Plant Physiol. (1986) 82, 346–350
0032-0889/86/82/0346/05/$01.00/0

Bacteroids Are Stable during Dark-Induced Senescence of Soybean Root Nodules[1,2]

Received for publication March 24, 1986 and in revised form May 27, 1986

GAUTAM SARATH, NANCY E. PFEIFFER[3], C. S. SODHI[4], AND FRED W. WAGNER*
Department of Agricultural Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68583-0718

ABSTRACT

Physiological and biochemical markers of metabolic competence were assayed in bacteroids isolated from root nodules of control, dark-stressed, and recovered plants of *Glycine max* Merr. cv 'Woodworth.' Nitrogenase-dependent acetylene reduction by the whole plant decreased to 8% of control rates after 4 days of dark stress and could not be detected in plants dark stressed for 8 days. However, in bacteroids isolated anaerobically, almost 50% of initial acetylene reduction activity remained after 4 days of dark stress but was totally lost after 8 days of dark stress. Bacteroid acetylene reduction activity recovered faster than whole plant acetylene reduction activity when plants were dark stressed for 8 days and returned to a normal light regimen. Significant changes were not measured in bacteroid respiration, protein content, sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles, or in bacteroid proteolytic activity throughout the experiment. Immunoblots of bacteroid extracts revealed the presence of nitrogenase component II in control, 4-day dark-stressed, and 8-day dark-stressed plants that were allowed to recover under a normal light regimen, but not in 8-day dark-stressed plants. Our data indicate that dark stress does not greatly affect bacteroid metabolism or induce bacteroid senescence.

Root-nodule senescence results in the loss of symbiotic nitrogen fixation in legumes (14, 15). Although the physiological and biochemical events responsible for this loss have not been resolved (24), diminishing whole plant nitrogen fixation ability correlates with decreases in total root nodule protein and Lb[5] content (14, 24). Some of the changes that occur in soybean root nodules throughout growth and development have been previously monitored to identify early biochemical events of senescence (19). In addition to anticipated changes in total cytosolic protein and Lb, proteolysis increases when senescence begins. Measurement of bacteroid protein contents and proteolytic activities supported the contention that bacteroids do not undergo appreciable degradation during natural senescence (19).

An earlier report from this laboratory demonstrated that dark stress of soybean plants induces catabolic changes in root nodules which mimic natural senescence (18). Whole plant nitrogen fixation, total nodule cytosolic protein, and Lb levels all decrease while there is a 5-fold increase in proteolytic activity measured with an artificial substrate. Stress-induced senescence is reversible if plants are returned to normal growing conditions (18). Remarkably, restoration of AR occurs in the same nodule population, indicating that the effects of stress for most of the root nodules are reversible. Some root nodules (approximately 10–15%) do not recover and eventually senesce (18). Recovery of soybean root nodules from induced senescence is unexpected because these nodules are determinate and lack defined meristems when mature (11). In contrast, when legumes with indeterminate nodules, such as alfalfa, are subjected to stress, recovery of nodule function occurs by generation of newly infected tissue from an apical nodule meristem (27) rather than by regeneration of existing tissue.

Studies of the effects of imposed stress on nodulated plants have provided much information on the changes taking place in whole nodules. There is rapid attenuation of nitrogenase activity (AR activity), with subsequent declines in total nodule protein and Lb levels (3, 7, 18, 20, 22, 29). However, corresponding data on bacteroid status are incomplete. In legumes such as peas, which possess indeterminate nodules, bacteroid function generally responds in a similar manner to stress as the host plant (3, 4). In plants with determinate nodules, such as soybean, existing evidence suggests that bacteroids are stable during both stress-induced and natural senescence (9, 17, 19, 25, 26).

We initiated this study to measure bacteroid stability during senescence induced by dark stress and subsequent recovery in root nodules of soybean plants.

