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Diurnal Variation in the Functioning of Cowpea Nodules[1]

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ABSTRACT

Nitrogenase (EC 1.7.99.2) activity of nodules of cowpea (*Vigna unguiculata* [L.] Walp), maintained under conditions of a 12-hour day at 30°C and 800 to 1,000 microeinsteins per square meter per second (photosynthetically active radiation) and a 12-hour night at 20°C, showed a marked diurnal variation with the total electron flux through the enzyme at night being 60% of that in the photoperiod. This diurnal pattern was, however, due to changes in hydrogen evolution. The rate of nitrogen fixation, measured by short-term $^{15}N_2$ assimilation or estimated from the difference in hydrogen evolution in air or Ar:O_2 (80:20; v/v), showed no diurnal variation. Carbon dioxide released from nodules showed a diurnal variation synchronized with that of nitrogenase functioning and, as a consequence, the apparent 'respiratory cost' of nitrogen fixation in the photoperiod was almost double that at night (9.74 ± 0.38 *versus* 5.70 ± 0.90 moles CO_2 evolved per mole N_2 fixed). Separate carbon and nitrogen balances constructed for nodules during the photoperiod and dark period showed that, at night, nodule functioning required up to 40% less carbohydrate to achieve the same level of nitrogen fixation as during the photoperiod (2.4 *versus* 1.4 moles hexose per mole N_2 fixed).

Stored reserves of nonstructural carbohydrate of the nodule only partly satisfied the requirement for carbon at night, and fixation was dependent on continued import of translocated assimilates at all times. Measurements of the soluble nitrogen pools of the nodule together with ^{15}N studies indicated that, both during the day and night, nitrogenous products of fixation were effectively translocated to all organs of the host plant despite low rates of transpiration at night. Reduced fluxes of water through the plant at night were apparently counteracted by increased concentration of nitrogen, especially as ureides, in the xylem stream.

In addition to showing long-term changes in the economy of nodule functioning throughout development (7, 15), many legume symbioses exhibit pronounced diurnal fluctuations in nitrogenase activity as assayed by the reduction of acetylene (2, 6, 11, 16, 19). These fluctuations have been interpreted in relation to changes in the respiration of the nodulated root (7, 11), the carbohydrate status of nodules (5, 11, 19), and the accumulation and export of nitrogenous solutes by the nodules (11). Conversely, where significant diurnal variations in acetylene reduction were not observed, the symbioses studied were regarded not to be currently limited by availability of carbohydrate from the host plant (6, 21).

Although acetylene reduction assays may well offer a reliable measure of overall nitrogenase activity, and thus reflect the availability and rate of utilization of oxidizable substrate in the nodule, they fail to provide information on how electron flow through the enzyme complex *in vivo* is partitioned between nitrogen and proton reduction (17). Marked diurnal fluctuation in this partitioning could well be a significant factor in the daily economy of the nodule, particularly were times of low availability of carbohydrate to coincide with periods of more intense nitrogen fixation relative to H_2 evolution. This paper examines such a possibility in a symbiosis (*Vigna unguiculata*:*Rhizobium* strain 176A27) lacking an uptake hydrogenase. The experimental procedure used involved measurements of diurnal fluctuation in H_2 evolution in air or Ar/O_2 and of $^{15}N_2$ fixation in air to estimate separate rates of nitrogen and proton reduction, and diurnal variations in nitrogenase function were then related to overall daily operation of the plant's nodules under a regime of fluctuating temperature and illumination.

