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Reversible Dark-Induced Senescence of Soybean Root Nodules[1]

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ABSTRACT

Nodule senescence was induced in intact soybean [*Glycine max.* (L.) Merr., cv Woodworth] plants by an 8-day dark treatment. Dark-induced senescence resulted in the complete loss of acetylene reduction activity, a 67% loss of total soluble protein, and an almost complete loss in total leghemoglobin of nodule extracts. Isoelectric focusing gels demonstrated a preferential loss of certain proteins, which was correlated with an increase in endoprotease specific activity toward azocasein. Nodules were completely green after the 8-day dark treatment. If plants were returned to a normal photoperiod after 8 days in the dark, nodules recovered from the dark treatment in 12 to 16 days. Acetylene reduction activity returned to normal, and both total soluble protein and leghemoglobin were resynthesized while protease activity against azocasein decreased to the level of control nodules. The nodule population that had turned green after 8 days in the dark exhibited a progressive increase in red color starting nearest the exterior of the nodule, and after 16 days of recovery nodules were indistinguishable from control nodules maintained under a normal photoperiod.

Foliar dark treatment has been used to induce metabolic changes in legume nodules which are characteristic of nodule senescence (3, 15, 17, 28). Dark treatment of soybean plants caused an immediate decline in the level of nitrogenase activity, although at least 4 to 6 d of continuous darkness were required to completely inhibit N_2 fixation (17, 28). Declining glucose concentrations were closely correlated to the complete inhibition of nitrogenase activity (17), and although poly-β-hydroxybutyrate did not appear to be used as an alternate energy source for N_2 fixation, it might serve as a source of four carbon intermediates for bacteroid respiration (28). Dark treatment of soybeans for 8 to 11 d resulted in a 50% decline in total Lb[3] which indicated substantial changes occurring in host cell function. In contrast, no differences were detected in bacteroid nucleic acid content or size (17), which suggests that during dark-induced senescence of soybean nodules bacteroid integrity was maintained to a greater extent than host cell function.

The morphology of senescing legume nodules has been most effectively characterized in pea and alfalfa nodules which grow axially and possess a developing apical meristem, a symbiotic zone and a senescent zone connected to the lateral root (9, 16, 19, 27). Thus, a single pea or alfalfa nodule possesses zones of different age and symbiotic viability. Senescence of alfalfa nodules can be accelerated as a result of defoliation or prolonged darkness (4, 26, 27), and the capacity of a single nodule to fix nitrogen can be regained by virtue of reinfection of the meristematic tissue (27). The morphology of soybean nodules has been found to be quite different, however. Soybean nodules consist of only two types of tissue. Cortical tissue surrounds the central tissue, which consists of bacteroid-containing cells and interstitial cells. Bacteroidal tissue is arranged in concentric zones with the oldest, (*i.e.* senescent) cells at the center of the nodule (25). Thus, since soybean nodules are spherical and do not have detectable meristematic tissue, reinfection of a nodule should not occur. Therefore, it has been assumed that once nodule senescence begins (as evidenced by a decline in nitrogenase activity) it is irreversible unless new nodules are formed (1, 10, 12).

Initial experiments in this laboratory suggested that the metabolic changes observed in soybean nodules as a result of foliar dark treatment, including loss of nitrogenase activity, could be reversed. This study was conducted to investigate the changes in nodular metabolism which occurred as a result of dark-induced senescence, and to document the reversal of this process in soybean nodules.

MATERIALS AND METHODS

Special Chemicals. Arcillite was purchased from IMC Chemicals. TEMED and ammonium persulfate were obtained from Bio-Rad Laboratories, and carrier ampholytes were purchased from LKB. All other special reagents were from Sigma. Acrylamide and bis-acrylamide were recrystallized prior to use, and other reagents were used without further purification.

Seed Inoculation and Plant Growth. The methods used to prepare inoculum, to infect soybean (*Glycine max.* [L.] Merr., cv Woodworth) seeds and to grow soybean plants have been reported previously (14), except that *Rhizobium japonicum* (strain 61A89) was used, and each 15-cm pot contained only one plant. After plants had nodulated (when the first trifoliate leaf was fully expanded), they were repotted in sterile Arcillite to reduce the frequency of secondary nodulation. They were grown at 26° ± 2°C in an environmentally controlled chamber on a 14-h photoperiod. A combination of fluorescent (Westinghouse cool-white) and incandescent (Westinghouse-300 w) lighting was used, and light intensity averaged 700 μE $m^{-1} \cdot s^{-1}$. Plants were watered daily and given nitrogen free Hoagland solution twice weekly.