MATERIALS AND METHODS

Dark-Stress Protocol. Seeds of *Glycine max* Merr. cv 'Woodworth' were surface sterilized and inoculated with a suspension of *Rhizobium japonicum* strain 61A89 at the time of planting. Plants were grown in sterile 'Turface' (Loveland Grass Pad Network, Omaha, NE) and maintained on a nitrogen-free nutrient solution at 26°C/16 h day and 21°C/8 h night photoperiod. Light intensity at pot height averaged 550 μmol m^{-2} s^{-1} PAR. Plants at the R-1 stage of growth (12) (approximately 35 d old) were placed in total darkness for up to 8 d, then allowed to recover for up to 17 d under normal growth conditions. Root nodules greater than 2 mm in diameter were harvested from plants at regular intervals during the experimental period, cleaned with ice-cold distilled H_2O, and maintained at 2°C until extracted.

Root Nodule Extraction. When necessary, extracts and manipulations of nodules were performed under anaerobic conditions in airtight glove bags (Instruments for Research and Industry, Cheltenham, PA) flushed with high purity N_2. Solutions were degassed then thoroughly flushed with N_2. All extractions were

[1] Supported in part by the United States Department of Agriculture under grant 83-CRCR-1-1293 and a grant from the Nebraska Soybean Development, Utilization and Marketing Board.

[2] Published as paper No. 8010, Journal Series, Nebraska Agriculture Research Division.

[3] Present address: Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0304.

[4] Present address: USAID/GUYANA, Agency for International Development, Washington, DC 20523.

[5] Abbreviations: AR, acetylene reduction; Lb, leghemoglobin.

performed at 4°C, essentially according to Bergersen and Turner (2).

Root nodules (3 g fresh weight) were crushed in an ice-cold mortar and pestle using 5 volumes per g nodule fresh weight of extraction buffer (0.1 M Na phosphate, 25 mM Na dithionite, 2 mM DTE, 0.3 M sucrose, 0.06% (w/v) insoluble PVP, pH 7.5). The brei was squeezed through 6 layers of cheesecloth into N_2-flushed stainless-steel centrifuge tubes and sealed under N_2 with gastight caps. After an initial centrifugation at 500*g* for 5 min to sediment plant debris and solid PVP, the supernatant fluid was centrifuged at 6000*g* for 10 min to sediment the bacteroids. The bacteroids were washed once in 50 mM Na phosphate, 0.3 M sucrose (pH 6.8), sedimented again at 6000*g* for 10 min, then resuspended in 50 mM Na phosphate, 25 mM Na-succinate, 0.3 M sucrose (pH 6.8), and used for measuring AR and respiration.

Acetylene Reduction. Whole plant AR was measured as reported previously (18). Bacteroid suspensions (3 ml) were dispensed into 21-ml glass vials and sealed under N_2 with rubber serum stoppers, then equilibrated at 22 ± 2°C. A mixture of acetylene (1.8 ml) and O_2 (to 2% gas phase) was injected into the vials. Methane (20.8 nmol) in the acetylene/O_2 mixture served as the internal standard. Vials were placed on a rotary shaker operated at 140 rpm. Triplicate gas samples withdrawn at 30 and 60 min were analyzed for ethylene as reported previously (18). Under these conditions of assay, acetylene reduction by bacteroid suspensions was linear for more than 150 min.

Bacteroid Respiration. Bacteroid respiration was measured according to the method of Klucas and Arp (15). Bacteroid suspension (1.8 ml) placed in 21-ml glass vials were equilibrated to room temperature (22 ± 2°C) and flushed with air. Respiration was measured as the release of $^{14}CO_2$ from exogenously supplied (1-^{14}C]succinate (1650 dpm/μmol in the final reaction mixture). Vials were placed on an orbital shaker operating at 140 rpm. Released $^{14}CO_2$ was trapped on filter paper discs (Whatman No. 3) wetted with 0.2 ml of 5% KOH. Reactions were terminated after 30 min by the addition of 0.2 ml of 2 N H_2SO_4. Radioactivity in the discs was counted in a Packard 24 CL/D liquid scintillation counter after the addition of 5 ml of a commercially available scintillation fluid (3a70; Research Product International Corp., Mount Prospect, IL). Calculated values for $^{14}CO_2$ released by bacteroid suspensions from exogenously supplied [^{14}C]succinate incorporated suitable controls and were corrected for quenching. Under these conditions of assay, release of $^{14}CO_2$ was linear for more than 45 min.