MATERIALS AND METHODS

Plant Material. Surface-sterilized seed of cowpea (*Vigna unguiculata* [L.] Walp. cv Caloona) was inoculated with *Rhizobium* strain 176A27 (Nitragin Co.). Ten d after sowing in sand, groups of five seedlings were transplanted to 3.5-L containers of N-free liquid culture (10) maintained in a naturally lit glasshouse. Fifteen d after establishment, the liquid cultures were transferred to a controlled environment cabinet with a 12-h day at 30°C and 800 to 1,000 $\mu E/m^2 \cdot s$ (PAR) and a 12-h night at 20°C. After 5 to 10 d growth in the cabinet, the lids of the culture containers were sealed at the edge and around the stem of each of the five plants with Terostat VII (Teroson Gmbh, Heidelberg, F. R. G.), and a moisturized stream of either CO_2-free air or CO_2-free Ar:O_2 (80%:20%, v/v) was passed through the enclosed gas space above the liquid culture at a flow rate of 50 to 100 cm^3/min. Plants were in mid vegetative growth and were fixing N at near maximum rates (9) when used.

Measurement of Hydrogen Evolution and Estimation of Nitrogen Fixation. Three sealed water cultures (total of 15 plants) gassed with CO_2-free air and three similar cultures gassed with CO_2-free Ar:O_2 were connected to an automated analysis system which sequentially sampled the effluent gas stream from each over a 6-min period. H_2 concentration of the gas stream was measured using a gas liquid chromatograph equipped with a 2-m column of molecular sieve 5A (100–120 mesh; Waters Associates) and a thermal conductivity detector. The chromatograph incorporated a motorized gas sampling valve which was controlled to sample 1.4 cm^3 at times coinciding with the sequential switching of the effluent gas streams from the six cultures. Measurement of H_2 evolution from each of the six cultures was thus made every 36 min for a period of 33 to 69 h. Values were integrated on a 3-h basis.

In the absence of hydrogenase reactions which utilize H_2, the rate of H_2 evolution into Ar:O_2 may be regarded as a measure of the total flow of electrons to nitrogenase functioning (17). In the present study, H_2 evolution in air and in Ar:O_2 was therefore used to estimate the rate of N_2 fixation for each 3-h period of the day and night. In air, the decrease in H_2 evolution was presumed to be

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due to electron flow to N_2 reduction, and, assuming that reduction of N_2 required three electron pairs compared to one for H_2 production, the following relationship was used to estimate the rate of N_2 fixation.

$$N_2 \text{ fixation} = \frac{H_2 \text{ evolution in } Ar{:}O_2 - H_2 \text{ evolution in air}}{3} \quad (1)$$

Measurement of CO_2 Evolution. The nodulated zone on the primary root of intact plants cultured as above was enclosed in a small (volume 10 cm^3) plastic cuvette as detailed previously (10), and the CO_2 evolved into a CO_2-free air or CO_2-free $Ar{:}O_2$ (80%:20%, v/v) stream was measured using an IR gas analyzer. The effluent stream from six such cuvettes was sampled sequentially as described above for H_2 assay. The total CO_2 evolved into the gas stream flowing through the cuvettes was due to respiration of both nodules and the segment of supporting root which was also enclosed. The separate respiratory contribution of the nodules in each time interval of the study was estimated by subtracting from the total CO_2 efflux the respiration rate of the root segment measured immediately following detachment of the nodules. As described previously (10), the small cuvettes enclosed all the nodules on the root system of a plant which was nodulated only on the primary root.

Plant Harvest and Analysis. Plants cultivated and maintained under the same conditions as those used for measurement of gas exchange were harvested at 4- to 5-h intervals during the study period for assay of ureides (8), soluble amino nitrogen (23), and nonstructural carbohydrate (3) in nodules. Dry weight and total N, measured by Kjeldahl digestion, were determined for nodules and for whole plants at the beginning and end (after 33 or 69 h) of periods of measurement of gas exchange.

Transpiration. The rate of water loss from liquid cultures identical to those used for measurement of gas exchange was estimated gravimetrically or as the change in volume of the culture solution at varying periods (3–6 h) throughout the photoperiod and dark period of diurnal studies.

Xylem Sap Collection. Five to 10 plants were decapitated at 3-h intervals throughout the study period, and root bleeding (xylem) sap was collected for assay of ureide (8) and total amino acid content (1).

