Plant Physiol. (1987) 85, 137–144
0032-0889/87/85/0137/08/$01.00/0

Carbohydrate Supply and N_2 Fixation in Soybean[1]

THE EFFECT OF VARIED DAYLENGTH AND STEM GIRDLING

Received for publication March 13, 1987 and in revised form May 29, 1987

KERRY B. WALSH*, J. KEVIN VESSEY[2], AND DAVID B. LAYZELL
Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

ABSTRACT

When arrival of shoot supplied carbohydrate to the nodulated root system of soybean was interrupted by stem girdling, stem chilling, or leaf removal, nodule carbohydrate pools were utilized, and a marked decline in the rates of CO_2 and H_2 evolution was observed within approximately 30 minutes of treatment. Nodule excision studies demonstrated that the decline in nodulated root respiration was associated with nodule rather than root metabolism, since within 3.5 hours of treatment, nodules respired at less than 10% of the initial rates. Apparently, a continuous supply of carbohydrate from the shoot is required to support nodule, but not root, function. Depletion of nodular carbohydrate pools was sufficient to account for the (diminishing) nodule respiration of girdled plants. Of starch and soluble sugar pools within the whole plant, only leaf starch exhibited a diurnal variation which was sufficient to account for the respiratory carbon loss of nodules over an 8 hour night. Under 16 hour nights, or in continuous dark, first the leaf starch pools were depleted, and then nodule starch reserves declined concomitant with a decrease in the rates of CO_2 and H_2 evolution from the nodules. Nodule soluble sugar levels were maintained in dark treated plants but declined in girdled plants. The depletion of starch in root nodules is an indicator of carbohydrate limitation of nodule function.

In legume nodules, maximal rates of dinitrogen fixation are achieved under conditions favourable to photosynthesis (13, 15, 21, 26). Recent studies of ^{14}C (6) or ^{13}C (12) transport from shoots of soybean fed labeled CO_2 have shown that nodules have a greater dependence than roots on photosynthate supplied recently by the shoot. $^{14}CO_2$ fixed in leaves moves into the phloem primarily as sucrose (3) and reaches the nodule predominately in this form (16). In nodules, the relative specific activities of the sucrose and respired CO_2 pools rose within 4 h to equal that of the steady state feed gas (12). In contrast, while the root sucrose pool displayed similar kinetics of labeling, root-respired CO_2 did not reach equilibrium within 10 h. This suggests that roots utilize a pool other than recent photosynthate for respiration. However, despite their utilization of current photosynthate from the shoot, soybean nodules are able to maintain a constant rate of N_2 fixation during the night, if temperature is held constant (24, 26). This suggests that carbon reserves within the plant can be mobilized in support of nodule activity.

In contrast, the location and the chemical nature of the carbon pool supporting nodule respiration during nonphotosynthetic conditions are not well characterized. Yet, the premise of detached nodule or excised root assay of whole plant N_2 fixation rate is that this pool is located in the nodule or root (14). Within soybean, several significant carbohydrate pools have been identified, including starch, sucrose, hexose and cyclitol pools in all plant tissues, and Phb[3] in nodule bacteroids. Leaf starch probably represents the largest storage pool of carbon within the soybean plant. Further, this pool fluctuates markedly in size on a diurnal basis, generally maintaining a constant rate of export from the leaf (7, 8). Nodule cyclitols and Phb pools have an extremely show turnover rate (11, 12, 16, 28) and are therefore unlikely to play a role in the support of nodule respiration. However, nodule starch and sugar levels decline under conditions of carbohydrate limitation (15, 22, 25) and are therefore possible candidates. Rainbird *et al.* (15) estimated that the depletion of nodule starch and sugar was sufficient to account for 50% of the nodule respiration over a night period.

This study examines the dependence of nodule function on various starch and soluble sugar pools within the plant under conditions of varied carbohydrate availability created by varying daylength or interrupting the phloem connection to nodulated roots. This is done to assess the role of carbohydrate limitation in determining nodule activity and to identify a pool in which fluctuations are indicative of the carbon status of the nodule.

MATERIALS AND METHODS

Plant Culture. Soybean seeds (*Glycine max* [L.] Merr. cv Maple Arrow) were inoculated by imbibing seed in a slurry of peat inoculum (Urbana Laboratories, Urbana, IL) of *Bradyrhizobium japonicum* USDA 16, a strain lacking uptake-hydrogenase activity (24). The cultivar Maple Arrow is daylength insensitive with respect to flowering (23). Thus, developmental changes in partitioning patterns should not have been invoked in the varied daylength experiments described below. Plants were grown in silica sand as previously described (16/8 h photoperiod, 450 $\mu E/m^{-2} \cdot s^{-1}$ at canopy level, 25 ± 1°C constant day/night) (24) except that they were watered daily with a N-free nutrient solution containing 1.0 mM K_2SO_4, 1.2 mM K_2HPO_4, 0.5 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 74 μM Fe(II) as Fe-sequesterene 330 (CIBA-Geigy Canada Ltd., Mississauga, Ont.), 4.5 μM H_3BO_3, 2.0 μM Na_2MoO_4 adjusted to pH 6.5 with KOH. The pots used for plant culture (24) were easily sealed at top and bottom for use as root system cuvettes in open gas exchange measurements. In all experiments, plants were between 25 and 35 d old, displayed 3 to 4 trifoliate leaves, and had leaf, stem + petiole, root, and nodule dry weights of approximately 470, 250, 350, and 70 mg, respectively. Plants used in the daylength treatment, although grown within the same growth cabinet as

[1] Supported by NSERC (Canada) Operating Grant to D. B. L., a Commonwealth Postgraduate Award to K. B. W., and a NSERC Postgraduate Scholarship to J. K. V.

[2] Present address: Department of Soil Science, North Carolina State University, Raleigh, NC 27695.

