Isolation and Cultivation in vitro of the Actinomycete Causing Root Nodulation in Comptonia

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subjected to electron ionization (EI) mass spectrometry. Synthetic bityrosine EI; 772 (M+), 713 ($M^+ - COOCH_3$), 676, 659 ($M^+ - NH_2COCF_3$), 653 ($M^+ - COOCH_3 - HCOOCH_3$), 627 ($M^+ - NH_2COCF_3 - CH_3OH$), 563, 546 ($M^+ - 2\ NH_2COCF_3$), 492 ($M^+ - COCF_3 - CH_3OCOCHNCOCF_3$) Peak 4 EI; 659, 627, 563, 546, 492.

Because of insufficient amounts of material for EI mass spectroscopy, the molecular ion and some of the major high-molecular-weight fragments seen in the synthetic bityrosine could not be detected in peak 4. However, no other fragments appear in the 490 to 700 *m/e* (mass to charge) region of peak 4 other than those present in the spectrum of the synthetic compounds. The data clearly support the conclusion that peak 4 is 3,3-bityrosine.

The only methods reported for the synthesis of bityrosine utilize either photolysis under anaerobic conditions (*15*) or hydrogen peroxide in the presence of peroxidase (*10, 14, 17*). Both of these routes of synthesis may be available to the lens.

It has been suggested that covalent cross links may exist between polypeptide chains in the old and cataractous human lens. Bityrosine can serve as such a cross link. However the linkage could also be within the same polypeptide chain or with a free tyrosine. It is not possible from the present observations to ascertain which of these situations describe the linkage in the lens protein nor has this problem been elucidated with any other native protein where such structures have been observed.

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References and Notes

1. K. Satoh, M. Bando, A. Nakajima, *Exp. Eye Res.* **16**, 167 (1973); K. J. Dilley and A. Pirie, *ibid.* **19**, 59 (1974).
2. A. Spector, D. Roy, J. Stauffer, *ibid.* **21**, 9 (1975).
3. R. Jacobs and D. L. Krohn, *J. Gerontol.* **31**, 641 (1976).
4. R. C. Augusteyn, *Ophthalmic Res.* **7**, 217 (1975).
5. A. Spector, J. Stauffer, J. Sigelman, *Ciba Found. Symp.* **19** (new series), 185 (1973); J. A. Jedziniak, J. H. Kinoshita, E. M. Yates, L. O. Hocker, G. B. Benedek, *Exp. Eye Res.* **15**, 185 (1973); A. Spector, S. Li, J. Siegelman, *Invest. Ophthalmol.* **13**, 795 (1974).
6. J. Dillon, A. Spector, K. Nakanishi, *Nature (London)* **259**, 422–423 (1976).
7. S. Garcia-Castineiras, J. Dillon, A. Spector, in preparation.
8. R. W. Cowgill, unpublished (presented at ARVO spring meeting 1974).
9. S. O. Andersen, *Biochim. Biophys. Acta* **93**, 213 (1964).
10. F. LaBella, P. Waykole, G. Queen, *Biochem. Biophys. Res. Commun.* **30**, 333 (1968).
11. P. Waykole and E. Heidemann, *Connect. Tissue Res.* **4**, 219 (1976).
12. M. Brenner, A. Niederwieser, G. Pataki, in *Thin-Layer Chromatography. A Laboratory Handbook*, E. Stahl, Ed. (Springer-Verlag, Berlin, 1969), p. 730.
13. H. Matsubara and R. M. Sasaki, *Biochem. Biophys. Res. Commun.* **35**, 175 (1969).
14. A. J. Gross and J. W. Sizer, *J. Biol. Chem.* **234**, 1611 (1959).
15. S. S. Lehrer and G. D. Fasman, *Biochemistry* **6**, 757 (1967).
16. P. A. Cruickshank and J. C. Sheehan, *Anal. Chem.* **36**, 1191 (1964).
17. R. Aeschbach, R. Amado, H. Neukom, *Biochim. Biophys. Acta* **439**, 292 (1976).
18. Supported by the National Eye Institute, NIH. S.G.C. and J.D. are national research service postdoctoral fellows of the National Eye Institute. We thank G. Jenkins and D. Adams for help and the Mass Spectrometer Section of the Columbia University Chemistry Department for technical assistance.
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Isolation and Cultivation in vitro of the Actinomycete Causing Root Nodulation in *Comptonia*

