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CARBOHYDRATES EVOLUTION IN SPINACH PLANTS DURING EARLY ACCLIMATION TO VARIOUS CHANGES IN PHOTOPERIOD

BY

Robert DEGLI AGOSTI *, Marc BONZON ** and Hubert GREPPIN **

ABSTRACT

In short days of 8 h light, the glucose, fructose, sucrose and starch contents in the primary leaves and petioles of spinach plants (*Spinacia oleracea* L. cv. Nobel) increase during the light and decrease during the dark. The transfer to continuous light results in a 5-8 fold increase in the hexose content, which begins 1-3 h after the end of the SD light period and reaches its maximum value after 16 h of total light. The sucrose content of petioles follows the same time-dependent changes, but to a lesser extent. In contrast, the malate and starch contents increase almost linearly with the day length. The sudden increase in hexose content can only be generated if light is given during a particular phase of the carbohydrate metabolism. The results show that part of the sugar metabolism in spinach leaves and petioles is under photoperiodic control and is endogenously organized in time.

KEYWORDS: *Spinacia oleracea;* Chenopodiaceae; carbohydrates; glucose; photoperiodism.

RÉSUMÉ

En jour court de 8 heures de lumière les teneurs en glucose, fructose, saccharose et amidon des pétioles et feuilles primaires de l'épinard (*Spinacia oleracea* L. cv. Nobel) augmentent pendant le jour et diminuent la nuit. Le transfert en lumière continue provoque une augmentation de 5 à 8 fois de la teneur en hexose. Cet effet débute dès 1 à 3 heures après la fin de la période lumineuse du jour court pour atteindre un maximum vers une durée totale de 16 heures de lumière. Le contenu en saccharose des pétioles montre une évolution similaire, mais avec une ampleur moindre. De manière contrastée, la teneur en malate et amidon augmente de façon pratiquement linéaire avec la longueur de la journée. L'augmentation brusque des hexoses ne peut avoir lieu que si la lumière est donnée pendant une phase particulière du métabolisme des sucres. Les résultats montrent qu'une partie du métabolisme des sucres dans les feuilles et pétioles de l'épinard est sous contrôle photopériodique et est organisé de manière endogène dans le temps.

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INTRODUCTION

In plants, light plays a pivotal role not only in photosynthesis, but also in photomorphogenetic and photoperiodic control of development. Thus, among other processes, the relative light or night length controls the flowering process in photoperiodically sensitive plants (Vince-Prue, 1975; Bernier *et al.*, 1981; Salisbury, 1981). In this context, it has been found that in some long-day plants (LDP) the lengthening of the light period results in an increase in soluble sugars in the leaves (Bernier *et al.*, 1981; Bodson, 1977). However, the data did not allow discrimination between a direct effect due to the lengthening of the photoperiod i.e. photosynthesis and an effect specifically linked to a photoperiodic action on sugar metabolism.

In spinach, the soluble sugars are sucrose, glucose and fructose, with starch being the reserve polymer. A sharp and large increase in hexose content has been observed 8 h after the transfer from short days (SD) of 8 h light to continuous illumination (Degli Agosti and Greppin, 1987*a*). Thus, it was of interest to follow the time-dependent changes in free sugars and starch content in the primary leaf and petiole during the very first hours of a change in day length in order to characterize a possible change in sugar metabolism.

MATERIALS AND METHODS

The long day plant *Spinacia oleracea* L. cv. Nobel (spinach) was cultivated on soil, four plants to a pot, in growth chambers under standard irradiation conditions (20 Wm^{-2}) provided by Sylvania fluorescent lamps ("day-light" F4OT12, 40 W), temperature ($20 \pm 0.5°C$) and humidity ($70 \pm 5\%$ and $50 \pm 5\%$ relative humidity during the day and the night, respectively). Vegetative plants were grown in short days (SD) of 8 h light (from 8 h to 16 h local time) and 16 h darkness. Subjecting the plants to continuous illumination after SD was called a transfer treatment (T).

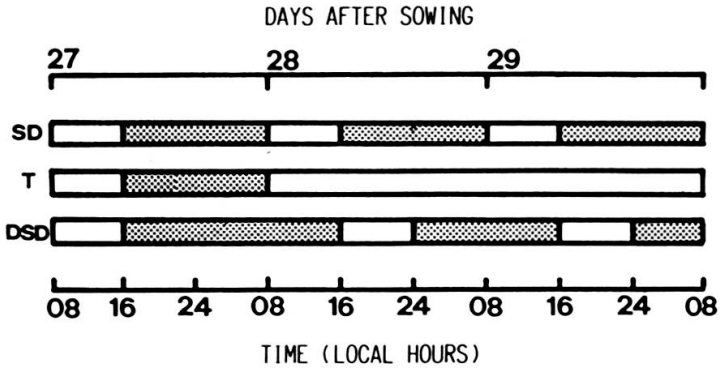

FIGURE 1.

Scheme illustrating the timing of the different photoperiodic treatments used in this study.

In the displaced short day experiments (DSD), the SD night was once extended to 24 h of darkness and the light period then given from 16 h to 24 h local time (see Fig. 1). Experimental treatments always started at the 28th day after sowing (Fig. 1). Only the light regimes were modified since removing the plants from one growth chamber to another resulted in a diminished or delayed response.