[3] Abbreviations: Phb, polyhydroxy-butyrate; DAP, days after planting; EAC, electron allocation coefficient.

those for the phloem interruption treatment, were slightly larger. The plants were crown nodulated, with approximately 40 nodules per plant. Nodule specific activity (H_2 evolution in N_2:0_2/g dry weight nodule) peaked 28 d after planting, although continued nodule growth allowed whole plant nitrogenase activity to increase until week 6 after planting.

Phloem Interruption Treatments. Translocation between shoot and roots was interrupted by (a) girdling, (b) stem chilling, or (c) leaf removal. In the girdling treatment, the phloem tissues were removed surgically from a 1-cm segment at the base of the stem. Xylary tissues were left intact and functional, since leaves maintained turgidity and maintained transpiration and photosynthesis at pregirdling rates for at least 10 h after girdling (data not shown). The stem chilling treatment employed a 3 cm long, plastic water jacket fitted to the lower stem. The jacket was connected in series to a recirculating water bath operating at 2°C. The girdling treatment was imposed at various times throughout the day/night period.

A 12-channel, open gas exchange system (24) was used to monitor CO_2 and H_2 evolution in air from nodulated roots of intact plants. Estimates of the relative contribution of roots and nodules to nodulated root respiration were determined for 5 plants at 0, 3.3, and 24 h after girdling. The steady state rate of gas exchange from a nodulated root was measured, the pot was removed from the gas exchange system, and the nodules were exposed by tipping sand out of the pot. Because the plants were largely crown nodulated, this procedure accessed 80 to 90% of the plant nodule mass. These nodules were excised, weighed, and frozen in liquid N_2. The denodulated roots of the otherwise intact plant were covered with sand, the pot was resealed, and steady state rates of H_2 and CO_2 evolution in air were measured. The difference between the initial and final gas exchange rates was attributed to the nodules which were removed. The denodulation procedure took less than 10 min to execute, and the plants showed no signs of stress subsequent to the procedure. A similar disturbance of nonnodulated, nitrate-grown plants did not affect the rate of root CO_2 evolution (data not shown).

Total electron flow through nitrogenase before and after the phloem interruption treatments was measured either as H_2 evolution in Ar:O_2 (9) or as acetylene reduction activity. H_2 evolution in Ar:O_2 was measured by switching the gas stream flushing the pot from air to an Ar:O_2 mixture (80:20) and recording the peak in H_2 evolution which occurred within the subsequent 10 min. This procedure was repeated with fresh plant material at various times following interruption of the phloem supply. H_2 and CO_2 evolution was also monitored from nodulated roots maintained in Ar:O_2 (80:20) before and after interruption of the phloem supply to the roots by girdling the lower stem.

Acetylene reduction activity was measured by switching the gas stream flushing the pot from air to N_2:O_2:C_2H_2 (70:20:10) and monitoring the changes in C_2H_4 concentration in the inflow and outflow gas streams every 30 s to 10 min over the subsequent 200 min. One-ml samples were taken from the gas streams and injected into a gas chromatograph (Shimadzu, model GC-8A) equipped with a poropak N column (60–80 mesh) and a flame ionization detector (24). When stable rates of ethylene production were obtained (within 30 min), the plant was girdled as described above.

The EAC of nitrogenase (9, 20) was calculated as:

$$EAC = 1 - \frac{H_2 \text{ evolution in air}}{H_2 \text{ evolution in Ar:}O_2}$$

At 0, 0.25, 1.0, 3.3, and 24 h after phloem interruption, five plants were removed from the gas exchange system and the root systems frozen in liquid N_2. Roots and nodules were later separated and ground in a cooled mortar for starch and soluble sugar analysis.

Varied Daylength Treatments. Plants selected from the general population (photoperiod: 16 h day/8 h night) at 28 d after sowing were placed in growth cabinets (constant 25°C) programmed for one of the following photoperiod treatments: 24/0, 16/8, 8/16, 0/24 (h day/h night). During the study period (28–31 DAP), three plants from each treatment were continually monitored for steady state rates of CO_2 and H_2 evolution in air from the intact nodulated roots. The plants monitored on the gas exchange system were replaced approximately every 4 h. Since all gas exchange measurements were carried out in the growth cabinet used for the 24/0 treatment, the shoots from plants of darkened chambers were darkened at appropriate intervals by placing over each plant a ventilated, 15-cm diameter PVC pipe sealed at the top.

At times throughout the study period (as indicated in Fig. 5), four plants were selected at random from each treatment. Each plant was divided into four fractions (leaves, stems + petioles, roots, and nodules), frozen in liquid N_2 and stored at −20°C. Prior to analysis, fractions from four plants were bulked and dried for 48 h in a forced air draft oven at 80°C. The proportion of dry:fresh weight was 0.23 for nodule, and 0.10 for root tissue. Determinations were made of fractions of individual plants harvested at the beginning and end of each photoperiod and at 16-h intervals in the continuous light and darkness treatments. Tissues were weighed, then finely ground in a Micro-Hammer Cutter mill (Glen Mills Inc., Maywood, NJ) prior to starch and soluble sugar analysis.

Tissue Analysis. Between 25 and 50 mg of dried tissue and between 250 and 400 mg of fresh tissue was extracted three times with hot 80% ethanol into a total volume of 10 ml. This ethanol fraction was dried and resuspended in deionized water and total soluble sugar content measured by the anthrone method (4) using sucrose standards. The ethanol-insoluble fraction was resuspended in 5 ml of deionized water and assayed for starch content using the enzymic method of Jones (10).

Statistical Analysis. Values presented in Figures 3, 4, and 5 are means of 5, 4, and 4 replicates, respectively, with an associated representation of one standard error of the mean. Statistical comparisons (Table II) were based on a 95% confidence interval.