Measurement of $^{15}N_2$ Fixation. Cuvettes were attached to the nodulated root regions of two plants as described above and connected in series to a closed gas exchange system incorporating the two cuvettes, a pump, and a small soda lime CO_2 absorber followed by a water bubbler. The gas within the system (260 cm^3) was cycled at a flow of 80 cm^3/min and, following a period of equilibration, 50 cm^3 was removed and, simultaneously, 50 cm^3 $^{15}N_2$ (95 atom % excess ^{15}N) was added. After 2 h, plants were separated into component organs and dried. Samples of the dry, finely milled material were taken for ^{15}N assay by MS following Kjeldahl digestion and oxidation of the resultant ammonia by the Rittenberg procedure (4).

RESULTS

Diurnal Variation in Nitrogenase Functioning. H_2 evolution, both in the presence and absence of N_2 in the gas stream surrounding nodulated roots, showed a marked diurnal variation with higher rates maintained during the warm photoperiod compared to the cooler dark period (Fig. 1). The separate components of nitrogenase functioning in air, H_2 evolution, and N_2 fixation, estimated (Fig. 2) from the data of Figure 1 using Eq. 1 above, indicated that the diurnal variation in nitrogenase functioning (H_2 evolution under $Ar{:}O_2$ in Fig. 1) was due almost entirely to changes in the rate of H_2 evolution. In contrast, the rate of N_2 fixation was not significantly different between the day and night despite the 10°C change in temperature. This was confirmed by

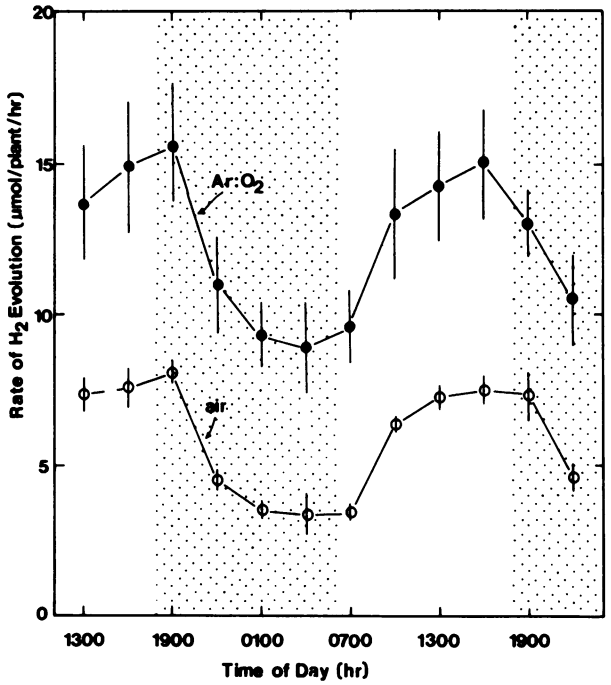

FIG. 1. Diurnal variation in rate of H_2 evolution by intact nodulated root systems of cowpea into a flowing stream of CO_2-free air or CO_2-free 80% Ar:20% O_2 (v/v). The stippled area represents night periods. Bars indicate ±SE of the mean from three replicates.