Gel Electrophoresis. Bacteroid samples were lysed either by being passed twice through a French Press operated at 20,000 p.s.i, or by sonication for 15 min in a melting ice bath. Samples were then centrifuged at 25,000*g* for 20 min to sediment debris. Protein content was determined by the method of Bradford (6) using commercial dye (Bio-Rad Laboratories, Richmond, CA).

SDS-PAGE was performed according to Laemmli (16) at room temperature using 5 to 25% linear gradient polyacrylamide gels.

Immunoblotting. Bacteroid protein samples were fractionated by SDS-PAGE on a 10% gel according to Laemmli (16) at room temperature. Proteins were electroeluted from the gel onto a nitrocellulose membrane. Transferred proteins were allowed to react with an antibody preparation raised in rabbits immunized to *Rhodospirillum rubrum* nitrogenase component II (a generous gift of Dr. Paul Ludden, Department of Biochemistry, University of Wisconsin-Madison). Bound antibodies were visualized with a Protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories).

Enzyme Assays. Proteolytic activity of crude bacteroid extracts was determined according to Pfeiffer *et al.* (19).

RESULTS AND DISCUSSION

Acetylene Reduction, Bacteroid Proteins, and Proteolytic Activities. Bacteroid AR declined by only 54% after 4 d but was lost after 8 d of dark stress. The recovery of AR in bacteroids isolated anaerobically began at about the same time as for whole plant recovery; however, the rate of recovery of nitrogenase activity in bacteroids was greater than the corresponding rate of recovery of whole plant AR (Fig. 1, A and B).

Whole plant AR was about 2.9 μmol/plant·min for plants at the start of the experiment. AR declined rapidly to 8% of initial rates after 4 d of dark stress and could not be detected in plants dark stressed for 8 d. A small increase in the rates of AR occurred when plants were allowed to recover under normal growth conditions for 6 d after being dark stressed for 8 d. After 14 d of recovery, whole plant AR was not significantly different from control plants maintained under normal growth conditions throughout the experimental period (Fig. 1B).

Nitrogenase component II in bacteroid extracts was monitored immunochemically to determine the presence of this protein during the experimental protocol. Nitrogenase component II isolated from *R. japonicum* bacteroids is composed of two subunits of approximately 30,000 D (30). We detected a major immunoreactive band of approximately 30,000 D and a few others of lower mol wt (Fig. 2). Nitrogenase component II is diminished in bacteroids isolated from 4-d dark-stressed plants and is totally absent after 8 d of dark stress (Fig. 2). However, nitrogenase component II reappears in bacteroids isolated from plants allowed to recover for only 6 d after 8 d of dark stress (Fig. 2). These data correlate well with the levels of bacteroid AR activity (Fig. 1A) and clearly demonstrate that AR activity at the