RESULTS

Changes in CO_2 and H_2 Evolution in Air Following an Interruption in Phloem Supply to the Root. When the nodulated roots of intact plants were sealed into the gas exchange system, steady state rates of H_2 and CO_2 evolution in air were obtained within 10 to 15 min. Interruption of the phloem supply to the nodulated roots by girdling, stem chilling, or leaf excision, had no effect on nodulated root respiration and H_2 evolution in air for 20 to 30 min. However after this period the rates declined sharply to approximately 35 and 10%, respectively, of the initial rates within 2 to 3 h from the start of treatment (Fig. 1, A and B). The rate of H_2 evolution in air continued to decline such that within 2 to 4 d no detectable H_2 was evolved from the nodulated roots (data not shown) Removal of the cold block reversed the effect, allowing full recovery of both CO_2 and H_2 evolution in air within 3 h (Fig. 1, A and B). Similar results were obtained when the phloem interruption treatments began during the day or the night periods, and no diurnal fluctuations in H_2 and CO_2 evolution in air were observed in the control plants.

The magnitude of the declines in CO_2 and H_2 evolution in air following an interruption of the phloem supply varied with nodulation status, age, or growth conditions of the plants. The declines were less in poorly effective symbioses, in older plants which were flowering or fruiting, in plants which were root chilled, and in plants grown under low irradiance (data not shown). A common characteristic of these treatments was a specific rate of H_2 evolution in air which was 10 to 50% of that

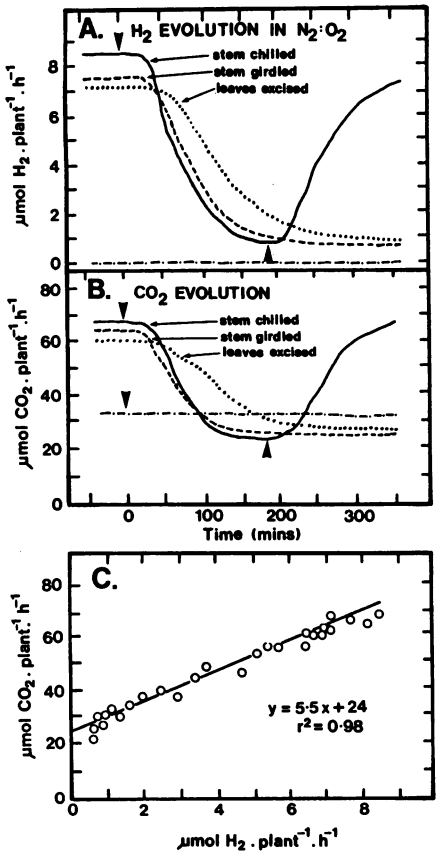

FIG. 1. Time course of H_2 (A) and CO_2 (B) evolution in air from nodulated soybean roots in which the phloem supply to the root system was interrupted by leaf excision, stem girdling, or stem chilling (as indicated on the figure). The effect of stem girdling of a nonnodulated, nitrate grown plant is also shown (-·-·-·-). The arrowheads denote the time (T = 0 min) at which the treatments were imposed (▼), and the time when the stem chilling treatment was removed (▲). Results are for individual plants, but are representative of many trials. The regression of CO_2 evolution against H_2 evolution in air (C) was obtained from the rates of gas exchange at specific times following the start of the stem girdling treatment.

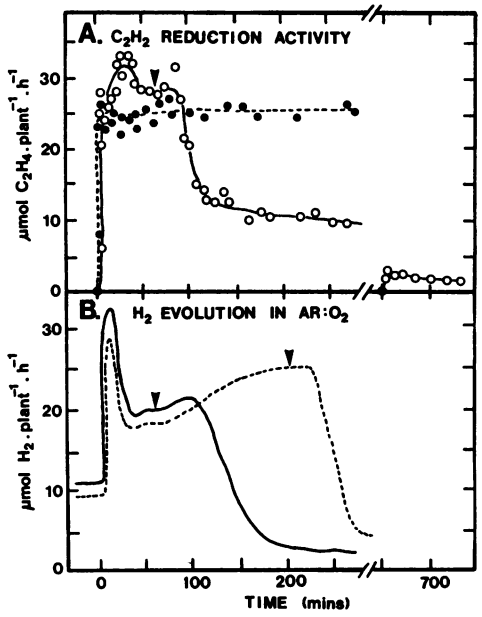

FIG. 2. Effect of stem girdling on the time course of total nitrogenase activity measured as C_2H_2 reduction (A) and H_2 evolution in Ar:O_2 (B). At T = 0 min, the nodulated roots were exposed to either C_2H_2:N_2:O_2 (10:70:20) or Ar:O_2 (80:20) and when relatively stable rates were obtained, the lower stems were girdled (solid arrowheads). An ungirdled, control plant is shown in part A (●). In part B, one plant (- - -) was girdled 150 min after the other (——), and serves as a control for the effect of continuous exposure to Ar:O_2 over this interval.

in the highly effective, vegetative plants used in the experiments reported in Figure 1.

Effects of Interrupting Phloem Supply to Nodulated Roots on Total Nitrogenase Activity. To determine whether the changes in H_2 evolution in air following phloem interruption were associated with changes in EAC or changes in total nitrogenase activity, measurements were made of C_2H_2 reduction activity and H_2 evolution in Ar:O_2 before and after stem girdling (Fig. 2). When the nodulated roots were exposed to 10% C_2H_2, the rate of C_2H_4 production increased rapidly and attained maximal activity within 5 to 8 min. If an C_2H_2-induced decline occurred in ethylene production, it was small, and a stable rate of nitrogenase activity was obtained within 30 min (Fig. 2A). In the control plants, this stable rate of C_2H_4 production was maintained for at least 3 h. By comparison, following a stem girdling treatment, the rate of ethylene production was stable over approximately 30 min and then declined to approximately 30% of the initial rate within the subsequent 60 min. By 10 h following the girdling treatment, C_2H_2 reduction activity was less than 10% of the initial rate (Fig. 2A).