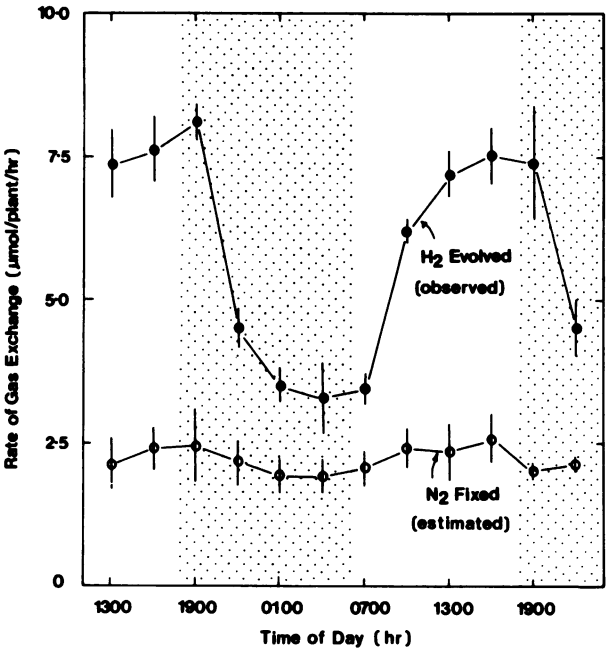

FIG. 2. Diurnal variation in rate of H_2 evolution and estimated N_2 fixation by intact nodulated root systems of cowpea. N_2 fixation was calculated from the data of Figure 1 using Eq. 1. The stippled area represents night periods. Bars indicate ±SE of the mean from three replicates.

measurement of $^{15}N_2$ uptake over 2 h in mid-photoperiod (1100–1300 h, 30°C) or mid-dark period (2300–0100 h, 20°C); the mean rates (±SE) were 1.13 ± 0.19 and 1.21 ± 0.16 μmol $^{15}N_2$ fixed/plant·h, respectively (calculated from the data of Table I).

The assumption that the difference between H_2 evolution in $Ar{:}O_2$ and in air was equivalent to the allocation of electrons to N_2 reduction in air was tested by comparing estimates based on

Table I. *Nitrogen and ^{15}N Content of Plant Parts and Percentage Distribution of ^{15}N after Exposure of the Nodulated Root Segments of Intact Cowpea Plants for 2 Hours with $^{15}N_2$ (Average 20.7 at % ^{15}N) Gas during Two Periods in the Diurnal Cycle (Day 30°C:Night 20°C)*

Nodulated root segments were enclosed in 10-ml volume cuvettes incorporated in a closed gas exchange system. Mean values (±SE) are from four separate experiments in each case.

Plant Part	Total N		Total ^{15}N		Distribution of ^{15}N	
	1300 h	0100 h	1300 h	0100 h	1300 h	0100 h
	mg/plant		*μg/plant*		*%*	
Leaves	4.27 ± 0.11	4.86 ± 0.38	25.92 ± 3.68	35.58 ± 4.82	41.0	52.5
Stem and petioles	0.93 ± 0.05	1.04 ± 0.05	9.96 ± 1.30	10.42 ± 1.40	15.8	15.4
Nodules	0.86 ± 0.05	0.93 ± 0.05	12.62 ± 1.54	10.36 ± 0.78	20.0	15.3
Roots	1.23 ± 0.05	1.26 ± 0.13	14.68 ± 3.82	11.40 ± 2.14	23.2	16.8
Total	7.25 ± 0.26	8.09 ± 0.61	63.18 ± 10.34	67.76 ± 9.14		

integrated measurements of H_2 evolution in Ar:O_2 and air over 69-h periods with direct measurements by Kjeldahl analysis of N_2 fixed over the same period. The mean values from four separate experiments were 2.48 ± 0.16 μmol N_2 fixed/plant·h based on measurements of H_2 evolution and 2.51 ± 0.22 μmol N_2 fixed/plant·h from direct assay of fixed N during the period indicating no significant difference between the two estimates.

Diurnal Variation in Respiration and Carbohydrate Content of Nodules. Under the conditions of diurnal change in illumination and temperature employed, the efflux of CO_2 by attached nodules enclosed in cuvettes showed a marked rhythm, with rates of respiration during the 30°C photoperiod being double those during most of the 20°C night period (Fig. 3). Nonstructural carbohydrate of the nodule, comprising soluble sugars and starch, also showed a diurnal variation (Fig. 3) falling from a level around 19 μmol sucrose eq/plant during the photoperiod to around μmol sucrose eq/plant at night. The pattern of change was, however, not the same as that of CO_2 efflux or of nitrogenase functioning under these conditions (Figs. 1 and 2).

