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Rhythms during extended dark periods determine rates of net photosynthesis and accumulation of starch and soluble sugars in subsequent light periods in leaves of *Sorghum** **

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Abstract. Photosynthesis and photosynthate partitioning in leaves of *Sorghum bicolor* (L.) Moench exhibited a cyclic dependence on the duration (10–62 h) of dark periods inserted prior to bright light test periods (550 μmol$\cdot$s$^{-1}\cdot$m^{-2}, photosynthetic photon flux). Maximum rates of net photosynthesis and of accumulation of starch and soluble sugars were, in the order given, two-, three- and fourfold greater than minimum values. Between 14 and 53% of photosynthate was retained in leaves depending on the length of the dark period. These changes were sufficient to account for the previously described stimulatory effect of short daylengths (i.e., long nights) on carbohydrate accumulation in leaves (N.J. Chatterton and J.E. Silvius, 1980, Physiol. Plant. **49**, 141–144). The free-running periods for the rhythmic dependence on darkness, determined either directly or by curve fitting, were about 24 h for net photosynthesis, 23 h for starch accumulation, and 26 h for soluble-sugar accumulation. The deviation from period lengths of 24 h for carbohydrate accumulation indicates that these rhythms are probably endogenous and circadian. Initial maxima were observed after 14 h of darkness for photosynthesis, after 18–22 h for starch, and after 26 h for soluble sugars. The differences in period length and phase indicate that at least three separate rhythms underlie the dependence of photosynthate partitioning in *Sorghum* on darkness. Periods of low leaf dry-matter accumulation coincided approximately with periods of high net photosynthesis. As a result, maximum photoassimilation and maximum export were synchronized and, furthermore, occurred at about the same time as expected light periods.

Key words: Photosynthate partitioning – Photosynthesis – Circadian rhythm – Photoassimilate export – Carbohydrate.

Introduction

In many plants, the proportion of photosynthate stored in leaves is considerably higher under short days than under long days (Chatterton and Silvius 1980a, b); the increased carbohydrate reserves are presumably beneficial during long dark periods. Although the mechanisms underlying the adaptation of photosynthate partitioning to daylength are unclear, carbohydrate storage is apparently regulated by factors in addition to that of a simple balance between source supply and sink demand (Baysdorfer and Robinson 1985), since short-day-grown plants are limited by photosynthate supply.

Typical of this response to daylength, the amount and type of carbohydrate stored in the leaves of the C_4 grass *Digitaria decumbens* Stent. during a photosynthetic period were influenced by both prior photoperiodic treatment (Britz et al. 1985a) and rhythmic events occurring during preceding dark or dim light periods (Britz et al. 1985b). *Digitaria decumbens* was used in these earlier experiments because photosynthate partitioning in this species changes overnight in response to altered daylength. However, the species is sterile and must be increased vegetatively in the greenhouse before transfer to controlled-environment chambers. In addition, its prostrate growth habit limits the amount of usable leaf material.

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* This paper is dedicated to Professor Wilhelm Nultsch on his 60th birthday

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Abbreviations and symbols: $DD_{(n)}$ = (the *n*th h of) continuous darkness; LT = local time of day; τ = free-running period length

Detailed studies were therefore initiated with *Sorghum bicolor*, another C_4 grass, which also responds rapidly to altered daylength (Britz 1986) and which can be grown quickly and reproducibly in large numbers from seed in controlled-environment chambers. A procedure useful in the evaluation of cyclic phenomena underlying photoperiodic responses (Vince-Prue 1975, pp. 155–199) was adopted for studies of photosynthate partitioning. After the plants achieved a predetermined developmental stage during normal light-dark cycles, the final dark period was extended for up to 62 h. Following various intervals, fresh groups of plants were reintroduced to the light during which time photosynthesis and carbohydrate accumulation were measured. Thus, all plants received the same amount of light and cyclic processes occurring during the dark period became apparent.