When the nodulated roots were exposed to Ar:O_2 (80:20), rates of H_2 evolution rose sharply and peaked within 5 to 8 min but then declined to approximately 66% of the initial rate within 30 min (Fig. 2B). The peak rate, being similar to the rate of C_2H_2 reduction, was taken to be equivalent to the undisturbed rate of nitrogenase activity (Fig. 2). Indeed, if returned to N_2:O_2 at this stage, H_2 evolution rate was unaltered. The decline in nitrogenase activity under Ar:O_2 presumably resulted from the decrease in nodule nitrogen assimilation in the absence of N_2 and is thought to be associated with a change in an O_2 diffusion barrier within the nodule (9). Subsequent to this decline, the rate of H_2 evolution in Ar:O_2 was either stable over the subsequent 3 to 4 h or increased gradually with time (Fig. 2B). However, rates declined sharply within 30 to 40 min of a girdling treatment, reaching a minimum rate of approximately 15% of the peak rate of H_2 evolution in Ar:O_2 within 2 h of girdling the stem.

In another experiment, nodulated roots were exposed to Ar:O_2 at various times following the girdling treatment and the peak values for H_2 evolution were used as an estimate of total nitrogenase activity at that time. Intact plants evolved H_2 at a specific rate (μmol/g dry weight nodule·h) of 93 ± 8 and 275 ± 31 (n = 9) under atmospheres of N_2:O_2 and Ar:O_2, respectively, thus displaying an EAC of 0.66 ± 0.03. Total nitrogenase activity and H_2 evolution in air declined following the girdling treatment, with 5% of the original rate remaining 10 h after girdling. However, the EAC of nitrogenase was relatively constant, displaying a mean EAC of 0.67 ± 0.03 (n = 9) over the decline in nodule activity induced by the girdling treatment.

Effects of Interrupting Phloem Supply to the Root System on Nodule, as Distinct from Root, Respiration. The contribution of nodule respiration to nodulated root respiration of healthy, vegetative plants was estimated at approximately 60% by assessing the difference in respiration following removal of nodules. However, 26 h after girdling, roots were estimated to be respiring at 70% of initial rates, while nodules were functioning at only 5% of initial rates (Fig. 3A). Therefore, the decline in nodulated root respiration following stem girdling was associated with nodule rather than root respiration. In addition, when nonnodulated, nitrate-fed plants were stem girdled, the respiration rate of roots

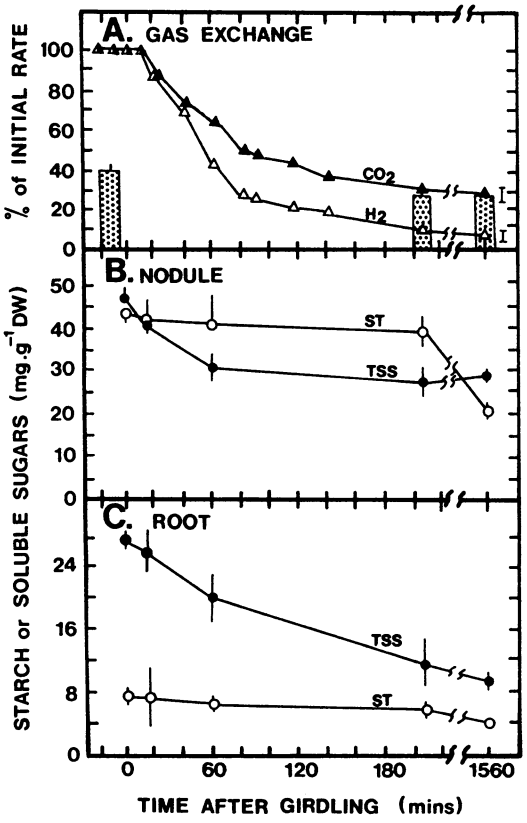

FIG. 3. Changes in nodulated root respiration (▲), H_2 evolution (△) and root only respiration (stippled bar, estimated following denodulation) (A); starch (○) and total soluble sugar (●) pools in soybean nodule (B) and root (C) tissues following a stem girdling treatment. Each value represents the mean of five determinations of nodules or roots from separate plants (±1 SE). For gas exchange measurements, the mean SE of all points is represented beside the CO_2 and H_2 profiles, respectively.

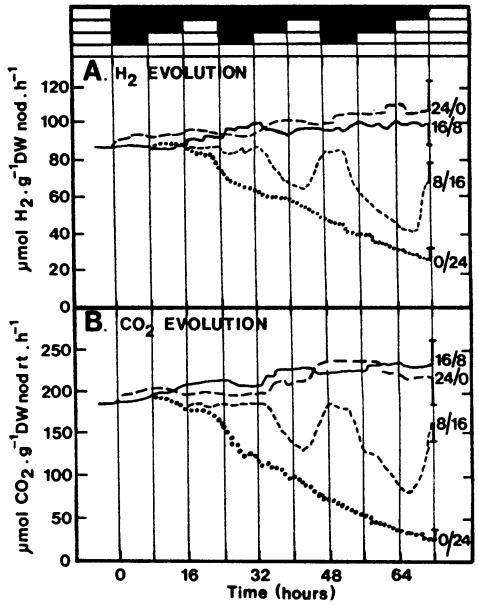

FIG. 4. Rates of H_2 (A) and CO_2 (B) evolution in air from nodulated root of soybeans treated to one of four photoperiodic regimes (as indicated: h day/h night). The blackened bars in the rows above the graph represent the timing and duration of the dark periods of each of the four photoperiodic regimes (in descending order of rows : 0/24, 8/16, 16/8, and 24/0). At $T = 0$ h the plants were 28 d old and were grown from sowing under a photoperiod of 16 h day/8h night. Each line represents the mean of the evolution rates of three plants. The average SE over the entire 72 h monitoring period is shown associated with each line.

was not affected for at least 5 h (Fig. 1B).

