropanol (aqueous), distilled water. For part of the material hexane/isopropanol was omitted from the sequence so that the least polar solvent was 100% isopropanol. The extracted mycelium was stained with Nile red and mounted as described above.

The stained mycelium was examined and photographed both by transmitted-light dark-field microscopy and by incident-light fluorescence microscopy, using a Leitz Ortholux equipped with a dark-field condenser, a Ploem-type vertical illuminator, and a Zeiss oil-immersion objective. For fluorescence microscopy an HBO 100 W mercury lamp was used with excitation filters BG 38, Baird interference 377-548 nm, and Zeiss wide-band interference 535 nm, the 580-nm dichroic beam splitter, and the 610-nm barrier filter.

Results

Nitrogenase activity, measured as the rate of acetylene reduction, was observed in Cc13 cultures grown without combined N at both 1 and 20 kPa O_2 , but arose first in the 1 kPa O_2 culture (Fig. 1) when vesicles were still small and only thinly enveloped (Fig. 2). Activity appeared 40 to 60 h after

introduction of N-free medium (Fig. 1). Vesicles examined at any given sampling time were larger and had thicker light-scattering envelopes, on the average at 20 kPa O_2 (Figs. 8, 10, 12) than at 1 kPa O_2 (Figs. 2, 4, 6), confirming the findings of Parsons *et al.* (1987). The light-scattering envelope extended around each vesicle and down its stalk, at least to the first septum.

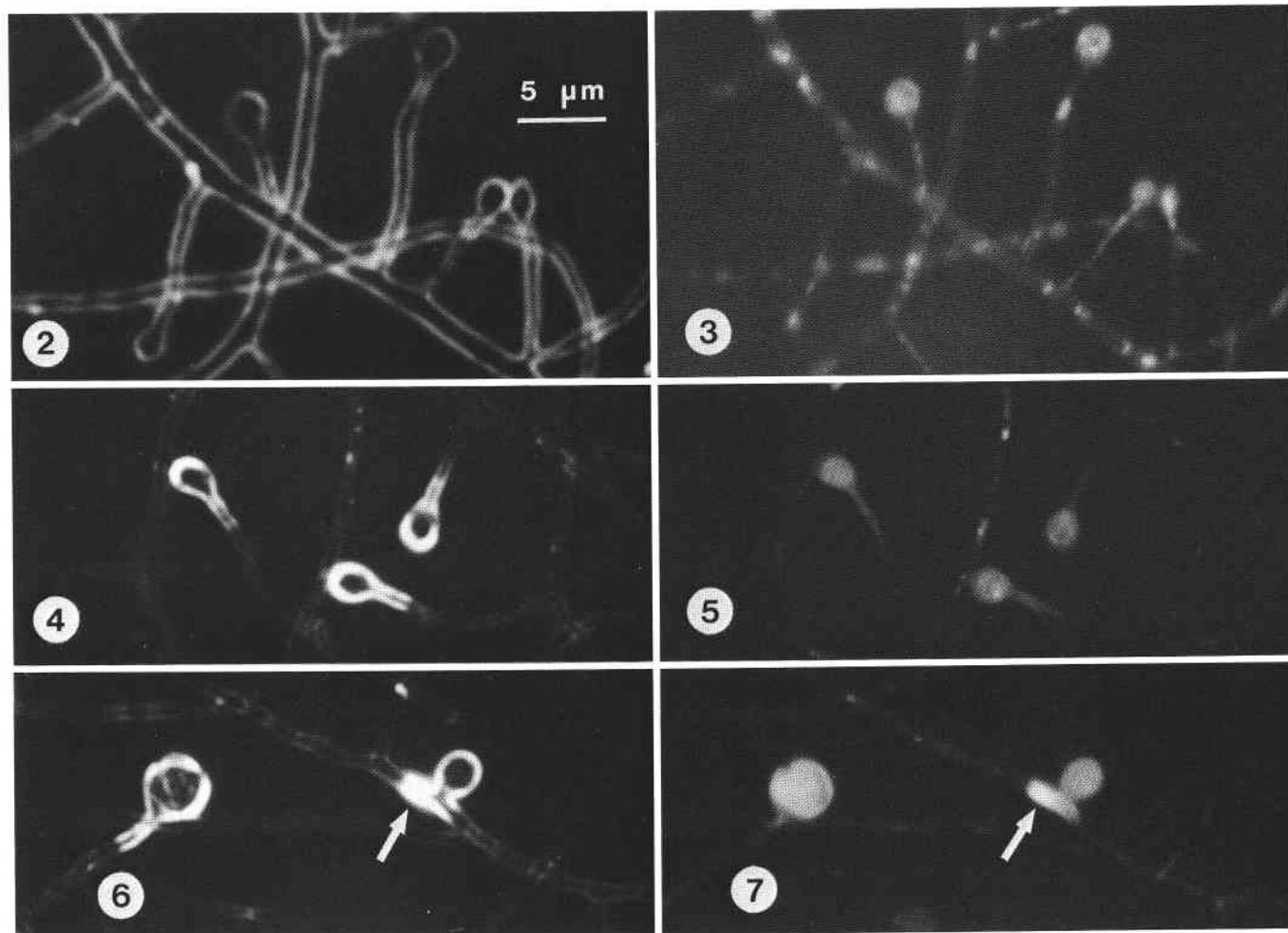
Nile red-stained vesicles, when excited with incident green light, fluoresced red (Figs. 3, 5, 7, 9, 11, 13, 15, 17). Mature vesicles were most intensely fluorescent, a peripheral ring up to 0.5 μm thick usually being somewhat brighter than the remaining central area. The ring of fluorescence occupied the same position as, but was always thinner than, the scattered-light ring observed by transmitted-light dark-field microscopy (Figs. 8-13). Fluorescence usually extended into the light-scattering vesicle stalk (Figs. 7, 11). There was no red autofluorescence of unstained vesicles excited with green light (but substantial yellow auto-fluorescence was produced with blue excitation).

