200 g/cm² and kept at +4°C for 4 days prior to development.

In a typical autoradiograph (Fig. 2) one can easily identify in each hemisphere retina (*R*) and lamina (*La*) (displaced on right half), medulla (*M*), lobula (*Lo*), and lobula plate (*LP*). [For an anatomical review of the dipteran brain see (*7*).] In the medulla of the right eye, the portion stimulated by the moving grating was heavily labeled, whereas the left medulla displayed a comparatively homogeneous labeling of the various layers throughout flicker-stimulated and nonstimulated portions. No clear differences could be detected between stimulated and nonstimulated regions in retina and lamina on either side.

This distribution of radioactive label qualitatively prevails in six sections of the animal of Fig. 2; it was also visible in four sections of another animal treated identically and in five sections from two animals stimulated for 14 hours. Only these four flies have been subjected to the procedure so far. Autoradiographs of unlabeled material showed negligible traces of chemography and pressure artifacts. We interpret our results as indicating that (i) the DG method may be successfully applied to insect nervous systems, (ii) there is distinct movement-specific activity in the medulla of *Drosophila*, and (iii) nonspiking cells such as receptors and monopolar cells in the lamina may be refractory to the DG method as we currently carry it out. We suggest that the movement-specific label in the medulla may perhaps originate from columnar cells corresponding to "elementary movement detectors," which have been hypothesized on the basis of behavioral experiments with *Drosophila* (*5*). Any further interpretation of the distribution of label in Fig. 2 must remain speculative. If the spatial resolution of about 5 to 10 μm obtained with the technique can be improved so the amount of label in individual cells may be quantitatively evaluated (*8*), it should be possible to clarify whether active brain regions are labeled as a result of enrichment of DG in glial cells or extracellular spaces or whether active neurons themselves show preferential uptake. In the latter case the method may prove useful for investigating the circuitry of identified neurons in invertebrate nervous systems.

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Cell-Free Nitrogenase and Hydrogenase from Actinorhizal Root Nodules

Abstract. *Field-grown alder (*Alnus glutinosa*) root nodules were disrupted in liquid nitrogen to release the actinomycete endophytes. The endophytes were broken by mild sonic oscillation and yielded a cell-free nitrogenase preparation capable of reducing acetylene and protons. In addition, the preparation carried a cell-free uptake hydrogenase.*

Certain nonleguminous plants form perennial nitrogen-fixing root nodules in symbiosis with actinomycetes (*1*). This association was designated actinorhizal at a recent symposium, held at Harvard Forest, Petersham, Massachusetts, on actinomycete-nodulated plants. Such nodulated plants are often pioneers in nutrient-poor soils and participate in soil enrichment early in some ecological successions (*2*). They serve in forest restoration or land reclamation that requires long-term nitrogen input (*2*). Recent isolation of the nitrogen-fixing prokaryotic symbiont and confirmation that it is an actinomycete (*3*) have stimulated a resurgence of interest in the actinorhizal nodules.

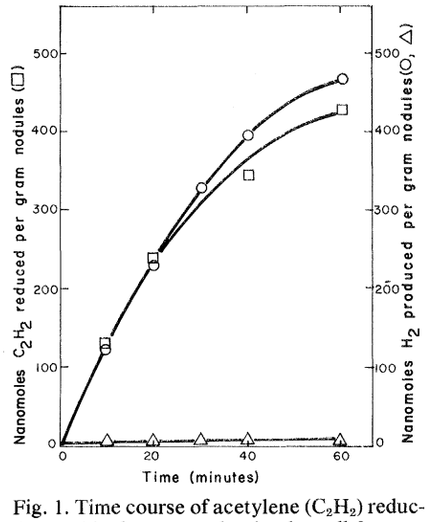

Fig. 1. Time course of acetylene (C_2H_2) reduction and hydrogen production by cell-free extracts from *Alnus glutinosa* root nodules. The assays were performed in 21-ml vaccine bottles at 30°C. The reaction mixture contained the same concentration of reactants as indicated in Table 1. The reaction was initiated by the addition of 1 ml of crude extract to give the reaction mixture a total volume of 2 ml. The gas phase consisted of 10 percent acetylene in argon for C_2H_2 reduction assays; C_2H_2 was omitted when hydrogen production was measured. Hydrogen was measured by gas chromatography with a thermal conductivity detector. Hydrogen production: ATP added (○); ATP omitted (△); C_2H_2 reduction (□).

There has been little biochemical study of the nodules, primarily because of the difficulties inherent in working with woody tissue containing an abundance of reactive phenolic substances that inactivate enzymes when cells are broken (*4*). Methods have been described for preparing particulate acetylene-reducing homogenates from actinorhizal nodules (*5*, *6*); however, there are no reports that enzymatically active cell-free extracts have been recovered. We report a technique for preparation of active cell-free nitrogenase and of an "uptake hydrogenase" from field-grown nodules of the European alder *Alnus glutinosa* L. Gaertn. The requirements for nitrogenase activity are similar to those for other nitrogen-fixing systems, again illustrating the highly conserved nature of the nitrogenase system among widely diverse prokaryotes.