A linear correlation existed between the observed rates of CO_2 and H_2 evolution from nodulated roots in air after stem girdling (Fig. 1C). The slope of this relationship indicated that 5.5 CO_2 were evolved per H_2 evolved in air. Given a constant EAC of 0.67, this was equivalent to a respiratory cost associated with nitrogenase activity of 8.5 CO_2 per N_2 fixed. The y intercept of 24 μmol CO_2/plant·h represents a component of nodulated root respiration not associated with dinitrogen fixation.

Changes in Carbohydrate Pools following Interuption of Phloem Supply to Roots. When deprived of shoot carbohydrate, the nodule and root soluble sugar pools were depleted within 3.5 h to 60 and 40%, respectively, of their initial levels (Fig. 3, B and C). Between 3.5 and 26 h after the stem girdling treatment, no significant changes were observed in the levels of soluble sugar in the nodule or root tissues. In contrast, the starch pool decreased in both root and nodule tissues in the period 3.5 to 26 h following the stem girdling treatment (Fig. 3, A and B).

Effects of Photoperiod on Nodulated Root Gas Exchange and Plant Carbohydrate Pools. In plants maintained at 25°C and a 16/8 h photoperiod, no diurnal fluctuations were apparent in the rates of nodulated CO_2 and H_2 evolution (Fig. 4, A and B). In the same photoperiod, soluble sugar pools showed little or no diurnal variation, while starch pools in leaves and roots varied from a low of 60 and 25 mg starch·g^{-1} dry weight, respectively, at the beginning of the photoperiod, to a high of 40 and 50 (mg starch·g^{-1} dry weight), respectively, at the end of the photoperiod (Fig. 5, A and C 16/8 photoperiod). By comparison, the starch pools of nodules and stems showed no diurnal fluctuation (Fig. 5D, 16/8). The starch contents reported here are comparable to those reported in the literature for similar tissues (7, 8, 11, 24, 26).

Plants placed in constant darkness maintained initial rates of CO_2 and H_2 exchange for approximately 23 h before the rates declined over the ensuing 50 h to between 14 and 29% of the initial rates (Fig. 4, A and B, 0/24 treatment). This decline was concurrent with both the depletion of leaf starch below 50 mg·g^{-1} dry weight (Fig. 5A, 0/24 treatment) and the initial decline in the starch reserves within the nodules (Fig. 5D, 0/24 treatment).

In plants grown under a 8/16 photoperiod, nodulated root gas exchange rates were constant until the later part of the second night period (Fig. 4, 8/16 treatment). The rates recovered partially during the following photoperiod, but an even more pronounced decline in nodule activity was observed during the third night of the 8/16 regime. During the final light period, the rates recovered once again (Fig. 4, 8/16). These changes in nodule activity were paralleled by changes in leaf and root starch pools (Fig. 5, A and C, 8/16 treatment), while the depletion of nodule starch was observed only during the second, 16-h night. The depletion of nodule starch reserves was not recovered during the subsequent light period (Fig. 5D, 8/16).

Finally, plants maintained under continuous light displayed rates of gas exchange (Fig 4, 24/0 treatment) which were similar to those obtained in the control plants (16/8 photoperiod). Under continuous light, the starch pools in the leaves, stems and nodules gradually increased such that after 48 h of irradiance the pool sizes were 2.2, 4.3 and 1.3 times the initial values, respectively (Fig 5A,B,D, 240/0). Similarly, values for soluble sugar were 2.1 and 2.4 times the initial values in leaves and nodules, respectively. No significant changes were observed in the root levels of soluble sugar or starch nor in stem soluble sugar content (Fig 5C, 24/0).