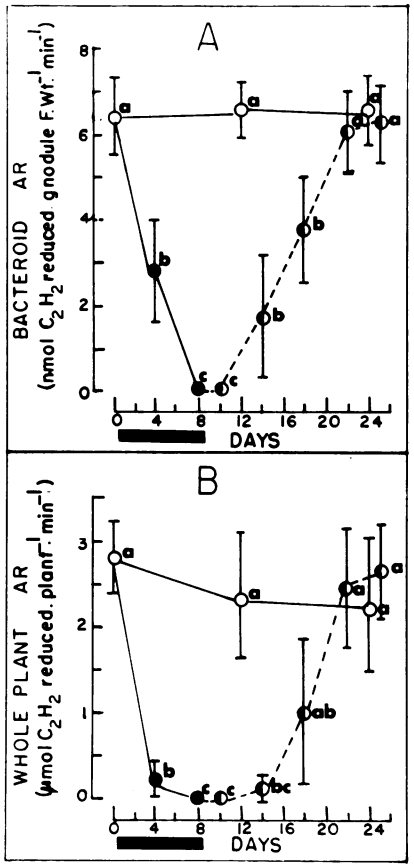

FIG. 1. Acetylene reduction by anaerobically isolated bacteroids (A); and by intact plants (B). (○) controls; (●) dark-stressed; (◐) plants allowed to recover after 8 d dark-stress. Bars show standard deviation. For bacteroid AR, data are means of duplicate assays from 3 replicate plants at each harvest date. For whole plant AR, data are the means of 3 replicate plants. Means followed by the same letters are not significantly different at the 5% level using Duncan's new multiple range test.