Diurnal Variation in Nitrogen Export by Nodules. The level and pattern of recovery of ^{15}N following supply of $^{15}N_2$ to nodulated plants indicated that fixation and export of newly fixed N from nodules occurred at the same rate at night as during the photoperiod (Table I). In each case, more than 80% of the ^{15}N was translocated out of the nodules with the distribution of label to organs between the two labeling periods being almost identical (Table I). Consistent with this pattern of equally intense export of N day and night, there was no marked diurnal variation in the soluble amino acid- or ureide-N pools of the nodule (Fig. 3).

As might be expected, transpiration during the night was relatively low compared to the photoperiod (Fig. 4). However, the concentration of nitrogenous solutes, and especially of ureides, in xylem sap was markedly elevated at night (Fig. 4).

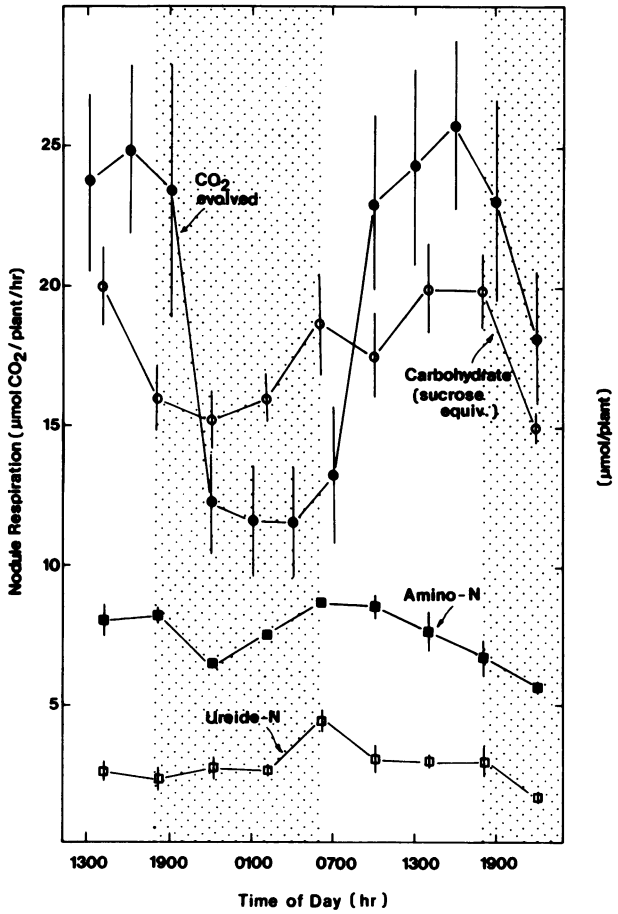

FIG. 3. Diurnal variation in CO_2 efflux, nonstructural carbohydrate, and soluble nitrogen levels in attached nodules of cowpea. Amino-N comprised amino acids, amides, and ammonia. Ureide-N comprised allantoin and allantoic acid. The stippled areas represent night periods. Bars indicate ±SE of the mean from six replicates and, where not shown, are within the dimensions of the symbols.

DISCUSSION

The validity of using the difference between H_2 evolution in the presence and absence of N_2 to estimate the rate of N_2 fixation rests on the assumptions that the symbiosis under study lacked an uptake hydrogenase (17) and that the total electron flux to its nitrogenase was the same in air as in Ar:O_2. Attempts to demonstrate H_2 uptake by intact attached nodulated roots or by detached nodules of the symbiosis used in this study were consistently negative indicating that the first of these assumptions was probably valid, just as has been shown previously for an association of the same *Rhizobium* strain (176A27) with a different cowpea host (18). In relation to the second assumption, close agreement was found between integrated estimates of N_2 fixation based on H_2 evolution of attached nodules in air or Ar:O_2, and measured gain in N of whole parent plants as determined by Kjeldahl analysis. Furthermore, these estimates of day/night rates of N_2 fixation (Fig. 2), determined indirectly from H_2 evolution, showed a similar lack of response to a 10°C change in temperature as did the day/night assays of $^{15}N_2$ fixation (Table I).