Abstract. *The soil actinomycete causing formation of nitrogen-fixing symbiotic nodules on roots of the woody angiosperm* Comptonia peregrina *(L.) Coult. (Myricaceae) has been isolated from surface-sterilized root nodules after incubation and enzyme maceration. The filamentous bacterium grows slowly in pure culture on a yeast extract medium, producing sporogenous bodies which form large numbers of ovoid spores. Reinfection of sand-grown or aeroponically grown seedlings of* Comptonia *was achieved repeatedly with inocula prepared from suspensions of the* Comptonia *isolate. The same actinomycete has been reisolated from these seedling nodules. The induced nodules are highly active in the acetylene-reduction assay, and plants grow vigorously without an exogenous supply of fixed nitrogen.*

Nitrogen is the mineral nutrient which most often limits plant production. Symbiotic nitrogen fixation by root nodules of angiosperms infected with soil microorganisms is a major source for replenishment of reduced nitrogen in the living world (*1*). Members of the legume family whose roots are infected by the soil bacterium *Rhizobium* are the primary source of nitrogen fixation in agricultural systems. Some nonlegume angiosperms that form root nodules when invaded by soil actinomycetes fix atmospheric nitrogen at rates comparable to legumes, contributing major amounts of reduced nitrogen to the forests, wetlands, fields, and other natural ecosystems where they abound. In this group are 15 genera distributed among six families with more than 160 known nodulated species; all are woody dicotyledonous plants, growing as shrubs or trees scattered around the world (*2*). The most intensively studied representatives in this group are species of the genus *Alnus*.

Study of the actinomycete-induced nodule symbioses has lagged behind research on legumes largely because of the repeated failure of attempts to isolate and grow the bacterial endosymbiont in pure culture. Claims of successful isolation and culture of actinomycetes from root nodules have been published but have remained unsubstantiated and largely unaccepted by workers in the field (*3*). We report here the isolation and culture of the actinomycetous endophyte from the root nodules of the "sweet fern" *Comptonia peregrina* (L.) Coult. of the family Myricaceae. The endophyte is a filamentous bacterium that grows slowly in complex media, producing numerous spores in sporogenous bodies. A suspension of spores and fragments of filaments prepared from pure cultures of the organism inoculated into an inorganic nitrogen-free nutrient medium provided to the roots of seedlings induces prolific formation of nodules (Fig. 1A) that fix atmospheric nitrogen, as judged by acetylene-reduction tests for nitrogenase (*4*) and by the vigorous development of the plants in the absence of added nitrogen in the nutrient medium.

The commonly accepted method of producing actinomycete-induced nodules experimentally involves inoculating roots of plants grown in sand or water culture with suspensions of ground-up nodules (*5*) or applying soil suspensions taken from areas where the nodulated host plants are growing (*6*). These methods are crude and the results are unpredictable and variable. Nodule suspensions from field-grown nodules contain a wide range of soil microorganisms as well as complex products, many possibly toxic, derived from the broken plant tissues. Soil suspensions are equally complex. Quispel (*7*) discussed the problems of endophyte isolation and described methods for assessing the effectiveness of suspension inoculation. Becking (*8*) attempted to culture the endophyte from *Alnus* within proliferating callus tissue cultures derived from surface-sterilized excised nodules. No evidence for sus-

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Table 1. Nodulation of *Comptonia* seedlings 4 weeks after inoculation. Plants were started in washed sand and received only nitrogen-free nutrient solution. All primary nodule lobes arising from a distinct infection site were counted as one nodule.

Treatment	Plants (No.)	Nodules (No.)	Plants nodulated (percent)
	Sand culture		
Uninoculated	21	5	10
Nodule suspension	41	4	5
Comptonia isolate	28	646	100
	Aeroponic culture		
Experiment 1			
Uninoculated	20	0	0
Comptonia isolate	23	40	91
Experiment 2			
Uninoculated	23	0	0
Comptonia isolate	40	725	100

tained growth of the endophyte in culture was obtained. Lalonde *et al.* (*9*) reported successful cultivation in pure culture of an actinomycete from *Alnus* which he showed by immunolabeling to be homologous with the host endophyte. However, he was unable to obtain reinfection of host plants with the cultured actinomycete.