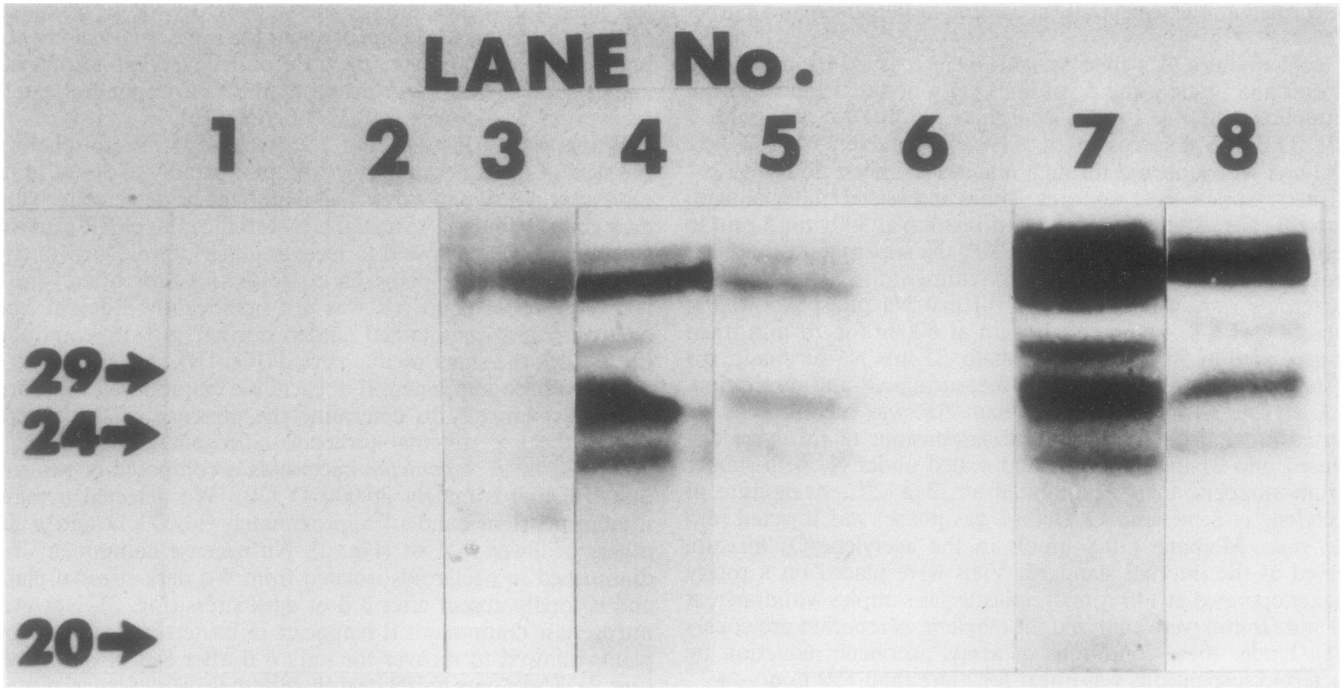

FIG. 2. Immunoblot of root nodule extracts reacted with an antibody preparation to *R. rubrum* nitrogenase component II. Two hundred μg protein per lane, except lane 3. Lane 1, root nodule cytosol from control plants (0-d); lane 2, root nodule cytosol from 8-d dark-stressed plants; lane 3, 1 μg nitrogenase component II from *R. rubrum*; lane 4, bacteroid extracts from d 0 control plants; lane 5, bacteroid extracts from 4-d dark-stressed plants; lane 6, bacteroid extracts from 8-d dark-stressed plants; lane 7, bacteroid extracts from 8-d dark-stressed plants allowed to recover for 6 d; lane 8, bacteroid extracts from 24-d control plants.

whole plant level is a poor measure of viable bacteroid nitrogenase. Similarly, in nodulated pea plants stressed with combined N, AR activity at the intact plant and nodule level decays much faster than that of bacteroids isolated from these nodules (13). Thus, nitrogenase activity appears to be regulated at both the nodule level and at the bacteroid level. Significant changes were not observed in one-dimensional SDS-PAGE profiles of bacteroid protein (Fig. 3). Bacteroid protein content was essentially unchanged during the experimental protocol with a mean value of approximately 5.6 mg protein/g nodule fresh weight. Proteolytic activities previously characterized in bacteroid extracts (19) were measured using artificial substrates. Significant differences in proteolytic activities were not observed in bacteroid extracts from root nodules of control, dark-stressed, or dark-stressed and recovered plants, when azocasein, L-leucine-β-naphthylamide and benzoyl-L-arginine-β-naphthylamide were used as substrates. These differences are interpreted to imply that there is little change in bacteroids with the imposition of stress. At the ultrastructural level only slight changes in bacteroid shape were observed, while host cells were extensively degraded (9). Therefore, nitrogenase component II might be selectively regulated in this system by repression of synthesis as a response to stress, as has been shown to occur in peas (4).

It is also possible that the turnover time of nitrogenase component II is between 4 and 8 d in soybean nodules and continued synthesis of this protein is dependent on the physiological state of the host cell. Bisseling *et al.* (5) have estimated that the turnover times for both nitrogenase components in pea root nodules to be between 2 and 3 d. In lupins the average turnover time of bacteroid proteins is approximately 5 d (10). Perhaps regulation of nitrogenase synthesis modulates nitrogenase function without changing other features of the symbiotic system under conditions of stress, when energy supply to the nodule is presumably limiting (7, 13, 22, 23).

Bacteroid Respiration. There was a decline in respiration rates in 8-d dark-stressed plants that were allowed to recover for various times; however, these changes were less than 10 to 15% of d 0 controls and were not significant (Fig. 4). *R. japonicum* bacteroids possess two different terminal oxidases (1). One of these oxidases, active at low pO_2 and coupled to ATP production, probably functions in bacteroids actively fixing nitrogen. The other oxidase is CO insensitive, uncoupled from ATP production and active at higher pO_2. Ching *et al.* (8) reported that ATP levels in nodules decrease with the imposition of dark stress. It is also known that functional nitrogenase requires high rates of ATP synthesis and is rapidly inhibited by increasing concentrations of ADP (30). Although we did not measure rates of ATP synthesis, the presence of AR activity in isolated bacteroids provides indirect evidence that bacteroid respiration in the presence of externally supplied succinate is coupled to ATP synthesis at least during the early phase of dark-induced senescence. However, bacteroids *in situ* may be under carbon stress due to inhibition of assimilate translocation from the darkened shoot system, although concentrations of carbohydrates in the nodule are thought to influence nitrogenase activity to a much greater degree than the rate of supply of carbohydrates from the shoot (21, 28). It thus appears that changes in bacteroid AR are due to a combination of plant cytosol related changes as well as changes in the amounts of nitrogenase proteins.