Material and methods

Sorghum bicolor (L.) Moench cv. Rio was grown in controlled-environment chambers (EGC, Chagrin Falls, Ohio, USA) at 27° C, 60% relative humidity and 0.035% CO_2 under 10-h nights and 14-h days with a photon flux (400–700 nm) of approx. 550 $\mu mol \cdot s^{-1} \cdot m^{-2}$ from cool-white fluorescent (F96T12/CW/VHO; Sylvania, Danvers, Mass., USA) and incandescent lamps (60 W), as measured with a LI-185A quantum radiometer (LiCor, Lincoln, Neb., USA). Lights-on was at 8:00 local time (LT) and lights-off at 22:00 LT. For each experiment, 1200 seedlings (8 per pot, $0.1 \cdot 0.1$ m^2, 0.2 m high) were started in vermiculite and watered daily with a complete nutrient solution (Robinson 1984). The plants were thinned in stages such that 10 d after sowing about 450 uniform seedlings remained (three or four plants each in 126 pots). The effective growing area was 2.25 m^2.

Continuous darkness (DD) at 27 ± 0.2° C was initiated at 22:00 LT on the 14th day after sowing. At this time the ligule of the fifth leaf (counting acropetally) was visible above the subtending leaf sheath in more than three-quarters of the plants. Further leaf development appeared to stop in DD. Starting at DD_{10} (i.e., 8:00 LT, a time corresponding to the end of the normal dark period) and continuing at 4-h intervals until DD_{62}, fourteen groups of nine pots with about 32 seedlings in each group were transferred in darkness to an illuminated chamber (550 $\mu mol \cdot s^{-1} \cdot m^{-2}$) similar to that for seedling growth (Fig. 1). Nine fifth leaves (one from a plant in each pot) were harvested for bulk analysis of total ethanol-soluble sugars (sucrose plus reducing sugars) and starch (Britz et al. 1985a) at each of two times, 1 and 6 h into the shifted light period. In between these times, CO_2-exchange rates were measured by infrared gas analysis (Model 865 CO_2 analyzer; Beckman Instruments, Fullerton, Cal., USA; Model ALL-10K mass-flow meter, Hastings Instruments, Hampton, Va., USA) for nine separate leaves (one from each pot) using the air-seal method (Wolf et al. 1969). In some cases, photosynthesis was calculated in terms of carbohydrate equivalents (Chatterton and Silvius 1979) for comparison with the accumulation of leaf dry matter, starch and soluble sugars. Photosynthesis values were stable over the 5-h interval between the two destructive harvests. Carbohydrate-accumulation data were expressed as rates, since this is the form commonly used, but the conclusions reached in this report would not be altered if the data were expressed as integrals.

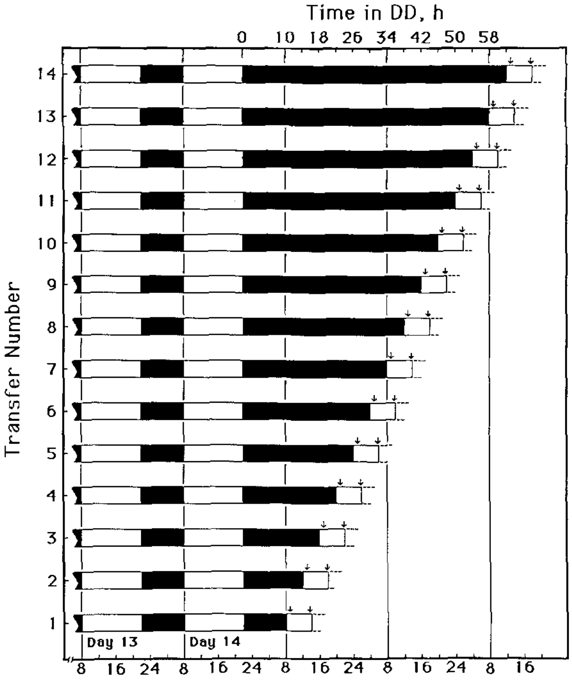

Fig. 1. Experimental protocol to determine the importance of dark-period duration on photosynthate partitioning in *Sorghum* leaves during subsequent light periods. Each bar represents a separate group of plants. *Clear portions* = light periods at approx. 550 $\mu mol \cdot s^{-1} \cdot m^{-2}$; *black portions* = dark periods. All groups were grown together from seed for 14 d in daily cycles of 14 h light and 10 h darkness. Lights-on was at 8:00 LT (*thin vertical line*) and lights-off was at 22:00 LT. After 22:00 LT on day 14 the growth chamber remained darkened for 62 h. Time of day (hours LT) is shown on the lower axis while the time in DD is shown on the upper axis. Starting at DD_{10} (8:00 LT) on day 15 and continuing at 4-h intervals until DD_{62}, nine pots were transferred in darkness to another chamber with similar conditions but at normal growth irradiance. Each group was kept in the light for 6 h after transfer with harvests at 1 and 6 h into the light (*small arrows*)

The experiment was repeated at four different times of year. Since none of the four replicates were significantly different, replicate values were averaged and plotted with respect to both the duration of incubation in DD and the time of day in hours LT at which the plants were transferred from DD to bright light.