Alnus glutinosa root nodules were collected from the University of Wisconsin Arboretum during the fall of 1978. Excised nodules were washed in 100 m*M* phosphate buffer containing 20 m*M* ascorbate at *p*H 7.0, and were stored frozen in liquid nitrogen. To release the endophyte, nodule samples (usually 5 g) were crushed to a fine powder in liquid

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nitrogen (*6*). After the liquid nitrogen had boiled off, the powdered nodules were suspended and centrifuged four times in 100 m*M* potassium phosphate buffer, *p*H 7.4, containing 10 m*M* sodium dithionite. The washing medium was changed to 20 m*M* tris-HCl, *p*H 7.2, with 20 m*M* ascorbate and 2 m*M* dithionite, and the homogenate was washed four additional times. Repeated washings freed the homogenate from most of the inhibitory phenolic compounds released during homogenization. Nitrogenase (C_2H_2 reduction) activity was stable even when degassed buffer alone was used in the later washings. This indicates that a high dithionite concentration is not needed in late washings to retain activity; it is needed for detecting activity in particulate homogenates (*5*). Microscopic examination of the homogenate showed many released vesicle and hyphal clusters of the actinomycete, together with large and small undisrupted clumps of nodule cells that often contained the unreleased endophyte.

For the preparation of cell-free extracts, the sedimented material from the washed homogenate was resuspended in twice its volume of the second washing medium, mixed with an equal weight of solid polyvinylpolypyrrolidone (PVP) (Sigma), and sonicated for 1.5 to 2.0 minutes. It is important that the sonication be mild, as prolonged sonication rapidly inactivates C_2H_2-reducing activity. The sonicated material was centrifuged at 20,000*g* for 20 minutes; microscopic examination of the supernatant revealed no intact organisms. The supernatant had an initial activity from 500 to 1000 nmole of acetylene reduced per gram of nodules per hour, or about half the activity of the particulate homogenates. However, the cell-free enzyme is somewhat unstable after isolation, as was shown by the nonlinear time course (Fig. 1); the C_2H_2-reducing activity was reduced by half during 1 hour at 30°C.

The cell-free nitrogenase from alder nodules required adenosine triphosphate (ATP), Mg^{2+}, and sodium dithionite to reduce acetylene (Table 1). Furthermore, the activity was lost upon exposure to oxygen and during boiling for 5 minutes. The ATP-generating system, including creatine phosphate and creatine phosphokinase, was required for maximum activity and did not inhibit activity as reported for crude homogenates (*6*). The addition of 0.5 m*M* Mn^{2+} and the activating factor from *Rhodospirillum rubrum* (*7*) did not enhance activity, evidence that a third component is not necessary for the functioning of the alder actinomycete nitrogenase. Thus, the requirements for activity by alder nitrogenase are much the same as those for other nitrogen-fixing prokaryotes.

Table 1. Requirements for acetylene (C_2H_2) reduction by alder endophyte nitrogenase. Assays were performed in 9-ml serum bottles at 30°C. The complete reaction mixture contained 5 m*M* ATP, 10 m*M* Mg^{2+}, 30 m*M* creatine phosphate, 0.05 mg of creatine phosphokinase, 20 m*M* $Na_2S_2O_4$, 25 m*M* Hepes, and 10 m*M* tris buffer, *p*H 7.4. Assays were initiated by addition of 0.5 ml of the crude extract to give a total reaction volume of 1 ml. The gas phase consisted of 90 percent argon and 10 percent acetylene generated from calcium carbide. Ethylene was measured by gas chromatography with a flame-ionization detector. Activity is based on fresh nodule weight because plant phenolics interfere with protein determinations. In the minus dithionite test, residual dithionite was first oxidized by methylene blue or 2-hydroxy-1,4-naphthoquinone; the assay reaction was allowed to proceed for 10 minutes, and then an excess of dithionite was introduced. Approximately 10 percent of the original activity was recovered after this treatment.

Assay conditions	Initial rate*
Complete assay mix	811
Minus ATP	0
Minus Mg^{2+}	0
Minus creatine phosphate	500
Minus creatine phosphokinase	509
Minus dithionite	0

*Nanomoles of ethylene per gram (fresh weight of tissue) per hour.

Nitrogenase reduces protons to hydrogen in an ATP-dependent reaction. Hydrogen production was detected in the crude homogenates and in the cell-free extracts; H_2 evolution was ATP-dependent, and occurred at a rate comparable to the rate of C_2H_2 reduction (Fig. 1).

In contrast to many legume root nodules, and some free-living microorganisms that fix nitrogen (*8*), all actinorhizal nodules examined have evolved little H_2 (*9*). Because we had shown that actinorhizal nitrogenase produces H_2, we looked for a highly active or tightly coupled uptake hydrogenase in the system and found H_2 uptake in cell-free extracts from the alder nodules (*10*). The relation between the uptake hydrogenase and ATP-dependent H_2 production by nitrogenase in the actinorhizal nodules is of interest, because part of the ATP expended for H_2 production (*9*) can be recovered by coupled hydrogenase-catalyzed reoxidation of H_2.

In the preparation of cell-free nitrogenase from actinorhizal plants, the problems caused by phenolics have been partly overcome by repeatedly washing the homogenate particles and by including PVP during sonication. The high concentrations of dithionite used previously to prepare homogenates (*5*, *6*) are not necessary throughout the washing process to retain activity, but dithionite is needed as reductant in demonstrating activity. The requirements for activity and the presence of ATP-dependent H_2 evolution demonstrate the similarity of the alder nitrogenase to other nitrogenase systems. Our experiments have shown some cross-reactivity of the alder nitrogenase system with the components from *Azotobacter vinelandii*, and this confirms the homology preserved in the evolutionary development of nitrogenases. Although work with the actinorhizal nodules is less convenient than work with some other nitrogenases, isolation of an active cell-free nitrogenase system from them opens the way to further biochemical investigations.

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