When compared with earlier results on this system, where dark stress induces senescence of root-nodule cells as determined ultrastructurally (9) and by the loss of total cytosolic protein and Lb (18), the data presented here clearly demonstrate that bacteroids and plant cells do not senesce simultaneously. Furthermore, recovery of host cell metabolic activity from dark-imposed stress leads to a renewed symbiotic relationship indistinguishable from control plants. It also appears that the functional state of the plant cell rather than the bacteroid determines AR activity at the whole plant level during dark-induced senescence and subsequent recovery in this system.

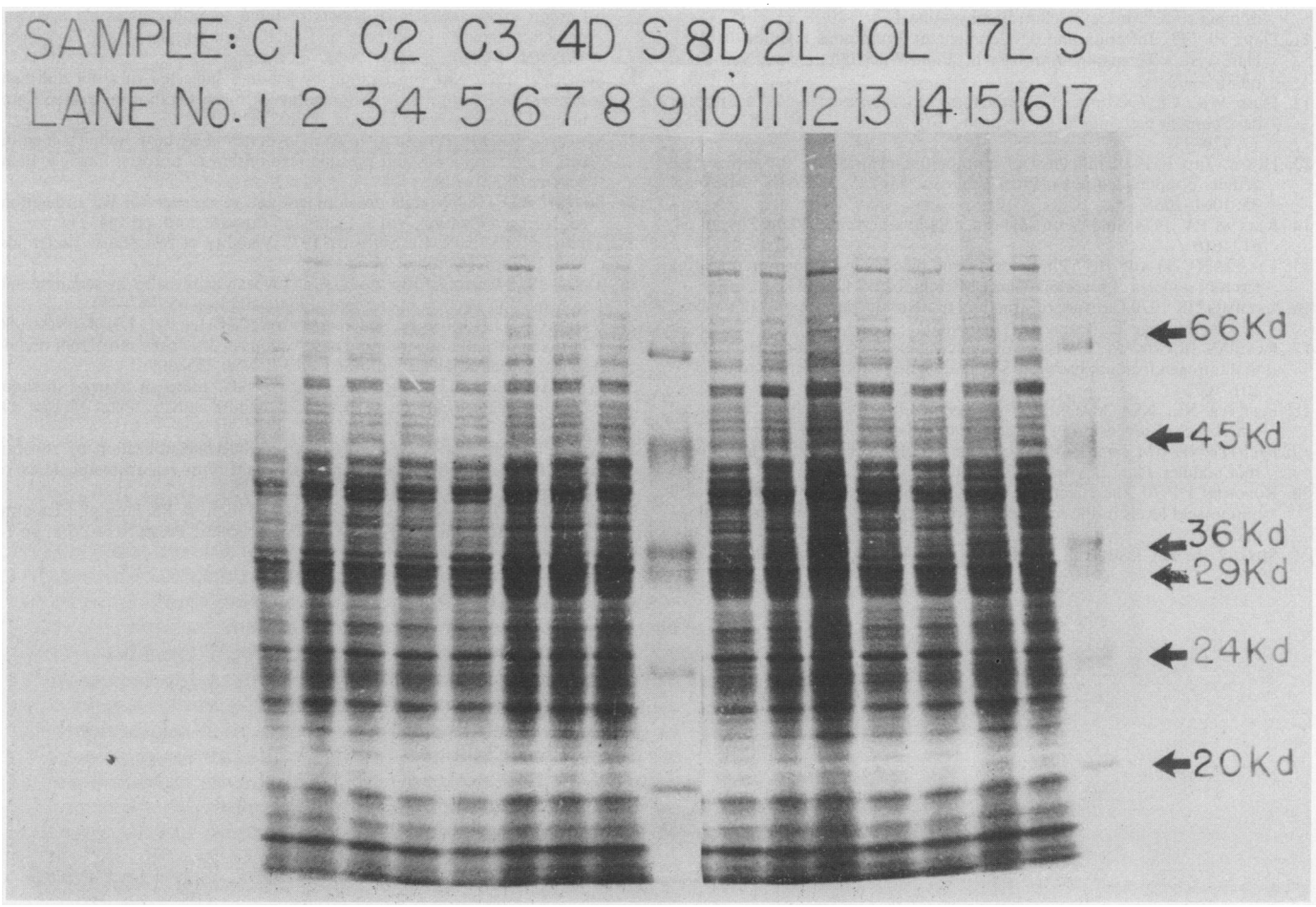

FIG. 3. SDS-PAGE profiles of bacteroid proteins. Conditions of electrophoresis as in "Materials and Methods." Eighty μg protein per lane. Lanes 1 and 2, 0 d controls (C1); lanes 3 and 4, 12 d controls (C2); lanes 5 and 6, 24 d controls (C3); lanes 7 and 8, 4 d dark (4D); lanes 9 and 17, molecular wt standards (S); lane 10, 8 d dark (8D); lanes 11 and 12, extracts from plants allowed to recover for 2 d (2L); lanes 13 and 14, extracts from plants allowed to recover for 10 d (10L); lanes 15 and 16, extracts from plants allowed to recover for 17 d (17L).

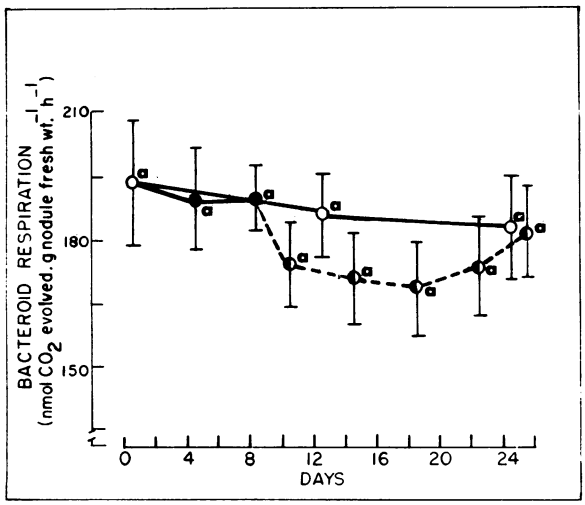

FIG. 4. Bacteroid respiration of exogenous [^{14}C]succinate in air. Symbols as in Figure 1. Data are the means of triplicate assays of 3 replicate plants at each harvest date. Means followed by the same letters are not significantly different at the 5% level, using Duncan's new multiple range test.