Results

Net photosynthesis, dry-matter accumulation and export. Rates of leaf net photosynthesis and dry-matter accumulation in the light varied rhythmically with respect to the duration of DD (Fig. 2). There was a large (40%) decline in photosynthesis following dark incubations between DD_{14} and DD_{30} with a subsequent much smaller increase

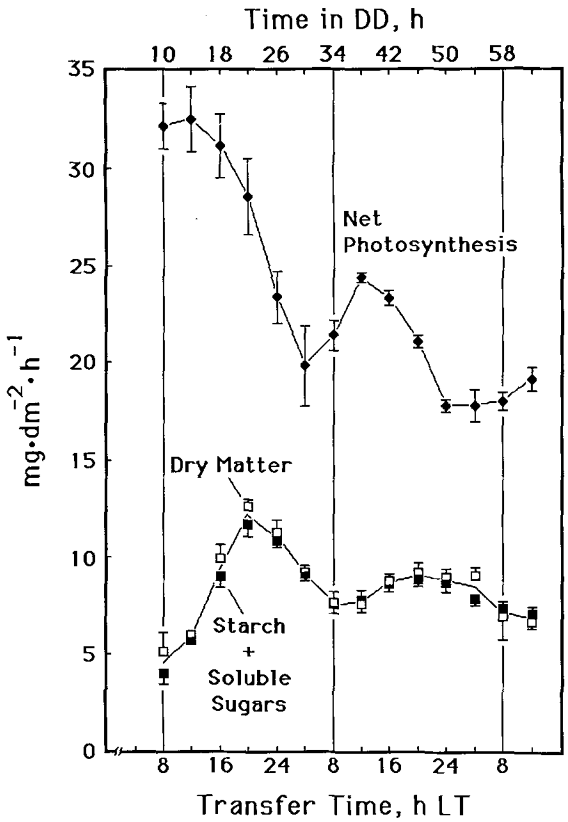

Fig. 2. Rates in bright light (550 μmol·s^{-1}·m^{-2}) of net photosynthesis (♦—♦), expressed as carbohydrate (Chatterton and Silvius 1979), and accumulation of leaf dry matter (□—□) and starch + soluble sugars (■—■) in fifth leaves of *Sorghum* with respect to the time of transfer from DD to light. Starch was determined as glucose equivalents × 0.9 while ethanol-soluble sugars were determined as sucrose. Each point is the average of four independent experiments. Bars = 1 SE

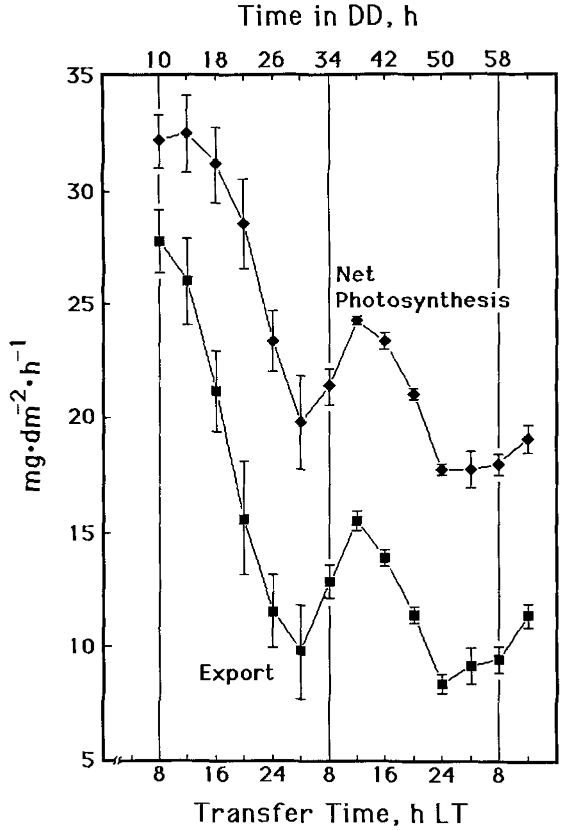

Fig. 3. Measured rates of net photosynthesis (♦—♦) and estimated rates of export (■—■) in *Sorghum* leaves, both expressed as carbohydrate. Export was calculated as the difference between net photosynthesis and starch + soluble-sugar accumulation. Data from Fig. 2; details as in Fig. 2