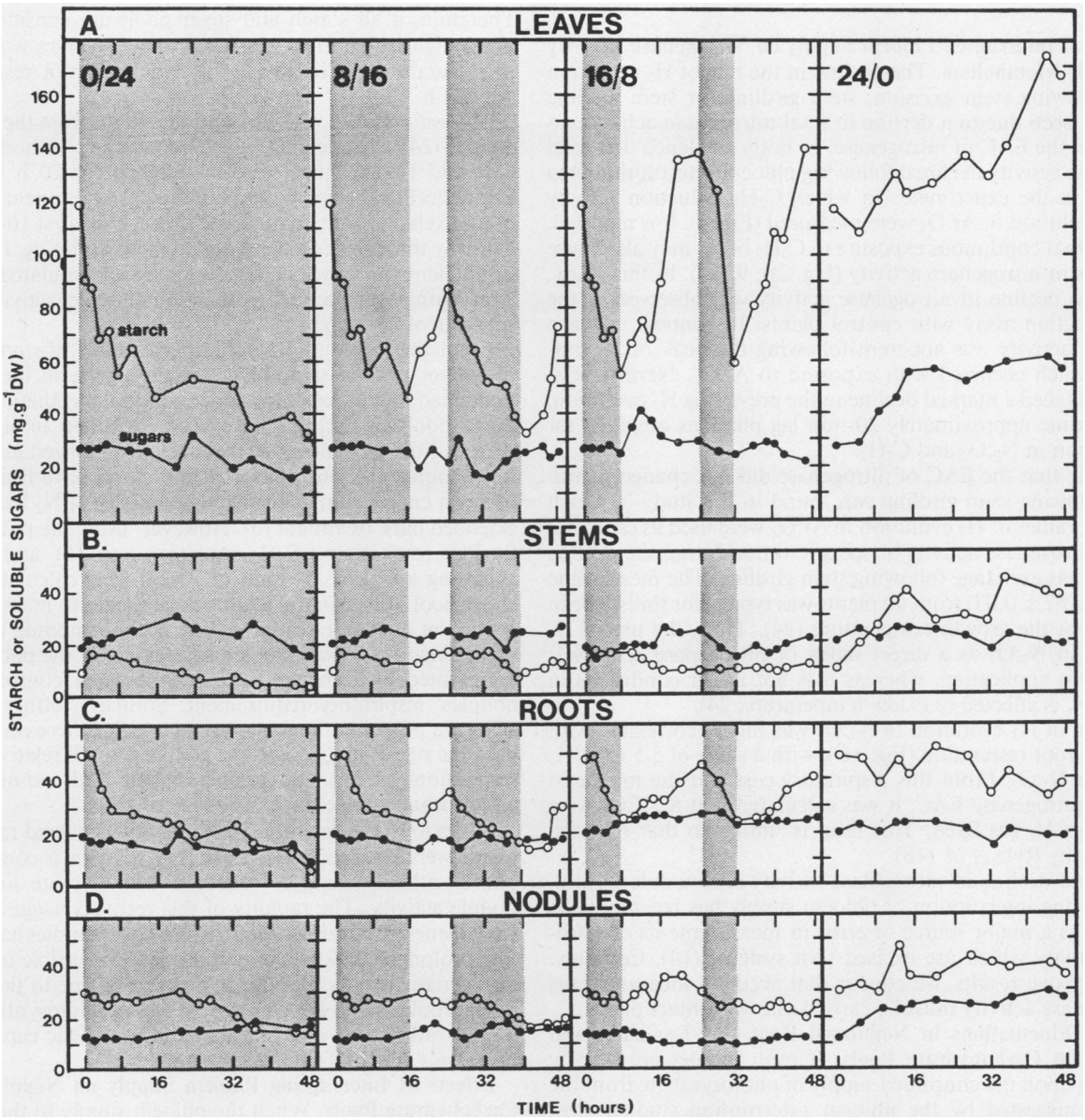

FIG. 5. Changes in the starch (O) and total soluble sugar (●) pools in leaves (A), stems (B), roots (C), and nodules (D) of soybean plants treated to one of four photoperiodic regimes (h day/h night): 0/24, 8/16, 16/8, or 24/0. Dark periods are indicated by stippling. At $T = 0$ h the plants were 28 d old and were grown from sowing with a photoperiod of 16 h day/8 h night. Four plants were bulked for each determination, except at harvests indicated in the "Materials and Methods" section. Average SE for starch content of leaves, stems, nodules, and roots were 8.8, 2.0, 2.3, 5.7 $mg \cdot g^{-1}$ dry weight, respectively, and for sugar content, 1.3, 1.2 0.6, 0.7 $mg \cdot g^{-1}$ dry weight, respectively.

DISCUSSION

The Role of Continued Phloem Supply in Supporting Nodule and Root Metabolism. The results of the leaf excision, stem girdling, and stem chilling studies demonstrated that nodulated root respiration and nitrogenase activity were highly dependent upon the continued supply of photosynthate from the shoot. Within 20 to 30 min following the disruption in the phloem supply to the N_2-fixing roots, a sharp decrease was observed in the rate of CO_2 and H_2 evolution in air (Fig. 1, A and B), H_2 evolution in Ar:O_2 (Fig. 2B), and C_2H_2 reduction activity (Fig. 2A). The nodule excision studies (Fig. 3A) indicated that the decrease in nodulated root respiration was almost entirely associated with nodule rather then root metabolism. This conclusion was consistent with the observation that nonnodulated roots maintained initial rates of respiration for more than 5 h following stem girdling (Fig. 1B).

The suggestion that nodules show a greater dependence than roots on the continued supply of photosynthate from the shoot is in agreement with the work of others (6, 12, 16). Reibach and Streeter (16) reported that the peak of activity from a pulse of $^{14}CO_2$ fed to the shoot was observed in the nodules approximately 3.5 h following the initiation of the shoot feed. Kouchi *et al.* (12) continuously fed ^{13}C-enriched CO_2 to soybean shoots and monitored the appearance of label in metabolite pools and respired carbon of nodulated and denodulated roots. The specific activity of the CO_2 respired from the nodules reached a stable value within 4 h from the start of the shoot feed. In contrast, root respired CO_2 was still increasing in specific activity 10 h after the start of the continuous shoot feed. These results suggest that either nodules possess a small carbohydrate storage pool relative to roots, or carbohydrate unloaded from phloem is a substrate preferred over storage carbohydrate in nodules but less so in

roots.

Effects of Interrupted Phloem Supply on Nitrogenase Activity and Nodule Metabolism. The decline in the rate of H_2 evolution in air following stem excision, stem girdling, or stem chilling may have been due to a decline in total nitrogenase activity, an increase in the EAC of nitrogenase, or both. Evidence that total nitrogenase activity declined following phloem interruption may be found in the experiments in which C_2H_2 reduction activity and H_2 evolution in Ar:O_2 were measured (Fig. 2). It is reported, however, that continuous exposure to C_2H_2 or Ar may also cause reductions in nitrogenase activity (Fig. 2B; 9, 14). In this study, little or no decline in nitrogenase activity was observed in the C_2H_2 reduction assay with control plants. In contrast, a sharp decline in activity was apparent following the peak of H_2 production which occurred with exposure to Ar:O_2. Nevertheless, girdling induced a marked decline in the prevailing H_2 evolution, with the same approximately 20-min lag phase as observed for H_2 evolution in N_2:O_2 and C_2H_2.

Evidence that the EAC of nitrogenase did not change significantly following stem girdling was found in the study in which only peak values of H_2 evolution in Ar:O_2 were used as estimates of total nitrogenase activity at specific times during the decline in nodule gas exchange following stem girdling. The mean value for EAC (0.67 ± 0.03) from all plants was typical for this soybean symbiosis at the growth temperature (24). Thus, the use of H_2 evolution in N_2:O_2 as a direct index of nitrogenase activity is valid in this application, whereas it is not under conditions in which EAC is affected (*e.g.* low temperature; 24).

The rate of H_2 evolution in N_2:O_2 was highly correlated with nodulated root respiration (Fig. 1C), with a slope of 5.5 CO_2/H_2 evolved in N_2:O_2. From this respiratory cost and the measured value for nitrogenase EAC, it was calculated that 8.5 CO_2 were evolved per N_2 gas fixed. This ratio is similar to that reported previously by Ryle *et al.* (18).