Our efforts to isolate the endophyte from root nodules of *Comptonia* were based largely on methods described by Quispel (*10*) for *Alnus*, involving surface sterilization of excised nodules followed by incubation on bacteriological media for 6 weeks to allow selection of sterile nodule pieces as the source of inoculum. Nodule material used in all of our experiments was taken from aeroponically grown (*11*) plants, alleviating the problem of adhering soil particles and greatly reducing the contaminating microorganisms typically found in soil and aerated water cultures (*12*).

Nodules 0.5 to 1.0 cm in diameter from 3- to 6-month-old aeroponically grown seedlings were cleansed thoroughly with water containing a drop of detergent, broken apart into individual lobes, and surface-sterilized in $HgCl_2$ (0.1 percent in 0.5 percent HCl) for 10 minutes and then rinsed several times with sterile, distilled water. Individual lobes were cultured for 6 weeks in M-3 incubation medium (*13*); pieces showing no contamination were selected for further study. Thin slices of the nodule lobes were cut aseptically with a microscalpel and incubated for 16 hours at 25°C in nutrient medium (*14*) containing 0.65*M* mannitol as osmoticum and 5 percent (weight to volume) Onozuka "SS" cellulase and 2 percent (weight to volume) Macerozyme R-10 pectinase (*15*) to degrade by enzyme action the walls of the nodule cortical cells containing the endophyte. After enzyme treatment, the nodule pieces were transferred to the same medium lacking enzymes and were teased apart with dissecting needles. Numerous endophyte clusters were released into the medium and were filtered aseptically from cell debris by passage through a 150-μm mesh nylon screen. The endophyte was washed twice by centrifugation and resuspension in fresh medium. In the first successful experiment, the final pellet containing endophyte material derived from five nodule lobes was suspended in 10 ml of liquid medium, and 2 ml of suspension was plated on each of five 6.0-cm Pyrex petri plates, sealed with Parafilm, and cultured in the dark at 25°C.

After 3 weeks of culture several small colonies of a microorganism with filamentous growth were observed in each plate with an inverted microscope. The morphology of this organism was unlike that of any of the known actinomycetes. Colonies were transferred to petri plates that were poured with M-3 incubation medium, where growth continued, and was accelerated. The growth of the isolate was exceptionally slow, however, producing colonies 1 to 2 mm in diameter after several months. Growth was slightly faster in standing liquid medium of the same composition. In subsequent growth tests a medium designated 6B broth (*16*) produced substantially improved growth. More recently we have found that all of the components of 6B broth except for 0.5 percent Difco yeast extract can be omitted without diminishing the growth rate of established cultures (*17*). A slightly slower growth rate was obtained on a completely defined culture medium (*18*). Even a 10 percent soil extract has proved suitable for slow growth of the isolate in axenic culture. The isolate grew optimally at *p*H 6.4 at 25° to 30°C; it appears to be microaerophilic, since it grows best at the bottom of tubes of liquid medium, fails to grow under anaerobiosis, and only very slowly in shaken flasks. Inoculum heated at 60°C shows survival after 60 minutes, with approximately tenfold reduction in numbers of colonies from 30 to 60 minutes.

A study of the morphological and developmental aspects of the actinomycetous "*Comptonia* isolate" was initiated. The isolate grown in liquid culture formed dense unpigmented colonies composed of branching, septate filaments. The diameter of the filaments varied between 0.2 μm in the peripheral filaments to about 1.2 μm in the older filaments near the center of the colonies. The thinner filaments showed Gram-negative staining, whereas the thicker filaments were Gram-positive. Growth of the isolate occurred only beneath the surface of agar medium; no surface or aerial filaments were produced.

Fructifications or sporogenous bodies (*19*) were produced in older regions of the filaments either directly from the axis of the filament (Fig. 1, B, D, and E) or on short, branch filaments (Fig. 1, C and E). The sporogenous bodies of either type developed from a locally thickened region of the filament which showed an increased frequency of transverse septation (Fig. 1B). Further enlargment of this region was followed by longitudinal septations (Fig. 1, C and D) which resulted in the production of roughly polyhedral spores 1.5 to 3.5 μm in diameter. The mature sporogenous bodies (Fig. 1E) may reach 25 by 35 μm. The intra-axial sporogenous bodies were much less common than the terminal structures and generally were of smaller dimensions. The Gram-positive spores did not appear to be contained within a sheath and separate readily when they mature, forming free-floating ovoid to spherical structures. There is no evidence of motility in either the spores or any other form of the isolate. Separation and concentration of these spores by selective filtration has allowed us to observe that they germinate in good numbers in plated agar yeast-extract medium.