and decrease. It is difficult to say from these data whether the rhythm was damping rapidly or whether a cyclic dependence on DD was superimposed on a monotonic downward trend as a result of increasingly longer dark periods. However, the relatively small effect of shortened daylength on the phase and magnitude of the rhythmic response of net photosynthesis (Britz 1986) is evidence that the rapid decline in photosynthetic rates following extended dark periods is clock-related and not simply a response to darkness. In any case, minima were observed about 24 h apart and occurred at times (4:00 LT) when the plants would have been in darkness had the normal LD cycle persisted. Likewise, the maxima occurred approximately at times when the plants would normally have been illuminated. A similar but less pronounced rhythm was also observed for *Digitaria decumbens* (Britz et al. 1985b).

Rhythmic changes in the accumulation of dry matter were smaller in magnitude and out of phase with changes in net photosynthesis. Thus, maximal accretion occurred when photosynthesis was relatively low, while minima occurred when photosynthesis was high. On the average, greater than 95% of the increases in leaf dry matter were accounted for by the sum of starch and stored-soluble-sugar accumulation. Dry-matter accumulation by a leaf was therefore the result almost entirely of carbohydrate accumulation which was not, however, a constant proportion of net carbohydrate assimilation. These results were also similar to those with *D. decumbens*, although the correlation between dry matter and carbohydrate was not as high in this species.

Export from the leaf was estimated as the difference between rates of net photosynthesis and accumulation of starch and soluble sugars. Calculated values for export followed photosynthesis closely (Fig. 3), since the changes in photosynthesis were much larger than those in carbohydrate storage. Relative export was about 86% of net photo-

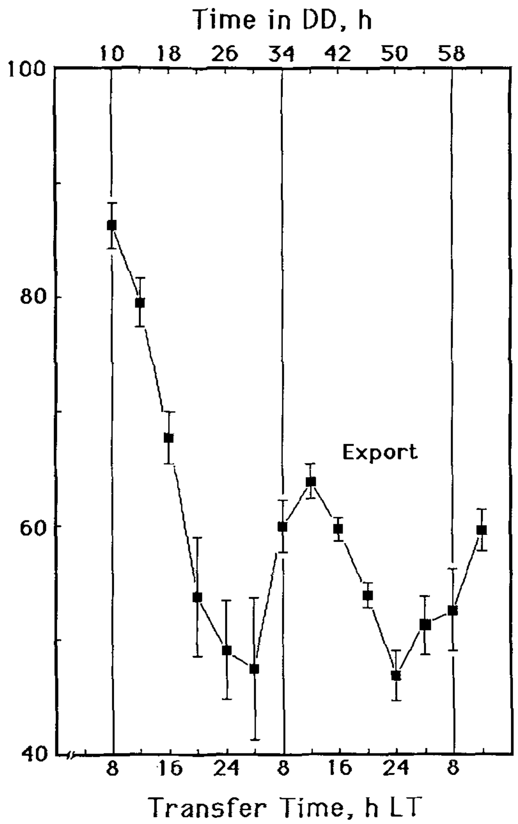

Fig. 4. Estimated export from leaves of *Sorghum* expressed as a percentage of the corresponding net photosynthesis values. Data from Figs. 2 and 3; details as in Fig. 2