When the fixed 118-h Cc13 samples from 20 kPa O_2 were extracted through a solvent series terminating with either isopropanol or hexane/isopropanol::3/2, and then stained with Nile red, mature vesicles still fluoresced red but lacked a distinct peripheral fluorescent ring and fluorescent stalk (Figs. 15, 17). In dark-field images of extracted mature vesicles a ragged scattered-light ring remained, borne, as in the fluorescence images, on an almost invisible stalk (Figs. 14, 16). Extraction with acetone produced essentially identical changes (not shown).

A major source of uncertainty in interpretation of the microscopic images is the fact that the fixed mycelium was whole mounted. The scattered-light image of the vesicle envelope, being generated by a partially out-of-focus, vertically curved shell of (presumably) high refractive index, was artifactually thick and varied in thickness depending upon which dark-field condenser, which objective, and which objective aperture was chosen; once such a choice was made, and then strictly adhered to, all the scattered-light images based on that particular choice were consistent with each other. By comparison, the fluorescence image of Nile red-stained vesicles was quite sharp and insensitive (in quality, not intensity) to changes of oil-immersion objective and objective aperture; however, since the vertical position of fluorescent material could not be defined, it is possible that Nile red in the envelope generated at least some of the overall fluorescence of each vesicle.

Discussion

Members of the Actinomycetales are characterized in part by their unique lipid, especially phospholipid, constituents (Lechevalier *et al.* 1982). *Frankia* is characterized by the presence of type I phospholipids (Lechevalier *et al.* 1982; Lopez *et al.* 1983). The phospholipids found in *Frankia* cultures are remarkably constant with respect to variations of nutrient medium, culture age, and morphogenetic state (Lopez *et al.* 1983). These lipids are presumed to be components of bacterial cell membranes or of storage inclusions. In the Nile red-stained preparations of *Frankia*, both diffuse and particulate fluorescent material in vegetative hyphae (e.g., Figs. 3, 5) presumably represents the typical polar lipids referred to above. An important question is whether the Nile red-stained material associated with the vesicle envelope is an unusual lipid or belongs to the type I phospholipids charac-



FIGS. 2-7. Paired dark-field (left) and Nile red fluorescence (right) images of *Frankia* strain HFPCc13 at different times following transfer from NH_4^+ -containing medium to N-free medium at 1 kPa O_2 . Figs. 2, 3: 47 h. Figs. 4, 5: 67 h. Figs. 6, 7: 118 h. In Fig. 6 there is a strongly light-scattering hyphal wall segment (arrow) subjacent to a developing vesicle; in Fig. 7 the same wall segment (arrow) exhibits strong Nile red fluorescence.

teristic of the genus. Chemical analysis of lipids extracted from vesicle-enriched cultures grown under raised $p\text{O}_2$ should make possible the distinction between the constitutive lipids and specialized lipids associated with the vesicle envelope (analogous to those of cyanobacterial heterocysts).