The development and morphology of the sporogenous bodies we have observed in pure cultures of the *Comptonia* isolate bear a striking resemblance to what seems to be a comparable stage in the endophyte of *Alnus glutinosa* observed in sections of mature nodules by van Dijk and Merkus (*19*) and referred to by them as "spindle formation." It is noteworthy that spindle development in the *Alnus* endophyte is not present in all nodules (*1, 19, 20*) and that equivalent structures have not been reported in the root nodules of *Comptonia*. The vesicular or terminal, club-shaped form of the endophyte has not been observed in our

cultures and may be formed only in the symbiotic association. The *Comptonia* isolate has been grown continuously in pure culture as described on a variety of media for more than a year.

Since others had isolated and cultured actinomycetes from nodules of *Alnus* species but were unable to show their activity in inducing nodulation, it was important for us to demonstrate the infectiveness of the *Comptonia* isolate for nodulation and the effectiveness of the experimentally induced symbiosis in fixing atmospheric nitrogen.

Infection of *Comptonia* seedlings with a pure culture of the isolate was attempted once a sufficient quantity of the isolate had been grown in culture. Seedlings (6 weeks old) growing in sand were supplied with nitrogen-free Hoagland's solution (*21*). A small amount of the isolate (< 0.05 cm^3 packed cell volume) was washed, crushed with a glass rod, resuspended, and poured into the sand. Other flats of seedlings were either uninoculated or inoculated with a crushed nodule inoculum (0.5 g ground in 20 ml of distilled water). The root systems were examined after 4 weeks, and the number of nodules was recorded; all of the primary nodule lobes thought to arise from a discrete infection site (*22*) were counted as one nodule (Table 1). We attribute the small numbers of nodules in the controls to contamination due to a common underlying container for the sand flats.

A similar test was made with *Comptonia* seedlings grown aeroponically. Sand-grown seedlings were transferred to an aeroponic tank, the roots were allowed to develop further for 1 week in a nitrogen-free nutrient mist; then the liquid in the tank was inoculated with a suspension of the *Comptonia* isolate. The roots were examined at regular intervals (Table 1). The earliest nodules formed appeared at 8 days, an infection period comparable to *Rhizobium* infection in legumes grown under these conditions.

The nitrogenase activity of nodulated plants infected by the *Comptonia* isolate was determined by the acetylene-reduction assay. Acetylene-reduction rates for several nodulated root systems excised just below the cotyledons and incubated in an atmosphere of 90 percent air and 10 percent acetylene were substantial. Ethylene production varied from 9.60 to 22.20 μmole/hour per gram (fresh weight) of nodules sampled either from sand-grown or aeroponically grown plants with nodules attached to roots or excised. A portion of one of the seedling root systems grown in sand is shown in Fig. 1A.

The *Comptonia* isolate we have established in pure culture is highly active in initiating nodule formation. The nodules formed are highly effective in acetylene reduction to ethylene and can be assumed to possess substantial levels of nitrogenase. Thus we believe we have fulfilled Koch's postulates with respect to isolation, independent culture, and demonstrated reinfection of the host plants. The final step has been to reisolate the organism from nodules on plants infected with the cultured endophyte. Success in this has been achieved with the use of a modification of the method described for the initial successful isolation. Nodules on plants grown aeroponically and infected with a suspension of the *Comptonia* isolate were excised, surface-steri-