The pattern of diurnal change of CO_2 release from nodules (Fig. 3) was similar to that of total electron flux to nitrogenase (Fig. 1), resulting in a relatively constant relationship between respiration and nitrogenase function. The average value for CO_2 evolved/$2e^-$ utilized by nitrogenase during the photoperiod was 1.69 ± 0.09 and for the night period, 1.39 ± 0.18. The relationship between CO_2 release and N_2 fixation, however, showed a marked

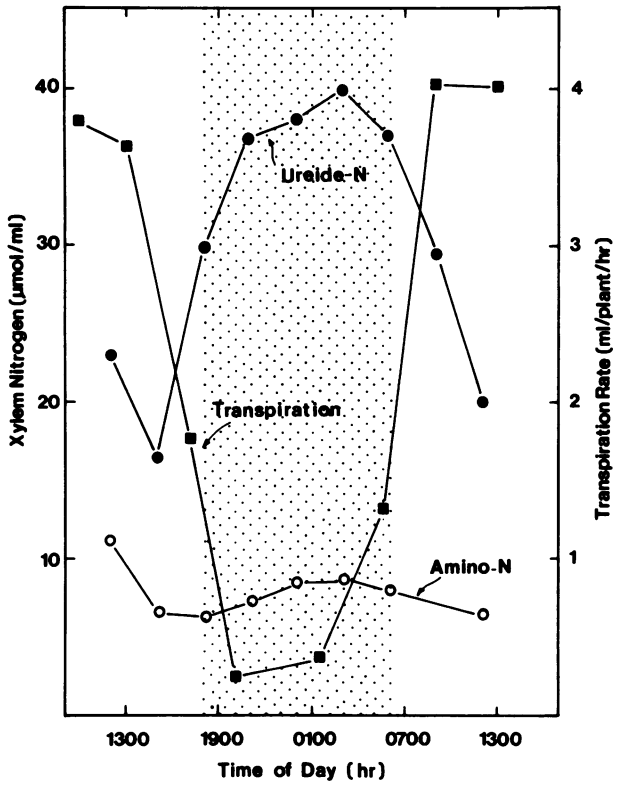

FIG. 4. Diurnal variation in the concentration of amino-N (amino acids, amides, and ammonia) and ureide-N (allantoin and allantoic acid) of root bleeding xylem sap and in transpiration rate of cowpea plants. The stippled area represents the night period.

diurnal variation with almost twice the level of respiration occurring per unit N_2 fixed during the photoperiod than at night. The mean values were 9.74 ± 0.38 and 5.70 ± 0.90 mol CO_2 evolved/mol N_2 fixed during the photoperiod and night period, respectively. Similarly, an earlier study of the diurnal functioning of pea (*Pisum sativum*) nodules found high rates of N_2 fixation maintained during cool nights despite a decline in the respiratory activity of the whole root system compared to the photoperiod (11).

The levels of nonstructural carbohydrate (starch, sugar) in nodules varied during the study period, and although the diurnal pattern of change was not synchronized with that of CO_2 release (Fig. 3), or with that of nitrogenase functioning (Figs. 1 and 2), nodules contained about 4 μmol sucrose eq/plant less at night than during the photoperiod (Fig. 3). Complete oxidation of this amount of sugar in respiration would yield some 48 μmol CO_2. Inasmuch as the total CO_2 efflux from the nodules during the night period was more than 100 μmol, nodules continued to import a significant amount of translocated carbohydrate at night, albeit at a reduced rate compared with the day. Thus, available stored reserves of the nodule only partially satisfied the demand for carbon during the night period.