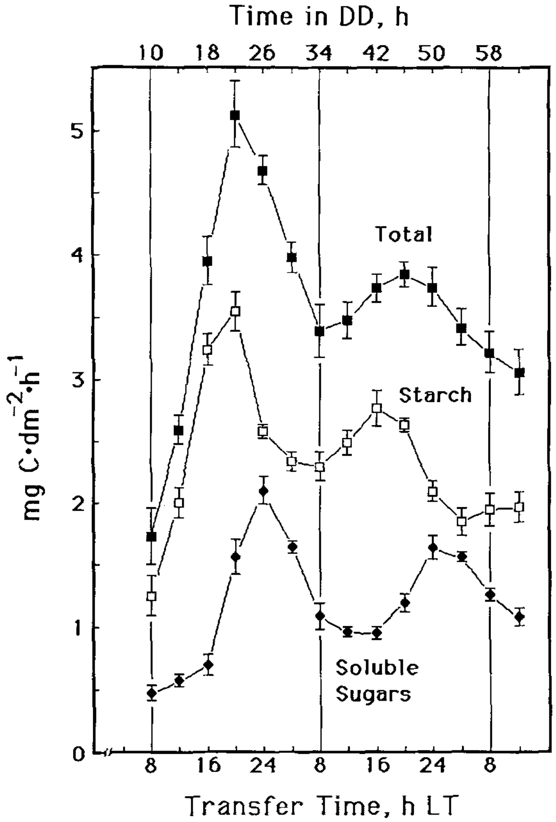

Fig. 5. Rates of accumulation in *Sorghum* leaves for starch (□—□), soluble-sugars (◆—◆) and their sum (■—■) expressed as C. Details as in Fig. 2

synthesis for plants transferred into bright light after the standard 10-h night but declined to about 47% as a result of the large increase in starch and soluble-sugar accumulation with progressively longer durations of DD (Fig. 4). Relative export recovered to about 63% of net photosynthesis during the second maximum. The rhythm persisted at least into a third cycle with a period close to 24 h.

Starch and soluble-sugar accumulation. The rhythm of carbohydrate buildup was the result of quantitatively and qualitatively different patterns for the accumulation of both starch and soluble sugars (Fig. 5). Both parameters were low for the initial transfer to light at DD_{10}, as would be expected for plants grown under long days. However, rates of starch accumulation in the test light periods increased about threefold above these initial values as the preceeding dark period was extended to DD_{18}. Soluble-sugar storage, initially much lower than that for starch, began to increase only after much longer dark preincubations (DD_{18}) and reached a maximum after DD_{26} with rates about fourfold above initial values. By this time, the starch-accumulation response was already well past its peak. Note that maximum rates of sugar accumulation approached minimum rates of starch accumulation at certain times in the cycle. Both rhythms persisted for at least one more cycle, but they dampened substantially.

The difference in phase between the two rhythms appeared to be greater for the second maximum. Two methods were adopted to quantify these observations (Table 1). The first method consisted of assigning a time for each maximum which corresponded either to the transfer time at which the highest rate was observed, or to the average of two or more times if the corresponding rates bracketed a peak and were within 10% of each other. The time difference in hours between the two maxima was τ. The second method involved fitting all 14 time points for each rhythm to a cosine curve (Halberg et al. 1967). Period lengths which provided the best fit were estimated by correlation coefficient and analysis of variance. Although only two cycles with 4-h resolution were available for each rhythm, cosine curves were reproducibly and significantly ($P<0.001$) fitted to all four replicates. Better fits to the data were obtained with cosine curves summing first-, second-

Table 1. Dark preincubation times for maximal rates of light-driven starch and soluble-sugar accumulation in leaves of *Sorghum* with corresponding period lengths (τ)

Parameter	Expt. No.	Max_1 (h)		Max_2 (h)	Period length (h)	
Starch	1	20^a	20.5^b	44^a	24^a	24.0^b
	2	22	21.5	40	18	23.25
	3	20	20.5	42	22	23.0
	4	20	21.5	44	24	23.25
	$\tau_{av} \pm 1$ SE				22 ± 1.4	23.4 ± 0.2
Soluble sugars	1	26^a	26.3^b	52^a	26^a	27.5^b
	2	26	26.0	52	26	26.25
	3	26	26.6	50	24	26.75
	4	26	26.4	52	26	25.75
	$\tau_{av} \pm 1$ SE				25.5 ± 0.5	26.6 ± 0.4
	$\Delta\tau \pm$ CL				3.5 ± 3.4^c	3.2 ± 1.6^d

[a] Based on the time in darkness required for the maximum rate or the average time if two or more values bracketing a peak were within 10%

[b] Based on the best fit to a cosine curve $\{F(t_i) = c_0 + c \cos[(2\pi/\tau)t_i + \phi]\}$ where c_0 = mesor value, c = semiamplitude and ϕ = phase angle