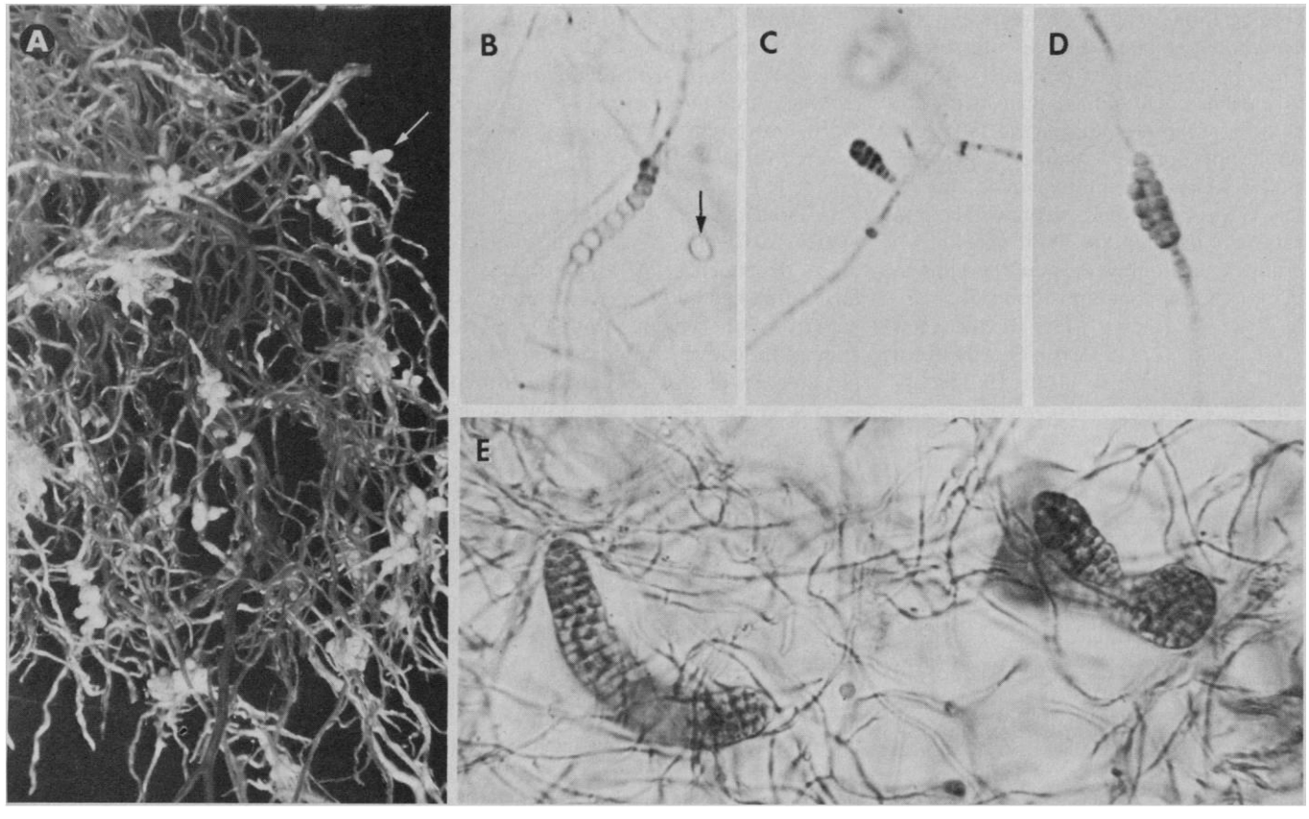

Fig. 1. (A) *Comptonia* seedling root system from sand culture 4 weeks after inoculation with a suspension of the *Comptonia* isolate. Arrow shows one of the 55 nodules present on this root system (× 2). (B to E). Photomicrographs of filaments and colonies of the *Comptonia* endophyte (all × 1200). (B) Early stage in development of the intra-axial sporogenous body. The filament is locally thickened and has numerous transverse septa. Free spore (arrow) was released from a mature sporogenous body. (C) Sporogenous body developing from a short branch filament. Thickening and transverse septa are seen. First longitudinal septum is visible at the distal end. (D) Later stage of sporogenous body development, showing longitudinal septation along most of the length of the filament. (E) Portion of well-developed filamentous colony with nearly mature sporogenous bodies showing the segmentation derived from early transverse septation. Fully mature sporogenous bodies lose the close coherence of spores, which become rounded and easily disaggregated.

lized as before, and then, without the usual preliminary incubation, were immediately subjected to sectioning, enzyme maceration, and teasing apart. Clusters of endophyte filaments were mechanically separated by filtration, repeatedly transferred by micropette until free of cell debris, and then pipetted into 6B broth in 6-cm petri plates; the plates were sealed with Parafilm and incubated in the dark at 25°C. Within 3 weeks, a small number of the clusters had formed; these were typical colonies of the actinomycete. These colonies could be subcultured on yeast-extract medium. Thus, the same isolation procedure has been utilized successfully both in the initial isolation and in the subsequent recovery of the same organism, demonstrating unequivocally the effectiveness of the technique and the certainty of the identity of the microorganism.