Dark-Induced Nodule Senescence. Plants used for dark-induced senescence were in the R-1 stage of reproductive growth (early flowering) (6). A total of five experimental trials were conducted. In three trials, intact plants were placed in a dark chamber constructed from heavy black plastic. In the remaining trials, intact plants were placed inside cardboard boxes. Cardboard boxes were found to be more suitable dark chambers because they provided better ventilation, but no differences in either the physical condition of plants and nodules, or in the biochemical param-

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[3] Abbreviations: Lb, leghemoglobin; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Bz-, *n*-α-benzoyl-; -βNA, -β-naphthylamide; Lba, leghemoglobin a; Lbc leghemoglobin c.

eters of nodules was detected due to the two types of dark chambers. The temperature inside the dark chambers did not vary significantly from the temperature of the surrounding growth chamber (26° ± 2°C), where control plants were kept on a normal 14-h photoperiod. In all cases, plants were left in the dark for up to 8 d. Nodules from plants maintained in darkness for up to 8 d were harvested at the end of the dark treatment. Nodules were also harvested from plants that had been in the dark for 8 d and then allowed to recover for 4 to 16 d under a normal 14-h photoperiod. Nodules from control plants were harvested from the beginning of the experimental period, and at intervals throughout the course of the experiments. All nodules were frozen immediately and stored at −80°C until used. Crude bacteroid-free extracts ('cytosol') were prepared as described previously (19).

Nodule Color and Size. Information on the size and interior color of nodules during the course of dark-induced nodule senescence and recovery was obtained from nodules that were harvested from plants immediately after acetylene reduction measurements were completed. Every attempt was made to collect all nodules from the root system, and then nodules were separated by diameter into three size categories: <2 mm, 2 to 3 mm, and >3 mm in diameter. Inasmuch as very few nodules greater than 3 mm in diameter were found on the root systems of any of the plants, all subsequent measurements were conducted using the 2 to 3 mm nodules and with nodules smaller than 2 mm in diameter. Twenty nodules from each of the two size groups were selected at random, cut open, and judged for interior color as completely red, completely green, or greenish-brown with varying amounts of red. Observations on the 20 nodules taken at random from each size category were extrapolated to give an indication of nodule color for the entire root nodule population.

Acetylene Reduction. Nitrogen fixation was measured on intact plants using the acetylene reduction assay (7). Potted plants were placed into 30 × 43 cm autoclave bags (Bellco Glass, Inc., Vineland, NJ) and sealed just below the first node with self-adhesive foam tape (Camper Seal Tape; Macklanburg-Duncan Co., Oklahoma City, OK). Acetylene (250 ml) and methane (1.0 ml) were injected into the bag via a rubber septum taped securely to the autoclave bag. The volume of acetylene was calculated to produce 0.1 atmosphere of acetylene, and methane (41.6 μmol) served as an internal standard. Assays were performed at approximately the same time of day to minimize diurnal variation. Triplicate gas samples were removed at 30 and 60 min and analyzed for ethylene on a Perkin-Elmer 3920 B gas chromatograph equipped with a hydrogen flame detector and a 3 m × 0.32 cm teflon column packed with Porpak N (Waters Associates, Inc.). The temperature of the carrier gas was 89°C and the flow rate was 40 ml/min. Excised nodules were also assayed for nitrogenase activity using the acetylene reduction assay (22). Ten taproot nodules or 20 smaller nodules from the lower root were picked from washed roots and immediately placed into 125 ml Erlenmeyer flasks containing filter paper wetted with 0.5 ml H_2O to maintain a moist environment, and then sealed with a rubber sleeved serum stopper. Acetylene (10 ml) and methane (0.2 μmol) were injected through the rubber stopper, and triplicate gas samples were removed at 20 and 40 min, and analyzed as described for intact plants.

Enzyme Assays. Endopeptidase activities were measured as described previously (14) using azocasein and Bz-L-Arg-βNA as substrates, except that the azocasein assay was modified in the following manner. Nodule extract (150 μl) was added to 250 μl of azocasein (10 mg/ml in 50 mM Na-phosphate buffer, pH 7.0) and incubated in a shaking water bath for 1 h at 37°C in 1.8 ml Beckman microfuge tubes. The reaction was terminated with 1.2 ml 10% TCA. The tubes were centrifuged for 1 min at 8,730*g* in a Beckman Microfuge B, and 1.4 ml of supernatant fluid were added to 1.4 ml of 1.0 N NaOH. The absorbance of the resulting solution was read at 440 nm. One unit of endopeptidase activity was the amount of enzyme required to produce a change in absorbance of 1.0 (1.2 cm) in 1 h under the conditions of the assay. Aminopeptidase activities were measured with L-Ala-βNA and L-Leu-βNA as substrates (14). One unit of activity was the amount of enzyme required to liberate 1 μmol of the product (-βNA) per h under the conditions of the assay.