The rapid decline in nitrogenase activity and nodule respiration following interruption of phloem supply has recently been identified as a major source of error in measurements of nitrogenase activity which use excised root systems (14). Certainly, from the above results, we concur that accurate measurements of nitrogenase activity must be carried out with intact plants.

Diurnal Fluctuations in Nodulated Root Gas Exchange and Whole Plant Carbohydrate Pools. If root nodules are heavily dependent upon the continued supply of photosynthate from the shoot, as suggested by the phloem interruption studies, then maintenance of nodule metabolism during the dark period must be dependent on pools of carbohydrate in the shoot. Evidence that this is the situation may be found in the results of Figures 4 and 5. No diurnal fluctuations in H_2 or CO_2 exchange were observed in the absence of a dark period (24/0 treatment) or during a 8 h dark period which followed 16 h of light (16/8 treatment). During the 8 h dark period, starch reserves in the leaves and roots declined sharply but were restored in the subsequent light period (Fig. 5, 16/8 treatment).

When pool sizes of starch and sugar in plant tissues were calculated from the data of Figure 5, the leaves of the plants in the 16/8 treatment were estimated to contain 78 to 82% of whole plant reserves at the end of the light period (Table I). Over the subsequent night period, 55 to 58% of the leaf starch pool was consumed. Indeed, leaf starch was the only pool to demonstrate a diurnal change comparable to that required to support nodulated root respiration. In contrast, root starch, the only other pool in which a marked diurnal change was monitored, could account for only 27 to 33% of night, nodulated-root respiration.

At the measured initial nodulated-root respiration rate of 70 μmol CO_2/plant·h, the reserves present in the leaves, stems, roots, and nodules (Table I) at the onset of the dark treatment would be exhausted in 25.7, 1.1, 5.0, and 0.8 h, respectively. Therefore, if all starch and sugar pools are considered, and if shoot night respiration is ignored, these reserves would be able to maintain an optimal rate of nodulated-root respiration for only 33 h.

This estimate is consistent with the results from the continuous dark (0/24) treatment in which initial rates of nodulated root CO_2 and H_2 exchange were maintained for 20 h in the dark before declining (Fig. 4). Also, in the 8/16 treatment initial rates of gas exchange were maintained through the first 16-h night and half-way through the second night before declining. Presumably, insufficient carbohydrate reserves were accumulated during the intervening light period to maintain nodule activity over the subsequent 16-h night.

From these results it would appear that leaf-stored starch is the major pool in soybeans available for use in the support of nodulated root metabolism under nonphotosynthetic conditions. Large pools of cyclitols have been identified in soybean, but there is little evidence that these are metabolized as a carbohydrate source (12, 16). Bacterial Phb stores have been proposed to be an energy source for the maintenance of N_2 fixation under extended dark treatment (5). However, using the published data for Phb reserves and C_2H_2 reduction rates (5), and generously assuming a CO_2/C_2H_2 ratio of 2 (27), it is calculated that the entire pool of Phb in the plant was sufficient to maintain nodule activity at control rates for only 4 h. Since nodule activity was maintained for days under a dark treatment, the pool of carbon represented by Phb was insignificant when compared to the nodules' respiratory requirement. Similarly, although organic acids are probably the carbon pool which is accessed by bacteroids, the size of this pool in the nodule is small relative to nodule respiration (12, 16), and as such organic acids cannot represent a significant storage pool.

In the 8/16 treatment, nodule activity recovered rapidly when plants were returned to the light (Fig. 4). This is consistent with photosynthesis acting to relieve a carbohydrate limitation of nodule activity. The rapidity of this recovery suggests that bacteroid senescence had not occurred. Previous studies have reported that prolonged darkness may cause the irreversible inhibition of nitrogenase activity, but this appears to be due to the senescence of chloroplasts and the consequent loss of photosynthetic capacity (1), rather than due to the loss of metabolic capacity of the bacteroids (19) in dark treated plants.

Effects of Interrupting Phloem Supply on Nodule and Root Carbohydrate Pools. When the phloem supply to the nodulated roots was disrupted by stem girdling, the nodulated root was forced to draw upon its own reserves. Initially, soluble sugar reserves were rapidly consumed, declining within 200 min of girdling to 58 and 43% of their initial pool sizes in nodules and roots, respectively (Fig. 3). In the period from 3 to 26 h after girdling, the soluble sugar levels were relatively stable, but the starch pools continued to decline to 47 and 54% of the initial pool sizes in nodule and roots, respectively. These results are similar to those reported in a recent study (25) in which soluble sugar and starch levels in nodules declined to 25 and 80%, respectively, of the initial level within 3 h of shoot excision. Over the same period, C_2H_2 reduction activity was inhibited to 37% of initial rate. Similarly, in chickpea kept darkened for 48 h, nodules possessed 69% of their original acetylene reduction activity, 40% of initial soluble carbohydrate content, and 74% of initial starch content (22).

The size of the nodulated root starch and sugar reserves were compared with the respiratory demands for carbohydrate in the girdled plant (Table II). In nodules, the mobilization of starch and sugar reserves was equivalent to that respired. However, in roots, starch and sugar utilization significantly exceeded respiratory carbon loss over the first hour (213% of respired carbon). The carbon lost from root starch and soluble sugar pools but not

Table I. *Comparison of the Size of the Carbohydrate Pools in Plant Organs, Their Depletion over an 8 h Dark Period, and the Measured Rate of Carbohydrate Consumption by Nodulated Roots during the Same Dark Period*

The values shown here were taken from the data of Figures 4 and 5 for the first and second dark periods in the 16/8 photoperiod treatment. The pool sizes of starch and soluble sugar were measured at the end of the light period, and consumption was measured as the depletion in these pools over the subsequent dark period. Comparison is made with the nodulated root respiratory carbon loss over each night period.