Acknowledgments—We would like to thank Drs. S. J. Danko, R. V. Klucas, and J. P. Markwell for critically reading the manuscript.

LITERATURE CITED

1. APPLEBY CA 1977 Function of P-450 and other cytochromes in *Rhizobium* respiration. *In* H Degn, D Lloyd, GC Hill, eds, Function of Alternative Terminal Oxidases, FEBS 11th Meeting, Copenhagen, Vol 49, Coll B6. Pergamon Press, New York, pp 11–20
2. BERGERSEN FJ, GL TURNER 1973 Kinetic studies of nitrogenase from soybean root-nodule bacteroids. Biochem J 131: 61–75
3. BISSELING T, RC VAN DEN BOS, A VAN KAMMEN 1978 The effect of ammonium nitrate on the synthesis of nitrogenase and concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. Biochim Biophys Acta 539: 1–11
4. BISSELING T, W VAN STAVEREN, A VAN KAMMEN 1980 The effect of waterlogging on the synthesis of the nitrogenase components in bacteroids of *Rhizobium leguminosarum* in root nodules of *Pisum sativum*. Biochem Biophys Res Commun 93: 687–693
5. BISSELING T, J VAN STRATEN, F HOUWAARD 1980 Turnover of nitrogenase and leghemoglobin in root nodules of *Pisum sativum*. Biochim Biophys Acta 610: 360–370
6. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
7. CHEN PC, DA PHILLIPS 1977 Induction of root nodule senescence by combined nitrogen in *Pisum sativum* L. Plant Physiol 59: 440–442.
8. CHING TM, SA HEDTKE, SA RUSSEL, HJ EVANS 1975 Energy state and dinitrogen fixation in soybean nodules of dark grown plants. Plant Phyisol 55: 796–798
9. COHEN HP, G SARATH, K LEE, FW WAGNER 1986 Soybean root nodule ultrastructure during dark-induced stress and recovery. Protoplasma. In press
10. COVENTRY DR, MJ DILWORTH 1976 Synthesis and turnover of leghemoglobin