The current taxonomic status of the actinomycetous root nodule endophytes is felt by many authors to be quite uncertain (*1, 19, 23*). Becking (*24*) applied the generic name *Frankia* to the endophytes of nonleguminous nodules and designated specific epithets referable to the host species or to original designations. According to his taxonomic treatment, the *Comptonia* isolate would be referable to *Frankia brunchorstii*. With the successful establishment of this organism in pure culture, we prefer to reserve judgment on the appropriate scientific designation. Better defined chemotaxonomic characters of the *Comptonia* isolate will help to establish its affinities with the known groups of actinomycetes and perhaps to clarify its taxonomic position (*25*).

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References and Notes

1. A. Quispel, in *The Biology of Nitrogen Fixation*, A. Quispel, Ed. (North-Holland, Amsterdam, 1974).
2. G. Bond, in *Symbiotic Nitrogen Fixation in Plants*, P. S. Nutman, Ed. (Cambridge Univ. Press, London, 1976), p. 443.
3. The list of unsuccessful attempts at reinoculation with cultured microorganisms isolated from surface-sterilized nodules of *Alnus* and other nodulated nonlegumes is long, dating back to 1910. These results and the less numerous claims of success have been thoroughly reviewed by G. Bond [in *Symbiotic Associations*, P. S. Nutman and B. Mosse, Eds. (Cambridge Univ. Press, London, 1963), p. 72], by S. Uemura [in *Bull. Govt. Forest Exp. Stn. Meguro* **167**, 69 (1964)], by G. Bond [*Annu. Rev. Plant Physiol.* **18**, 107 (1967)], by A. Quispel (*1*, p. 499), and by W. W. Fletcher and I. C. Gardner [*Ann. Microbiol. (Milan)* **24**, 159 (1974)].
4. R. W. F. Hardy, R. D. Holsten, E. K. Jackson, R. C. Burns, *Plant Physiol.* **43**, 1185 (1968).
5. G. Bond, W. W. Fletcher, T. P. Ferguson, *Plant Soil* **5**, 309 (1954).
6. G. Bond, *New Phytol.* **70**, 1 (1971).
7. A. Quispel, *Acta Bot. Neerl.* **3**, 495 and 512 (1954).
8. J. Becking, *Nature (London)* **207**, 885 (1965).
9. M. Lalonde, R. Knowles, J. André Fortin, *Can. J. Microbiol.* **21**, 1901 (1975).
10. A. Quispel, *Acta Bot. Neerl.* **4**, 671 (1955).
11. R. Zobel, P. Del Tredici, J. G. Torrey, *Plant Physiol.* **57**, 344 (1976).
12. B. Bowes, D. Callaham, J. G. Torrey, *Am. J. Bot.* **64**, 516 (1977).
13. Composition of M-3 (grams per liter): $CaCO_3$, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $MnSO_4 \cdot H_2O$, 0.025; H_3BO_3, 0.10; $ZnSO_4 \cdot 7H_2O$, 0.010; $Na_2MoO_4 \cdot 2H_2O$, 0.00025; $CuSO_4 \cdot 5H_2O$, 0.000025; Difco yeast extract, 0.5; Edamin, 1.0; thiamin hydrochloride, 0.0001; nicotinic acid, 0.0005; pyridoxine hydrochloride, 0.0001; mannitol, 1.0; sucrose, 20.0; agar, 10.0; *p*H adjusted to 7.0.
14. B-D medium [P. Goforth and J. G. Torrey, *Am. J. Bot.* **64**, 475 (1977)] used as a base supplemented with 1 m*M* each of L-glutamic acid, L-aspartic acid, glycine, L-arginine, L-asparagine, L-glutamine, and urea, and (per liter) 2.0 mg of naphthaleneacetic acid and 1 μg of zeatin.
15. These hydrolytic enzymes of the cell wall were manufactured by Yakult Biochemical Co., Inc., of Japan.
16. G. M. Ludemann, *J. Bacteriol.* **96**, 1848 (1968).
17. Stock cultures are maintained in 0.5 percent Difco yeast extract in distilled water at *p*H 6.4 in unshaken flasks or test tubes 5 to 6 cm deep with medium. Subculture is effected by crushing washed colonies in a tissue homogenizer with distilled water and inoculation of new cultures with a drop of the suspension. Cultures 4 to 6 weeks old are routinely used for subculture or plant inoculation.
18. Medium composition (milligrams per liter): $CaCl_2 \cdot 2H_2O$, 200; KH_2PO_4, 200; $MgSO_4 \cdot 7H_2O$, 200; NaCl, 100; H_3BO_3, 1.5; $ZnSO_4 \cdot 7H_2O$, 1.5; $MnSO_4 \cdot H_2O$, 4.5; $NaMoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.04; thiamin hydrocholoride, 0.1; nicotinic acid, 0.5; pyridoxine hydrochloride, 0.5; and 10 m*M* succinic acid, 2 m*M* L-glutamine, 0.5 m*M* myoinositol, and 0.1 m*M* Fe · EDTA; *p*H 6.4.
19. C. van Dijk and E. Merkus, *New Phytol.* **77**, 73 (1976).
20. R. Schaede, *Planta* **19**, 389 (1933).
21. D. R. Hoagland and D. I. Arnon, *Univ. Calif. Agr. Exp. Sta. Circ. 347* (1938).
22. D. Callaham and J. G. Torrey, *Can. J. Bot.* **55**, 2306 (1977).
23. G. Bond, in *The Biology of Nitrogen Fixation*, A. Quispel, Ed. (North-Holland, Amsterdam, 1974), p. 342.
24. J. H. Becking, *Int. J. Syst. Bacteriol.* **20**, 201 (1970).
25. The *Comptonia* isolate described here has been used successfully in our laboratory to induce the formation of root nodules with high acetylene-reduction activity in seedlings of *Myrica gale* and *M. cerifera* and in the laboratory of M. Lalonde, Kettering Research Laboratory, Yellow Springs, Ohio (personal communication) in seedlings of *Alnus crispa* and *A. glutinosa*.
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Membrane Enzymes: Artifacts in Arrhenius Plots Due to Temperature Dependence of Substrate-Binding Affinity