If, as indicated in Figure 2, N_2 fixation continued at high rates during the night, then nitrogenous solutes would be expected to accumulate in the nodule (11) especially with reduced water movement in xylem (Fig. 4). This, however, did not occur to a significant extent, and only small fluctuations in ureide and amino acid pools of the nodule were observed during the diurnal period (Fig. 3). The results of the $^{15}N_2$ experiments supported these observations. Similar total amounts of ^{15}N were recovered in plants fed in the day and at night (Table I) with, in each case, more than 80% of the fixed label translocated out of the nodule. Moreover, the distribution of ^{15}N between host organs was almost identical day and night (Table I), indicating that the pattern and rates of solute transfer were similar despite a sharp decline in transpiration rate at night (Fig. 4) and coincident rises in the N content of root bleeding xylem exudate (Fig. 4). Thus, it appears that continued translocation of N to shoots at night was maintained, counteracting reduced fluxes of water through xylem by greatly increased concentrations of N in the moving stream. Similar studies with pea (11) also concluded that high rates of N export from nodules occurred at night although, in this case, a greater proportion of fixed N accumulated nightly in the nodules.

Using values for N_2 fixation and H_2 evolution (Fig. 2), CO_2 efflux (Fig. 3), changes in nonstructural carbohydrate levels (Fig. 3), and pool sizes of nitrogenous solutes in nodules (Fig. 3), together with the ratio of C/N in exported solutes (from data of Fig. 4), separate C and N balances for nodules during the photoperiod and during the dark period were constructed. Using the data from 1300 to 1400 h of the photoperiod and from 0100 to 0200 h of the dark period as examples of the extremes in diurnal functioning in this study, the calculated balances are depicted in Figure 5, A and B. The thickness of arrows for C and N are roughly equivalent to the quantities involved (expressed in units of weight), and expressed in terms of the fixation of 100 g N_2 (the actual rate of fixation in each case was 56 μg N_2/h·plant).

Small gains were made in the carbohydrate and nitrogenous solute pools of the nodule in the photoperiod, while a small loss of soluble N occurred in the night period. In both cases, the nitrogenous products of nitrogenase activity were depicted as being effectively exported from the nodule. Clearly, at night, nodule functioning showed a considerably greater economy of C utilization. The 100 g N_2 fixed required 586 g C as imported sugar in the photoperiod compared to 352 g C, or around 40% less, in the night period (Fig. 5).

On the basis of H_2 production requiring 4ATP and $2e^-$/mol H_2 and N_2 reduction requiring 12ATP and $6e^-$/mol N_2 (14), energy demands of nitrogenase, in terms of the two periods shown in Figure 5, might be expected to be greater by about 35% during the day than in the night period. Assuming oxidative phosphorylation to operate with a $P/2e^- = 3$ at all times in the nodule, nitrogenase function (N_2 fixation and H_2 evolution) would theoretically account for 309 g C as CO_2 evolved in Figure 5A (day) and 229 g C in Figure 5B (night). The difference between these two estimates (80 g C) is only 35% of the difference between observed CO_2 evolution at the two times (227 g C) indicating that processes other than H_2 evolution could have also contributed to utilization of the 'extra' energy apparently made available in the photoperiod. Alternatively, the nature of respiratory pathways in the nodule could have varied on a diurnal basis, resulting in differing efficiency for coupling of ATP and reductant generation to oxidation of the available substrates, or there could have been marked diurnal variation in the activity of C conservation mechanisms such as CO_2 fixation (14).

Clearly, diurnal variation of total electron flux to nitrogenase, as has, for example, been observed in C_2H_2 reduction assays (for review, see 12), might, at least in part, be due to changes in H_2 evolution rather than result from fluctuating N_2 fixation. The findings are obviously of some significance in interpreting long-term studies of N_2 fixation in the field as well as conclusions about the C and N economy of nodules or nodulated root systems based on integrated C_2H_2 reduction assays. Previous studies based on continuous collection of CO_2 and direct measurement of N_2 fixation by N increment in the plant (9, 10, 14, 15) would have accommodated the effect of diurnal variations in respiration, in nitrogenase activity, and in the partitioning of electrons between N_2 and proton reduction on the C and N economy of nodules.