Protein and Leghemoglobin. Total soluble protein in the cytosol was determined by the Bradford dye-binding procedure using Bio-Rad dye reagent (2). BSA was used as a protein standard. Total Lb in nodule extracts was determined by the pyridine hemochrome assay (20) by mixing 0.1 ml nodule extract in 1.4 ml H_2O with an equal volume of 4.4 M pyridine in 0.2 M NaOH. Concentrations were estimated from (dithionite-reduced minus $Fe(CN)_6^{-3}$-oxidized) difference spectra at 556 and 539 nm (ΔA_{mM} = 23.4) in a Cary 219 spectrophotometer. Conversion to mg Lb/ml was made using an average mol wt of 16,375 for Lb (5). In addition, the relative concentrations of Lba and Lbc were estimated from scans of polyacrylamide gels. Lba and Lbc were separated in bacteroid-free nodule extracts in cylindrical 6.5% polyacrylamide gels by the method of Laemmli and Favre (11) except that SDS was omitted. Aliquots (50 μl) of nodule extract were applied to the gel tubes and after electrophoresis (2 h at 1.5 mamp/tube) the gels were scanned at 340 nm. In separate experiments using different volumes of purified Lba and Lbc, peak areas for Lba and Lbc were proportional to sample volume; thus, relative concentrations of Lba and Lbc could be calculated between samples. The relative concentrations of Lba and Lbc were quantitated by integrating the peak areas.

Isoelectric Focusing. Isoelectric focusing of nodule extract was performed in 5% acrylamide gels (29), using a pH gradient of 3.5 to 10, and then stained with Coomassie blue R-250 (0.02% in H_2O).

Statistical Analysis. Statistical analyses of nodule numbers and total weights were conducted using analysis of variance of a two-factor factorial conducted in a completely randomized design (23). Least squares means and their associated standard errors were computed and pair-wise *t* tests were used to detect significant differences among the treatments. Least squares means were necessary since the treatments varied in numbers of observation per mean. Results of biochemical assays were reported as mean values only.

RESULTS

Properties of the Experimental Plants. At the time when young soybean plants were transplanted into sterile Arcillite, the roots were extensively nodulated. Growth and development of the plants was normal until they were subjected to the experimental treatment at the R-1 stage of reproductive growth (early flowering) (6). Although the plants were repotted in sterile Arcillite, total nodule weight and numbers continued to increase during the experimental period, which was a time span of 24 d (Table I). The increase in the number of nodules was probably due to continuing infection and to the fact that very young nodules on the initial control plants could not be harvested or counted due to their small size. However, there was no significant increase in nodule weight or number between plants that were kept in the dark for 8 d as compared to controls maintained under a normal photoperiod for 8 d, or in plants that underwent the dark treatment and subsequent recovery, as compared to control plants maintained under normal light conditions for the duration of the experimental period (20–24 d) (Table I). The experimental protocol was repeated three times over a period of several months, and the values shown in Table I are a composite of all the data. Differences in growing conditions such as plant density led to slight differences in plant size and the extent of nodulation which resulted in the variation seen in Table I for nodule number and weight, although all plants were just beginning to flower when the dark treatment was initi-

Table I. *Nodule Weights and Numbers per Plant from Soybeans Subjected to Dark Treatment and from Control Plants Maintained under Normal Light Conditions*

Plants were grown hydroponically in Arcillite, and roots were harvested carefully to obtain the maximum number of nodules possible.

Treatment[a]		Total Nodule Weight[b]		Nodule Number[b]	
Days in dark	Days in light	Mean	SE	Mean	SE
		g			
0	0	3.99 A	0.49	228 A	22
8	0	4.45 A	0.51	247 A	23
0	8	4.67 A	0.41	262 A,C	18
8	12–16	6.48 B	0.36	348 B	16
0	20–24	6.49 B	0.44	312 B,C	19

[a] Day 0 taken as the first day of the experiment, when plants were beginning to flower. The entire experimental protocol was repeated three times using 1 to 3 soybean plants for each treatment in each of the repeated experiments.

[b] Values represent the least squares mean and SE of nodules collected from between 6 and 11 plants obtained from analysis of variance of a two-factor factorial. Means denoted by the same letters are not significantly different at the 5% level of probability.

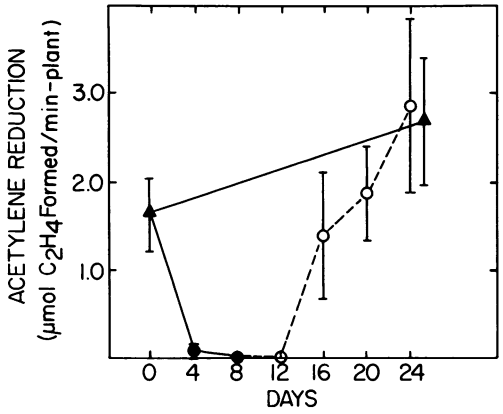

FIG. 1. Acetylene reduction by intact soybean plants.(▲), Control plants; (●), plants kept in total darkness; (○), plants returned to normal photoperiod after 8 d in the dark. Bars represent the mean and SD of assays from 2 to 5 plants.

ated. Nodule number and weight within a treatment from a single experiment showed very little variation (data not shown), but nodule number and total weight varied between growth experiments.