Abstract. *For the membrane sodium-stimulated magnesium-adenosinetriphosphatase of* Acholeplasma laidlawii *B both the* V_{max} *and* K_m *values in the Michaelis equation vary strongly with temperature. Simulations of Arrhenius plots show that an enzyme with a temperature-dependent* K_m *can yield a variety of Arrhenius plot artifacts, most notably erroneous "breaks," if activity is assayed at a fixed substrate concentration.*

In studies of membrane-bound enzymes, Arrhenius plots of the temperature dependence of enzyme activity have often been utilized to correlate the activity of the enzyme with the phase state of the membrane lipids. In particular, any abrupt changes in the measured activation energies E_a are often attributed to a lipid phase transition; it has been suggested that such breaks in Arrhenius plots arise from a change in the enzyme conformation or in the nature of the rate-limiting step of the overall catalyzed reaction (*1*). In a study of the membrane Mg^{2+}-adenosinetriphosphatase of *Acholeplasma laidlawii* B (*2*), we have found that the variation with temperature of its substrate-binding affinity can strongly influence the behavior of Arrhenius plots, altering the measured E_a values and break temperatures and even creating artifactual breaks in some cases if enzyme activity is assayed at a single fixed substrate concentration. As we will demonstrate in this report, such effects can be so pronounced that they should be accorded more serious consideration in experimental design than is generally the case at present.

Acholeplasma laidlawii B cells were cultured as described elsewhere (*3*) in the presence of oleic, elaidic, or palmitic acid to replace approximately 70 percent of the normal membrane fatty acids with the exogenous species, thus producing three cell populations of widely varying membrane fluidity (*4*). Cells were harvested and membranes were prepared by osmotic lysis as described elsewhere (*5*). To assay the adenosinetriphosphatase activity, membranes (100 to 200 μg of protein per milliliter) were incubated with 50 m*M* NaCl, 15 m*M* $MgSO_4$, 50 m*M* tris buffer (*p*H 7.4), and various concentrations of adenosine triphosphate (ATP) at various temperatures; portions of the reaction mixture were quenched by mixing with an equal volume of 0.5 percent dodecyl sulfate solution and assayed for phosphate by the method of Atkinson *et al.* (*6*). Protein was assayed by the method of Hartree (*7*).

To ensure that all factors other than ATP concentration were optimal in our assays, we began by reexamining the ionic requirements of the enzyme. As previously reported (*2*), the enzyme has an absolute requirement for Mg^{2+}. How-

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