[c] 90% confidence limits (CL)

[d] 99% confidence limits

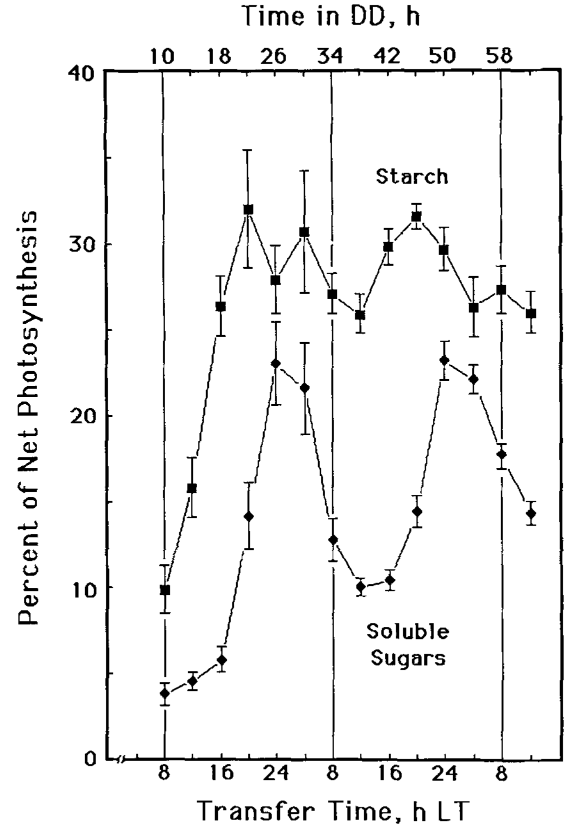

Fig. 6. Rates of starch (■—■) and soluble-sugar (◆—◆) accumulation in *Sorghum* leaves expressed as a percentage of net photosynthesis. Details as in Fig. 2

and third-order harmonics, but these curves did not yield appreciably different estimates of τ. As a result, only data from single harmonic curves are shown in Table 1. Both methods confirmed the difference in phase and indicated a statistically significant difference in τ of about 3 h. Rhythmic changes in rates of net photosynthesis were not analyzed with this technique because of the rapid decline in average photosynthetic rates with increasing duration of the dark period.

The rhythm in rates of soluble-sugar accumulation persisted when expressed as a percentage of net photosynthesis (Fig. 6), although the first maximum was shifted to a later time as a result of the rapid decline in photosynthetic rates between DD_{22} and DD_{30}. Less damping was observed for the second peak after correction for reduced rates of net photosynthesis. Values ranged from 4% to almost 25% of net photosynthesis. However, rhythmicity in starch accumulation was greatly reduced when the rates were expressed as a percent of net photosynthesis. After DD_{18} there was less than a 5% change in relative starch accumulation. While this result might indicate that starch accumulation was a function of rhythmicity in photoassimilation, other experiments showed that both starch and soluble-sugar accumulation can be phase-shifted with respect to photosynthesis, ruling out a direct connection (Britz 1986).

Discussion

Cyclic processes during the dark period influenced both the assimilation and partitioning of photosynthate within *Sorghum* leaves during subsequent light periods. In fact, incubation of plants in DD for up to 62 h before transfer to bright light indicated the participation of three different and, to some extent, independent rhythms. Thus, the rhythmic dependence of starch and soluble-sugar accumulation on DD were out of phase (Fig. 5) and appeared to have different τ (Table 1). In addition, incubation of plants in continuous dim light (50 $\mu mol \cdot s^{-1} \cdot m^{-2}$) instead of DD completely suppressed rhythmicity in soluble-sugar accumulation but only partially suppressed it in starch (Britz 1986). The rhythmic response of net photosynthesis, unlike that for carbohydrate accumulation, was not affected by prior short-day treatment or incubation in continuous dim light (Britz 1986). However, in spite of these differences, maximum absolute and relative carbohydrate export (Figs. 3, 4) were synchronized with times when the plants would have been in the light, had they remained in a normal light-dark cycle. A similar pattern was also observed for *Digitaria* (Britz et al. 1985b).