Dark-Induced Senescence of Soybean Nodules. Soybean plants at the R-1 stage of reproductive growth (early flowering) were removed from the normal 14-h photoperiod and placed in total darkness for up to 8 d. After the 8-d dark treatment, plants were returned to a normal 14-h photoperiod for up to 16 d. Throughout the course of the experiment, control plants were kept on a normal 14-h photoperiod, and observations of nodules from these plants served as control values. During the period of dark stress, the acetylene reduction activity of intact plants was reduced to 5% of the initial control in 4 d, and was below detection after 8 d (Fig. 1). Eight d of continuous darkness resulted in considerable leaf yellowing, with leaf color ranging from yellow to pale green. The exterior of the root nodules looked normal, but examination of the interior revealed that virtually 100% of the nodules were green inside, presumably due to the loss of Lb (Fig. 2, A and B). When plants were returned to a normal 14-h photoperiod after 8 d in the dark, the yellow leaves dropped from the plant but the pale green

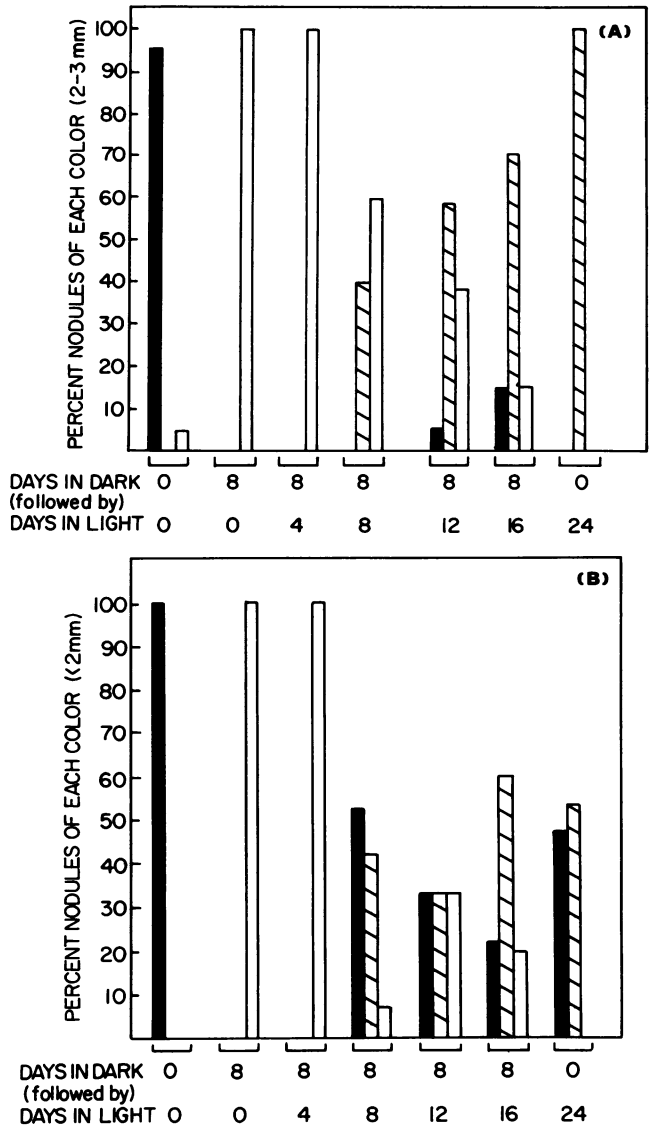

FIG. 2. Interior color distribution of soybean root nodules (A) 2 to 3 mm in diameter and (B) less than 2 mm in diameter harvested from control plants at the beginning and end of the experimental period, after 8 d in the dark, and following a recovery period of 4, 8, 12, and 16 d under a normal photoperiod. Two plants were harvested at each experimental period, and twenty nodules of each size from each plant were cut open and judged for interior color. Nodules were either bright red (solid bar), red with varying amounts of green or brown toward the center (hatched bar), or completely green (open bar).