Although the present data do not identify the factor or factors in nodule functioning which regulate the efficiency of N_2 fixation, studies with isolated, purified nitrogenase have indicated that, in

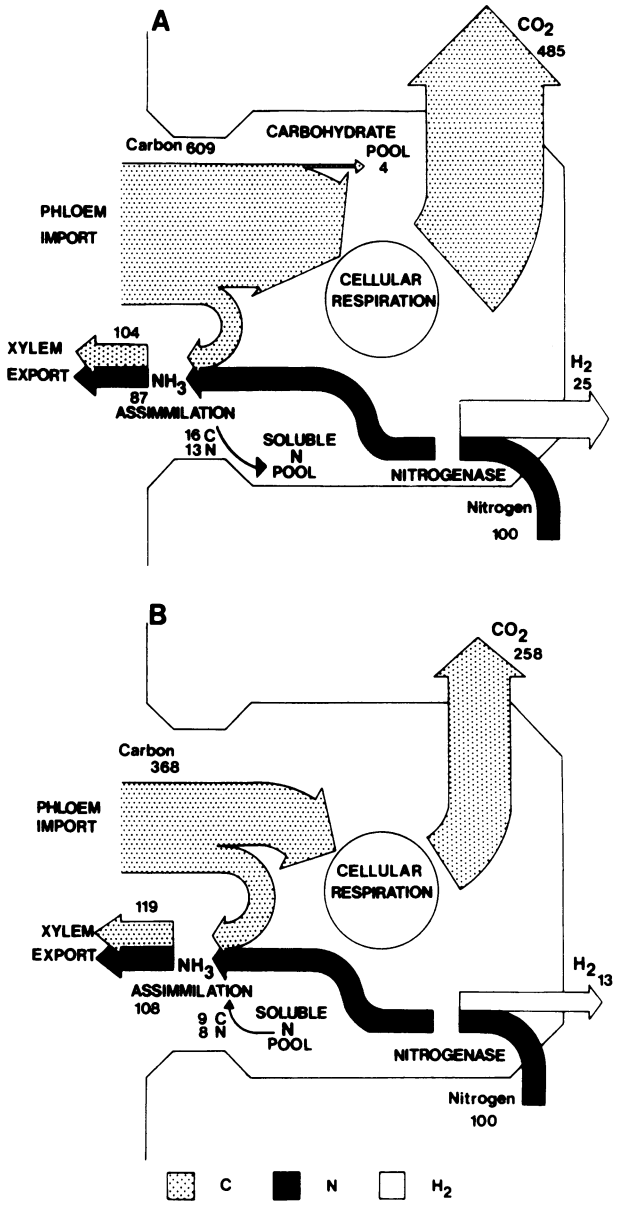

FIG. 5. C and N balance of nodules of cowpea for 1300 to 1400 h of the photoperiod (A) and 0100 to 0200 h of the dark period (B) as examples of the extremes in diurnal functioning. The thickness of arrows for C and N are drawn proportional to the weights of each and relative to the fixation of 100 units N_2. Differences in H_2 production drawn on the same scales as C and N would not be obvious; therefore, for convenience, the thickness of the arrows depicting H_2 is proportional to $\times 10$ the weight of H_2.

addition to a direct effect of temperature on the enzyme (20), ATP level, ATP/ADP ratio, pH, and the ratio of the two component proteins affect the distribution of electrons between proton and N_2 reduction (for review, see 13). All or any of these factors could vary in the nodule on a diurnal basis. Schweitzer and Harper (19) have shown that diurnal variation in C_2H_2 reducing activity of soybean was due to an effect of temperature rather than illumination, and changes in temperature could modulate many of these possible regulatory factors *in vivo* through altered respiration rate or effects on other energy-requiring reactions of the nodule. It is of interest that Walker *et al.* (22) have recently found that the ratio of H_2 produced to N_2 fixed by *Azotobacter chroococcum* was sensitive to O_2 level.

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