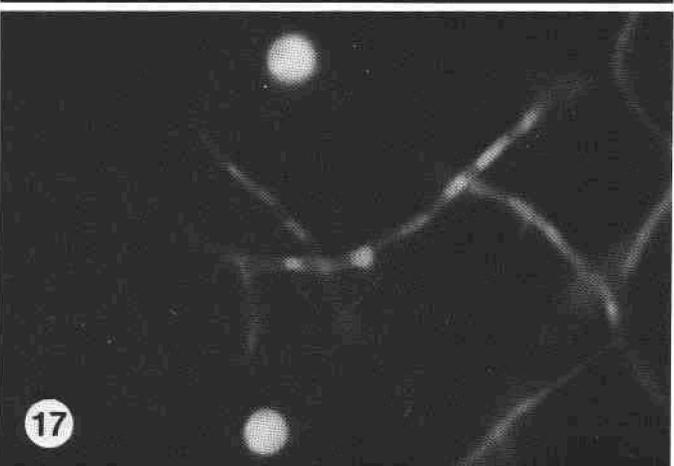
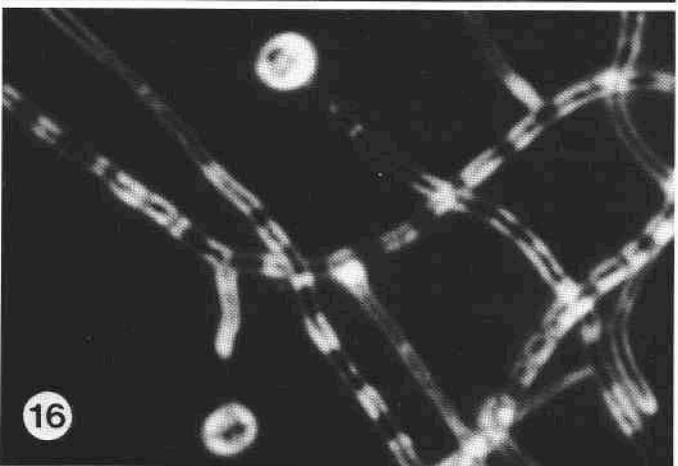
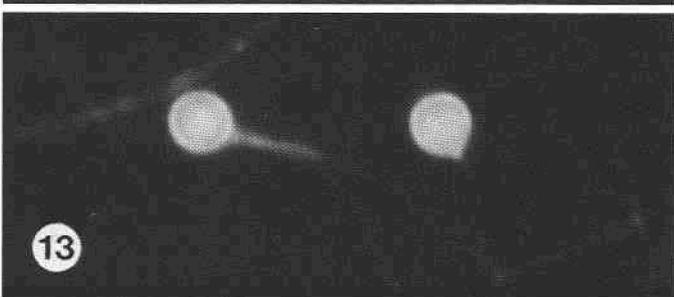
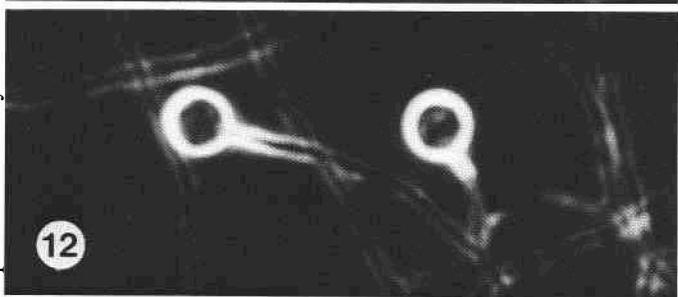
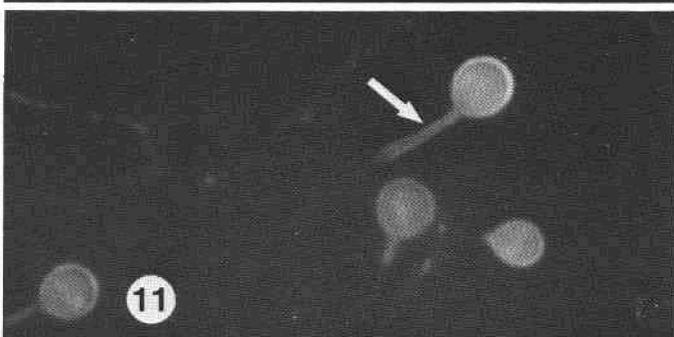
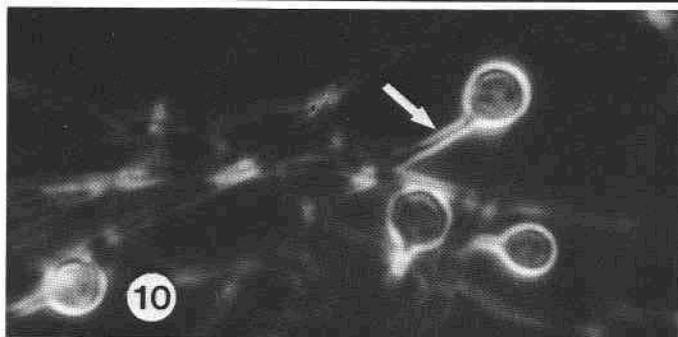
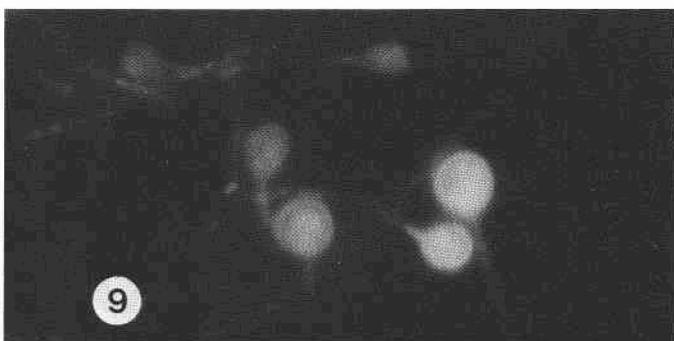
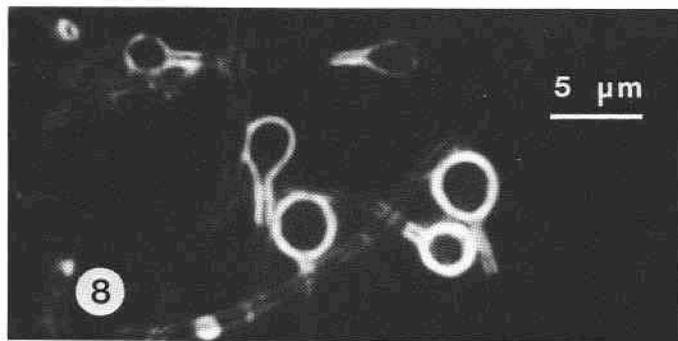
The results reported here, based on the use of the dye Nile red as a fluorescent hydrophobic probe for lipid, are consistent with the view of Torrey and Callaham (1982) that the envelope of *Frankia* vesicles consists largely of lipid and serves as a diffusion barrier protecting nitrogenase against O_2 . Shown by Parsons *et al.* (1987) to be light-scattering under dark-field illumination, the vesicle envelope proved also to be stainable with Nile red. We observed that the peripheral fluorescent ring of Nile red-stained mature vesicles was no longer visible after exposure of fixed mycelium to even a relatively polar solvent, namely isopropanol.