leaves returned to a normal dark green color. Nodules from plants returned to a normal 14-h photoperiod were still quite green after 4 d, but then a progressive change in nodule color was observed, regardless of nodule size (Fig. 2, A and B). After a recovery period of 8 d, many nodules appeared to regain their red color, although green pigment was still observed at the center of most nodules. In the case of larger nodules (2 to 3 mm in diameter), new nodule growth would be extremely unlikely, because newly formed nodules would not be 2 to 3 mm in diameter after only 8 d of growth. After 16 d of recovery, over 70% of the large nodules (2 to 3 mm in diameter) were almost completely red, with only a small amount of green or brown pigment in the center of the nodule. Nodules from control plants examined at the beginning of the experiment were uniformly bright red, but nodules from control plants at the end of the experimental time period (24 d) had begun to turn

slightly green or brown in the center, probably as a result of the normal aging process, and were indistinguishable from recovered nodules. Nodules less than 2 mm in diameter, which were most likely a result of secondary infection, also turned green following the 8-d dark treatment (Fig. 2B). Thus, dark treatment resulted in greening of the entire nodule population, regardless of nodule size. These smaller nodules subsequently recovered in a manner similar to that of the larger nodules, except that a higher percentage of completely red nodules was observed during the recovery period. Since the total nodule number was increasing during the course of the experiment, it is likely that these small completely red nodules represented new nodule formation during the recovery period. About 50% of the small nodules from control plants at the end of the experiment (24 d) had turned slightly brown or green in the center, similar to the larger nodules from control plants. Acetylene reduction of the intact plants also increased progressively during the recovery period (Fig. 1), and after 16 d of recovery was not significantly different from that of control plants.

Excised Nodules. Taproot nodules and nodules from the lower roots (presumably the result of secondary nodulation) were removed from the plants and assayed for nitrogenase activity by the acetylene reduction method. The specific activity of both taproot nodules and smaller secondary nodules from control plants decreased as the nodules aged during the 24-d course of the experiment (Table II). Excised nodules from plants kept in the dark for 8 d lost the ability to reduce acetylene, but when these plants were returned to normal light conditions, both taproot nodules and secondary nodules regained the ability to reduce acetylene. After 11 to 18 d of recovery, the specific activity of both nodule types was actually higher than the corresponding activities of nodules from control plants (Table II). The color distribution of these nodules was similar to that of the root populations as a whole shown previously in Figure 2, A and B. Thus, taproot nodules from plants which were recovering from dark-induced senescence and which had red perimeters and green centers also had active nitrogenase activity.

Effect of Dark-Induced Senescence on Nodule Proteins. The amount of soluble protein in nodule cytosol measured per g fresh weight of nodule decreased by 30% when plants were kept in total darkness for 4 d, and after 8 d of dark treatment, soluble protein decreased by nearly 70% (Fig. 3A). Nodule protein levels continued to decrease during the first 4 d of recovery in a normal photoperiod, but after 8 d of recovery nodule protein had started to increase, and by 16 d the soluble protein concentration of nodules from recovered plants was similar to the protein concentration of control plants maintained on a normal photoperiod (Fig. 3A). The concentration of total Lb measured as pyridine hemochrome also decreased as a result of dark treatment (Fig. 3B). Four d of dark treatment resulted in only a slight decrease in Lb content, but after 8 d of darkness, Lb concentrations were below detection limits. Thus, Lb appeared to be maintained for an initial period in the dark after acetylene reduction was lost, but was then degraded more rapidly than total soluble protein during the last 4 d of dark treatment. The Lb content of nodules began to increase after only 4 d of recovery while total protein was still declining. Thus, this increase may have represented heme synthesis only, and not functional Lb. The Lb content of nodules during recovery from dark-induced senescence continued a steady increase, and after 16 d of recovery it was very similar to the content in nodules from control plants (2.8 and 3.3 mg, respectively). Quantitation of Lba and Lbc separated by polyacrylamide gel electrophoresis demonstrated that both fractions decreased by 80% during dark-induced senescence and returned to normal levels in nodules from plants allowed to recover in the light. Differences in degradation rate in the dark or resynthesis during recovery were not detected between the two fractions.

The proteins in extracts from nodules harvested during dark-induced senescence and recovery were separated by isoelectric focusing (Fig. 4). As expected from the decrease in total soluble