It is not certain that the response of photosynthesis to dark-period length represents an endogenous rhythm because the period is close to 24 h and thus could be driven by cryptic environmental signals with 24-h periodicity. However, the patterns of both soluble-sugar and starch accumulation have τ different from 24 h which indicates that these rhythms are likely to be endogenous and circadian. Further experiments should be performed for durations longer than two cycles and with transfers at 2-h intervals in order to determine period lengths more accurately. Although the experiments were limited by growth-chamber space and time for analyses, the application of novel infrared spectroscopic methods for the estimation of carbohydrates in leaf samples (Henry 1985) indicates that it may be practical to expand the scope of investigation.

Another question to be resolved is whether carbohydrate accumulation was in all cases linear over the 5-h interval between these harvests. The first harvest was made 1 h into the light period because delays between the onset of the light period and the initiation of starch synthesis have been reported (Fondy and Geiger 1982). A lag longer than 1 h would result in an underestimation of rates. Changes in partitioning could therefore be more dramatic than reported. Although starch accumulation in soybean was initiated promptly at times in the cycle when rates were high (Chatterton and Silvius 1979) a more detailed kinetic analysis of carbohydrate accumulation in *Sorghum* at selected times in the cycle should be obtained.

It is also necessary to reconcile differences in phase, τ and sensitivity to light for soluble-sugar and starch accumulation. The possibility of separate and perhaps interacting clocks has interesting implications for the regulation of metabolism, especially as carbohydrate accumulation appears to be regulated independently of net photosynthesis and sink demand (which presumably increases during DD). However, since sucrose synthesis in C_4 grasses probably occurs in the mesophyll and starch is deposited mainly in the bundle sheaths (Furbank et al. 1985; Leegood 1985), it is conceivable that the rhythms controlling sugar and starch accumulation are localized in different cell types. The existence of interacting clocks with different period lengths and dependence on environment in multi- and even unicellular plants is well established (Mayer and Sadleder 1972; Kiefner et al. 1974; Bollig 1975; Hoffmans-Hohn et al. 1984).

Rhythmicity in carbon metabolism has long been known. Crassulacean acid metabolism (CAM) has been proposed to involve separate rhythms for phosphoenolpyruvate carboxylase, malic enzyme and vacuolar uptake of malate (Kluge 1977; Queiroz 1979). In fact, in early work on CAM-related rhythms of CO_2 evolution in *Bryophyllum* several features, such as synchronization to end-of-day signal and suppression in bright light (Wilkins 1959), were found which are also apparently found in *Sorghum*. Results indicating rhythmic changes in starch content in non-CAM plants kept in constant conditions (Pongratz and Beck 1978; Kerr et al. 1985) are not directly comparable with our results because effects of variable durations of darkness prior to bright light were not considered.

The importance of the dark period was shown in a previous study in which partitioning was almost identical following standard 18-h dark preincubations regardless of whether the preceeding light period was 6 h or 14 h (Britz 1986). These results are consistent with the involvement of circadian rhythms in the measurement of dark intervals, possibly similar to the photoperiodic regulation of flowering in some plants (Lumsden et al. 1982). Note that the phase of the rhythm controlling starch accumulation (Fig. 5) is such that threefold changes in rates can occur within a range of dark periods (10–18 h) corresponding to expected seasonal variations. A role for this response in the adaptation of plants to natural daylengths is thus indicated even though the ultimate physiological role is not clearly established. Photoperiodic effects on partitioning could influence vegetative or reproductive growth through the amount and timing of assimilate availability. Note that plants with high end-of-day starch tend to have higher ratios of shoot-to-root biomass (Chatterton and Silvius 1979; Huber 1983).

Nonetheless, the generality of rhythmic control over the daylength response of partitioning remains to be established. Preliminary experiments with another C_4 grass, *Digitaria decumbens*, indicated that substantial differences in rhythmicity may exist between related species (Britz et al. 1985b; Britz 1986). For example, following incubation in continuous dim light (40 $\mu mol \cdot s^{-1} \cdot m^{-2}$), starch accumulation in *Digitaria* (unlike that in *Sorghum*) retained a rhythmic dependence on time of transfer to bright light, but was almost 180° out of phase with net photosynthesis. Further work is underway to define the diversity of rhythmic control of photosynthate partitioning as well as to understand more precisely the interaction of these rhythms with light and the underlying changes in metabolism.

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