in lupin root nodules. Biochim Biophys Acta 447: 1–10
11. DART PJ 1977 Infection and development in leguminous nodules. *In* RWF Hardy, ed, A Treatise in Dinitrogen Fixation. Wiley Interscience, New York, pp 367–402
12. FEHR WR, CE CAVINESS, DT BURMOOD, JS PENNINGTON 1971 Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci 11: 929–931
13. HOUWAARD F 1978 Influence of ammonium chloride on the nitrogenase activity of nodulated pea plants (*Pisum sativum*). Appl Environ Microbiol 35: 1061–1065
14. KLUCAS RV 1974 Studies on soybean nodule senescence. Plant Physiol 54: 612–616
15. KLUCAS RV, D ARP 1977 Physiological and biochemical studies on senescing tap root nodules of soybeans. Can J Microbiol 23: 1426–1432
16. LAEMMLI UK 1970 Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature 227: 680–685
17. PAAU AS, JR COWLES 1979 Effect of induced senescence on parameters related to dinitrogen fixation, bacteroid size and nucleic acid. J Gen Microbiol 111: 101–107
18. PFEIFFER NE, NSA MALIK, FW WAGNER 1983 Reversible dark induced senescence of soybean root nodules. Plant Physiol 71: 393–399
19. PFEIFFER NE, CM TORRES, FW WAGNER 1983 Proteolytic activity in soybean root nodules. Plant Physiol 71: 797–802
20. ROPONEN I 1970 The effect of darkness on the leghemoglobin content and amino acid levels in the root nodules of pea plants. Physiol Plant 23: 452–460
21. SHEEHY JE, KA FISHBECK, TM DEJONG, LE WILLIAMS, DA PHILLIPS 1980 Carbon exchange rates of shoots required to utilize available acetylene reduction capacity in soybean and alfalfa root nodules. Plant Physiol 66: 101–104
22. SPRENT JI 1976 Nitrogen fixation by legumes subjected to water and light stresses. *In* PS Nutman, ed, Symbiotic Nitrogen Fixation in Plants. Cambridge University Press, London, pp 405–420
23. STREETER JG, ME BOSLER 1976 Carbohydrates in soybean nodules: identification of compounds and possible relationship to nitrogen fixation. Plant Sci Lett 7: 321–329
24. SUTTON WD 1983 Nodule development and senescence. *In* WJ Broughton, ed, Nitrogen Fixation, Vol 3. Clarendon Press, Oxford, pp 144–212
25. TSEIN HC, PS CAIN, EL SCHMIDT 1977 Viability of *Rhizobium* bacteroids. Appl Environ Microbiol 34: 854–856
26. TU JC 1975 Rhizobial root nodules of soybean as revealed by scanning and transmission electron microscopy. Phytopathology 65: 447–454
27. VANCE CP, LEB JOHNSON, AM HALVERSON, GH HEICHEL, DK BARNES 1980 Histological and ultrastructural observations of *Medicago sativa* root nodule senescence after foliage removal. Can J Bot 58: 255–309
28. WILLIAMS LE, TM DEJONG, DA PHILLIPS 1982 Effect of changes in shoot carbon-exchange rate on soybean root nodule activity. Plant Physiol 69: 432–436
29. WONG PP, HJ EVANS 1971 Poly-β-hydroxybutyrate utilization by soybean (*Glycine max* Merr.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol 47: 750–755
30. YATES MG 1980 Biochemistry of nitrogen fixation. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 1–63