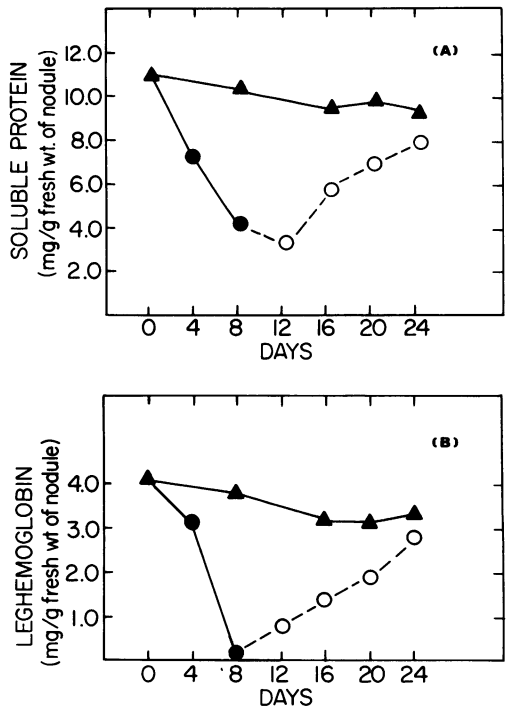

FIG. 3. A, Total soluble protein in nodule cytosol determined by the dye-binding assay. Mean protein concentration (▲) of nodules from plants maintained under a normal 14-h photoperiod, (●) from plants kept in total darkness, and (○) from plants kept in darkness for 8 d, then allowed to recover under a normal 14-h photoperiod for up to 16 d. B, Total leghemoglobin of soybean root nodule extract. Leghemoglobin was determined by the pyridine hemochrome method as described in "Materials and Methods." (▲), Lb concentration of nodules from control plants maintained under a normal photoperiod throughout the experiment; (●), Lb concentration of nodules from plants kept in the dark for 8 d; (○), Lb concentration from nodules of plants returned to a normal photoperiod after 8 d in the dark.

Table II. *Acetylene Reduction by Excised Taproot and Secondary Nodules from Soybean Plants Subject to Dark Treatment and from Control Plants Maintained under Normal Light Conditions*

Treatment[a]		Taproot Nodules[b]	Secondary Nodules[b]
Days in dark	Days in light		
		nmol C_2H_4 formed/min·g nodule fresh wt	
0	0–8	56.5 ± 15.5	98.0[c]
8	0	0	0
8	11–18	37.4 ± 23.6[d]	78.2 ± 38.5[d]
0	19–26	25.9 ± 7.0	25.5 ± 3.5

[a] Day 0 taken as the first day of the experiment, when plants were beginning to flower.

[b] Values represent the mean and SD of from 1 to 3 plants per treatment. Two samples each containing 10 taproot nodules or one sample containing 20 small nodules from the lower roots of each plant were assayed by acetylene reduction.

[c] This value represents a single observation.

[d] Relatively large variations in acetylene reduction activity of excised nodules were observed in nodules recovering from dark treatment because the percentage of viable nodules (red *versus* green) varied. Acetylene reduction could not be detected in green nodules (data not shown).