In mature vesicles Nile red staining was not restricted to the peripheral ring; the central area was also stained, usually less

intensely than the periphery. Moreover, the central area remained stainable after extraction with lipid solvents. Our evidence does not exclude the possibility that the lipid solvents caused the envelope to collapse inward rather than to dissolve. Nile red being a hydrophobic rather than a lipid-specific dye (Greenspan and Fowler 1985, Table 4), we suggest that the solvent-resistant, stainable components of the vesicle are hydrophobic domains of intracellular proteins and inclusions.

Although in *Casuarina* root nodules Cc13 fixes N_2 without forming vesicles (Zhang and Torrey 1985), in pure culture its capacity to fix N_2 at $p\text{O}_2$ greater than 0.3 kPa depends upon induction of vesicles (Murry *et al.* 1985). Protection of vesicle nitrogenase against O_2 in pure culture has been attributed to the laminate envelope acting as a passive barrier to diffusion of dissolved O_2 (Torrey and Callaham 1982; Murry *et al.* 1985; Parsons *et al.* 1987). The evidence for lipid being a major component of the envelope is not merely the envelope's laminate structure in frozen-fractured material, but also its

FIGS. 8-17. Paired dark-field (left) and Nile red fluorescence (right) images of *Frankia* strain HFPCc13 at different times following transfer from NH_4^+ -containing medium to N-free medium at 20 kPa O_2 . Figs. 8, 9: 47 h. Figs. 10, 11: 67 h. The uppermost vesicle of Figs. 10 and 11 illustrates close correspondence between the scattered-light and Nile red images of the outer wall, in both the vesicle body and the stalk (arrows). Figs. 12-17: 118 h. Figs. 14, 15: mycelium extracted with isopropanol. Figs. 16, 17: mycelium extracted with hexane-isopropanol (3:2). After extraction, the vesicle stalk no longer scatters light, the peripheral scattered-light ring of the vesicle body has become patchy in form, and the peripheral Nile red fluorescence ring of the vesicle body is no longer distinguishable.



apparent dissolution by lipid solvents commonly used to dehydrate fixed specimens for embedding and sectioning (Torrey and Callahan 1982; Lancelle *et al.* 1985). A predominantly lipid envelope could be expected to have a refractive index above 1.43 (Lange 1956), which together with the envelope's laminar inhomogeneity would cause it to scatter light in aqueous media, as has been observed (Parsons *et al.* 1987).

In any case, the present evidence strongly supports the view that the vesicle envelope, which is progressively elaborated in N₂-fixing *Frankia* grown under increasing pO₂, consists of lipids arranged in a multilaminate structure. Its chemical nature and final proof of its role as a physical barrier to direct entry of O₂ into the vesicle remain to be demonstrated.

Acknowledgements

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