Plant Organ	Night 1		Night 2	
	Pool Size	Consumption	Pool Size	Consumption
	mgC/plant			
Leaf	29.8	16.4	36.9	21.5
Stem	1.3	0.0	2.0	0.0
Root	5.8	2.8	6.1	3.2
Nodule	0.9	0.3	0.9	0.0
Total	38.0	19.4	45.0	24.7
Nodulated root respiration		9.3		11.8

Table II. *Comparison of the Nodulated Root Respiration Rate with the Starch and Total Soluble Sugar Reserves which were Mobilized from Root and Nodule Tissues following a Stem Girdling Treatment*

The values (units of μg carbon/plant$^{-1}\cdot h^{-1}$) are calculated from the results of Figure 3.

Plant Organ	Interval	C Mobilized from			Respired C
		Starch	Soluble sugars	Total	
	min		*μg C/plant·h*		
Nodule	0–15	86	791	877	1040
	15–60	67	471	538	708
	60–210	43	86	129	141
	210–1560	35	−6	29	40
Root	0–15	112	936	1048	728
	15–60	205	1440	1645	693**
	60–210	54	657	711	610
	210–1560	13	20	33	505**

** Significantly different ($P < 0.05$) from the paired value of total mobilized C.

respired may have been (a) loaded into the xylem and exported to the shoot or nodule, (b) excreted to the rhizosphere, or (c) retained in the root but chemically transformed. Conversely, in the period 3.5 to 26 h, root respiration exceeded the decrease in starch and soluble sugar.

Carbohydrate Supply as a Limitation to Nodule Activity. Minchin and *et al.* (14) have reported that soybean plants in which nodule metabolism was inhibited by prolonged dark treatment recovered to approximately the initial rate of C_2H_2 reduction within 20 h following a return to a range of light intensities. They concluded that even under optimal conditions for photosynthesis and plant growth, nodule function was limited, and that the limitation was of phloem-carbohydrate supply rather than of intrinsic nodule metabolism (21). Williams *et al.* (26) increased photosynthesis by increasing the pCO_2 surrounding the leaves and found that nodule specific activity was not affected. Since leaf starch pools accumulate at a rate such that export rates from the leaf are constant under high pCO_2 (8), it may be concluded, again, that translocation rate is limiting nodule activity. Unfortunately, neither study monitored nodule carbohydrate pool size during the imposed treatments. In the present study, plants maintained in continuous light accumulated starch and sugars in the nodule (Fig. 5, 24/0 treatment) and did not show significantly higher rates of nitrogenase activity than the control plants (Fig. 4). These results suggested that nodules were not limited by phloem supply but by the ability to utilize the available photosynthate.

However, to maintain maximum rates of gas exchange, the soybean nodules of the present study required a continuous supply of photosynthate from the shoots (Fig. 1). The leaf starch pool provided this carbohydrate in the dark periods (Fig. 5; Table I), and nodule starch storage pools were not depleted until shoot starch reserves were reduced below approximately 50 mg·g^{-1}. In stem girdled plants, the starch and soluble sugar pools were depleted in concert with the inhibition of nodule metabolism. That the soluble sugar pools in the nodules were stable under an extended dark treatment but mobilized in the girdled plants probably reflects the time required to equilibrate these two pools. However, these results are consistent with the concept that nodule carbohydrate reserves are only exploited under conditions in which external, phloem-supplied carbohydrate is limited. Thus, when these reserves are used, nodule metabolism is severely restricted.

Because the nodule carbohydrate pools are small in relation to the respiratory demand of active nodules (Table I; Fig. 3), the absolute size of these pools cannot be used as a reliable indicator of the carbohydrate limitation of the nodule. Previous workers (17, 22, 25) have claimed that nodules are not carbohydrate limited, since carbohydrate reserves remained in the nodule under conditions in which nodule activity was declining. These studies have not considered the size of the nodule starch/sucrose pool relative to the nodule respiratory need, the distribution of these reserves between nodule cortical, infected and uninfected cells, or the possibility that nodule starch reserves might not be mobilized at a rate sufficient to maintain maximal rates of nodule metabolism.

In this study, when nodulated roots were denied the support of shoot reserves (extended darkness or girdling), and when nodule starch reserves demonstrated a net utilization, nodule activity was declining. Therefore, a better indicator of nodule carbohydrate limitation would be evidence of starch mobilization rather than the absolute amount of starch present in the nodules. This proposal is consistent with the literature documenting fluctuations in nodule carbohydrate pools. When conditions are imposed with the aim of starving the root system of carbohydrate, reductions in nodule activity (generally acetylene reduction) are observed concomitant with, or prior to, changes in nodule carbohydrate levels (11, 22, 25). In cowpea, a diurnal fluctuation in nodulated root respiration was paralleled with a fluctuation in nodule carbohydrate status (15).

We have also observed that in root-chilled or poorly effective plants, girdling induces proportionally less of a decline in nodule respiration. As the specific activity of the nodule is low in these

plants, remobilization of the starch reserves in the nodule following girdling is presumably adequate to maintain pregirdling rates of respiration. Nodules of such plants possess large starch reserves (24). Similarly, in plants grown under very low light intensities, or in fruiting plants, girdling also induced proportionally less of a decline in nodule activity. In these cases, the nodules are likely to be severely carbohydrate limited already (2), and therefore a further decline in photosynthate supply would be expected to have little effect.

The evidence presented here is consistent with a limitation of nodule activity by carbohydrate supply following girdling or extended darkness. Untreated plants did not appear to be limited because increased carbohydrate supply (extended light) did not increase nodule activity. However, the nature of the treatments which were imposed could complicate this interpretation. Dark treatment, for example, will inhibit transpiration rate, which may affect nitrogenase activity if fixation products accumulate in the nodule. Also, in treatments which caused variations in the rate of nodule respiration, the possible role of a variable O_2 diffusion barrier (9) must be considered.

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