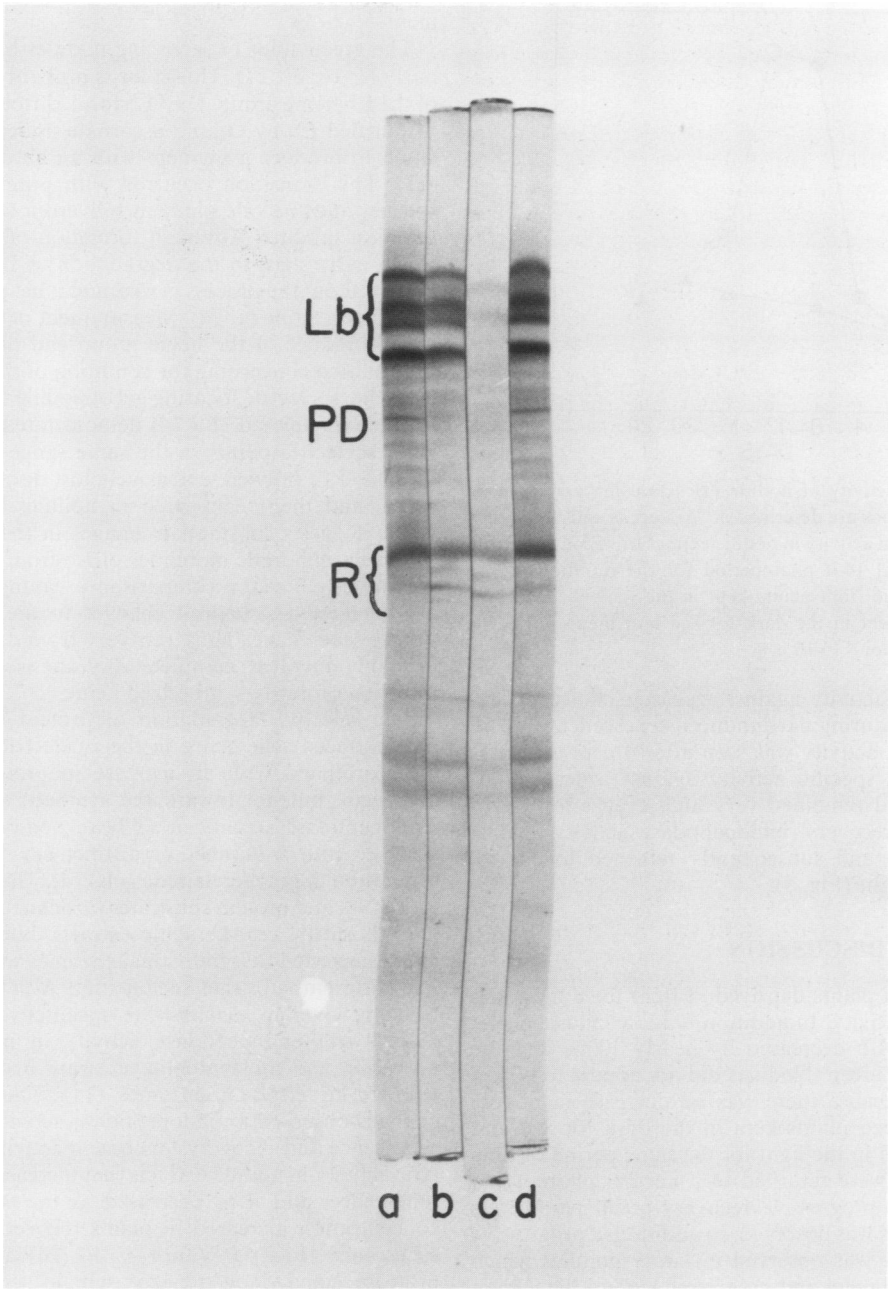

FIG. 4. Isoelectric focusing gels, pH 3.5 to 10.0, of nodule extracts. Soybean nodules were induced to senesce in the dark, and then allowed to recover under a normal 14-h photoperiod. (a), Extract from nodules of a plant harvested at the beginning of the experimental period (early flowering); (b), extract from nodules of a plant harvested after 4 d in the dark, and (c), after 8 d in the dark; (d), extract from nodules of a plant allowed to recover for 16 d under a normal photoperiod after 8 d in the dark. (R), protein bands that were resistant to degradation; (PD), protein bands that were preferentially degraded; and (Lb), vicinity of leghemoglobin bands.

protein observed in senescent nodules, nearly all protein bands stained less intensely in nodule extracts from the 8-d dark treatment when equivalent sample volumes per g fresh weight were applied to the gels. Although differences in staining intensity of the gels could only be interpreted on a qualitative basis, it was clear that some proteins were degraded much more rapidly than others, while other proteins appeared to be remarkably resistant to degradation. The proteins that were rapidly degraded during the dark treatment reappeared when plants were allowed to recover under a normal photoperiod.

Effect of Dark-Induced Senescence on Peptidohydrolase Activities. We have recently characterized a number of proteolytic enzymes in soybean nodules (14). Since these enzymes may be responsible for the differential degradation of proteins observed in isoelectric focusing gels of nodule extracts (Fig. 4), their activities were measured during dark-induced senescence and recovery. Specific activities of aminopeptidases measured with either L-Leu-βNA and L-Ala-βNA as the substrates decreased from 0.40 ± 0.03 and 0.47 ± 0.09 units/mg soluble protein, respectively, to 0.29 ± 0.07 and 0.27 ± 0.06 units/mg soluble protein after the 8-d dark treatment, and then returned to initial values after 12 to 16 d of recovery. Endopeptidase activity measured with the synthetic substrate Bz-Arg-βNA followed a similar pattern (data not shown). Thus, the specific activity of aminopeptidases and endopeptidases against these synthetic substrates declined during dark-induced senescence in the same manner as total soluble protein

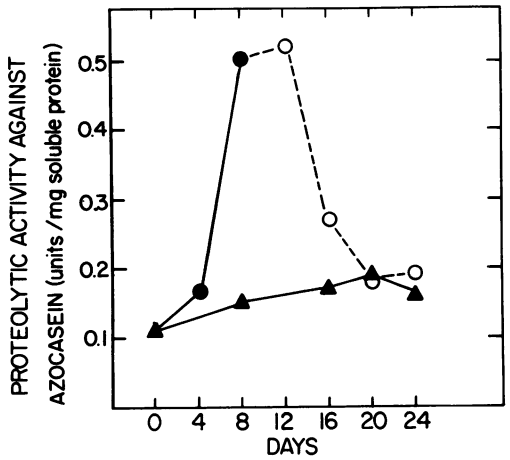

FIG. 5. Endopeptidase activity of nodule extracts using azocasein as the substrate. Assay conditions are described in "Materials and Methods." (▲), Mean value of azocasein activity in nodule extract from control plants maintained under a normal 14-h photoperiod for the duration of the experiment; (●), mean value from plants kept in the dark for 8 d; (○), mean values from plants kept in the dark for 8 d and then allowed to recover under normal light for 4 to 16 d.

and Lb. Endopeptidase activity against azocasein, however, exhibited a 4-fold increase during dark-induced senescence (Fig. 5). Only a small increase in activity was seen after 4 d in the dark, but then endopeptidase specific activity against azocasein increased dramatically, and remained very high even after 4 d of recovery. After 8 d of recovery, endopeptidase activity against azocasein had declined, and subsequently returned to control levels after 12 d in the light (Fig. 5).

DISCUSSION

Nodules from soybean plants deprived of light for 8 d had no detectable nitrogenase activity. In addition, soluble cytosolic protein decreased by 67%, Lb decreased by nearly 100%, and the nodules were completely green. Nodules did not appear to be lost from the root system because there was no decrease in nodule number or weight between plants kept in the dark for 8 d and control plants maintained in the light for the same period of time (Table 1). When plants were returned to a normal photoperiod after 8 d of darkness, a progressive recovery of all parameters assayed in nodule extracts was observed. In addition, a progressive recovery of nodule color was observed in larger nodules which started on the nodule perimeter and progressed toward the nodule center until the entire nodule was red and indistinguishable from control nodules of similar age and size. Although secondary nodulation was apparently occurring throughout the experimental period, as evidenced by the increase in nodule number between the initial light control and the light control harvested at the end of the experimental period (Table I), the recovery of nitrogenase activity following dark-induced senescence cannot be explained by the growth of new nodules alone. Acetylene reduction measurements with excised nodules demonstrated that both taproot nodules and small secondary nodules which had undergone dark-induced senescence and recovery were capable of N_2 fixation. In addition, examination of very small nodules (<2 mm in diameter) during the recovery period revealed the same color distribution as larger nodules. Assuming that very small nodules are most likely to be the youngest nodule population, it is clear that younger nodules were affected by the dark treatment in the same manner as the larger, presumably older, nodules. Thus, dark-induced senescence was observed in the entire nodule population, and both large and small nodules appeared to recover from the dark treatment.

The green color of senescing nodules has been reported previously (1, 16, 21, 27). This color is probably due to the destruction of the Lb heme group. Oxy-Lb formed from the coupled oxidation of purified Lb by O_2 and ascorbate undergoes a molecular transition to produce a pigment with an absorption peak at 675 nm (13). This transition occurred with pure Lb from a variety of species, and the bile pigment biliverdin was isolated as the degradation product. Although formation of this pigment may represent early steps in the degradation of Lb (13), little is actually known about the process *in vivo*, and it has generally been assumed to be irreversible (1, 24). Measurement of Lb (Fig. 3B) was based on absorbance of the heme group and does not give any direct information concerning the condition of the Lb apoprotein. However, the isoelectric focusing gel of nodule cytosol from plants kept in the dark for 8 d (Fig. 4) demonstrated that all protein bands with isoelectric points in the same range as the Lb components (pH 4.5–5.0) (8) were extensively lost during dark-induced senescence, and then reappeared in nodules from recovered plants, which suggests substantial changes in the Lb apoprotein *per se*. The electrophoretic mobilities of Lb from senescing cowpea nodules were reduced in comparison to younger nodules (24), which also suggested structural changes in the protein moiety of Lb during senescence. Thus, recovery from dark-induced senescence probably involves complete resynthesis of Lb apoprotein and insertion of newly synthesized heme.

The selective degradation of protein bands depicted during dark-induced senescence in the isoelectric focusing gels (Fig. 4) was correlated with an increase in proteolytic activity toward azocasein, but not toward the synthetic substrate Bz-Arg-βNA. Endopeptidase activity in soybean nodules has previously been resolved into a number of distinct enzymes by electrophoretic separation in polyacrylamide gels (14). The synthetic substrate Bz-Arg-βNA and protein substrates (azocasein and hemoglobin) were hydrolyzed by crude nodule extracts, but electrophoretic differences suggested that individual enzymes within the nodule extract had different substrate specificities. Although there appeared to be some overlap in substrate specificity, it was clear that the majority of endopeptidase activity in nodule extracts against azocasein and the synthetic substrate Bz-Arg-βNA could be attributed to different enzymes (14). This is supported by the changes observed in endopeptidase activity during dark-induced senescence and recovery. Azocasein activity was the only proteolytic activity in nodule extracts that increased during dark-induced senescence, and then decreased as the soluble protein and Lb concentration increased in plants recovering from dark-induced senescence (Fig. 5). Vance *et al.* (26) also reported increased protease activity toward azocasein in senescing alfalfa nodules after defoliation. The correlation between proteolytic activity against azocasein and selective protein degradation observed during dark-induced senescence suggests that this activity may play a key role in nodule senescence.

Dark-induced senescence of legume nodules has been used as a model for natural senescence by a number of investigators (3, 17, 18, 21, 28). Parameters such as nitrogen fixation, carbohydrate reserves, soluble protein, and Lb have responded in similar fashions during dark-induced and natural senescence, suggesting that dark treatment may be used as a model to study natural senescence. In addition, we have shown that the degeneration of nodule tissue caused by dark treatment is a reversible process. Because soybean nodules growing in the field may be subject to various environmental conditions such as water or heat stress or low-light intensities in rainy weather which could cause some loss of nodule function, the ability to recover from stress would be important. Thus, the documentation that soybean nodules can recover from dark-induced senescence without the reinfection of meristematic tissue that occurs in other legumes suggests that soybean nodules

may be capable of recovery from severe environmental stress without the formation of new nodules.

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