Soluble sugars and malate were extracted from petiole segments (1 cm) and from disks (0.3 cm in diameter) punched from the lamina. The tissues were heated to 100°C for 15-30 min in tightly sealed tubes containing 80% ethanol. This procedure allows complete and quantitative extraction of the metabolites. There was no degradation as shown by the total recovery of pure sugars or malate added before extraction. Glucose and fructose were assayed enzymatically (Bergmeyer *et al.*, 1974; Bernt and Bergmeyer, 1974) using a method modified for spectrofluorimetric measurement of NADPH (Degli Agosti and Greppin, 1987*a*). For glucose determination, the extracts were added to the assay buffer (pH 8.1) containing 50 mM Tris(hydroxymethyl)-aminomethane-HC1, 2 mM $MgCl_2$, 0.3 mM ATP, 0.05 mM NADP, 0.7 units ml^{-1} of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and the endogenous fluorescence level measured. The reaction was started by adding 3.5 units ml^{-1} (final concentration) of hexokinase (EC 2.7.1.1).

Fructose was measured in the same cuvette by adding 3.5 units ml^{-1} (final concentration) of phosphoglucose isomerase (EC 5.3.1.9). The NADPH fluorescence was monitored at 480 nm following an excitation at 340 nm and converted to units of sugars (µmoles) by using a calibration curve established with pure sugars. For sucrose determination, the ethanolic extracts were evaporated to dryness, dissolved in 100 mM NaOH and incubated 5 min at 95°C in order to destroy the reducing sugars (Van Handel, 1968). Quantitative hydrolysis of sucrose into glucose and fructose was achieved by adding 50 units ml^{-1} of invertase (EC 3.2.1.26) solubilized in 100 mM Na-acetate buffer (pH 4.5) and by incubating the mixture 20 min at 25°C. The pH was then adjusted to 8.1 with 2 M Tris buffer and the glucose produced was assayed as previously described. Starch determination was essentially carried out according to Jones (1979): the tissues were extracted twice in hot 80% ethanol (elimination of free glucose). The starch was then gelatinized by incubating the tissues in a 50 mM Na-acetate buffer (pH 4.5) for 1 h at 100°C. Amyloglucosidase (EC 3.2.1.3, 1.2 units ml^{-1}, final concentration) and azide (0.03 mM) were added, the tissues ground in a glass Ten-Broeck homogenizer and the samples incubated for 16 h at 40°C before measurement of the glucose released. Malate was assayed according to the enzymatic method of Williamson and Corkey (1969) adapted to spectrofluorometric NADH measurement. Results are expressed on a fresh weight basis, as the fresh weight varied no more than 7% during the experiments.

Invertase was extracted with a citrate (9 mM)-phosphate (82 mM) buffer (pH 7.0) containing 2 mM ß-mercaptoethanol. One gram of petiole segments was frozen in liquid nitrogen and added to teflon cells containing a tungsten-carbide ball pre-cooled

to −192°C. After the cells were shaken for 15 s in a Micro-Dismembrator (Braun, F.R.G.), 5 ml of the extraction buffer were added to the frozen powder and the cells shaken for a further 15 s. Their content was transferred to centrifuge tubes, allowed to thaw on ice and centrifuged for 20 min at 26,000 g. The supernatant was collected and filtered on a Sephadex G25 column (8 × 0.35 cm) equilibrated with the extraction buffer. The enzymatic activity was measured in citrate (47 mM)-phosphate (100 mM) buffer (pH 5.2) at 30°C. The reaction was started by adding sucrose at different concentrations (see results). Aliquots were sampled and their content in glucose and fructose immediately measured. The production of glucose was always equivalent to that of fructose and was linear over the range of measurements. Protein was estimated according to the Biorad micromethod (Bio Rad Laboratories, München, F.R.G.).

Amyloglucosidase was from Seikagaku Kogyo Co., Ltd (Japan). The other enzymes, ATP, NADP and NADH were from Boehringer (Mannheim, F.R.G.). All other chemicals were the highest grade available.

Each experiment was repeated at least 3 times. The pattern of the time course of the free sugar and starch content was stable from experiment to experiment. However, the absolute levels in free sugars or starch were strongly dependent on season. Therefore data shown are those from one representative time-series where the coefficient of variation ranges from 10 to 15% for each time-point.

RESULTS

The time-dependent changes in the glucose and fructose content in the primary leaves and petioles of 28 days old spinach plants are shown in Fig. 2 for two different photoperiodic treatments. In SD, the hexose content increases during the light phase and decreases in darkness. The transfer to continuous illumination is marked by a large increase in these soluble sugars during the first eight hours, followed by a decrease during the next eight hours with a subsequent increase again. In both the petiole and the lamina, the maximum increase reaches 980% and 1200% for the glucose and fructose content respectively, as compared to the level observed at the end of the SD light period.

The changes in the sucrose content in the petioles in SD as well as after the transfer to continuous illumination follow the same pattern as that observed for the hexoses (Fig. 3). Moreover, the time-dependent changes in glucose content (Fig. 3) show that the very beginning of the increase occurs between 16 and 20 h (local time), e.g. after zero to four hours of supplementary light. The sucrose content of the leaves increases during the light phase of the SD and remains almost constant after the transfer (Fig. 4). In contrast, petiolar starch content is strictly dependent on light and darkness, with a constant rate of accumulation in light (Fig. 5). In the leaves, this polymer accumulates in the light, but its rate of accumulation decreases slightly after transfer to continuous light (Fig. 5).

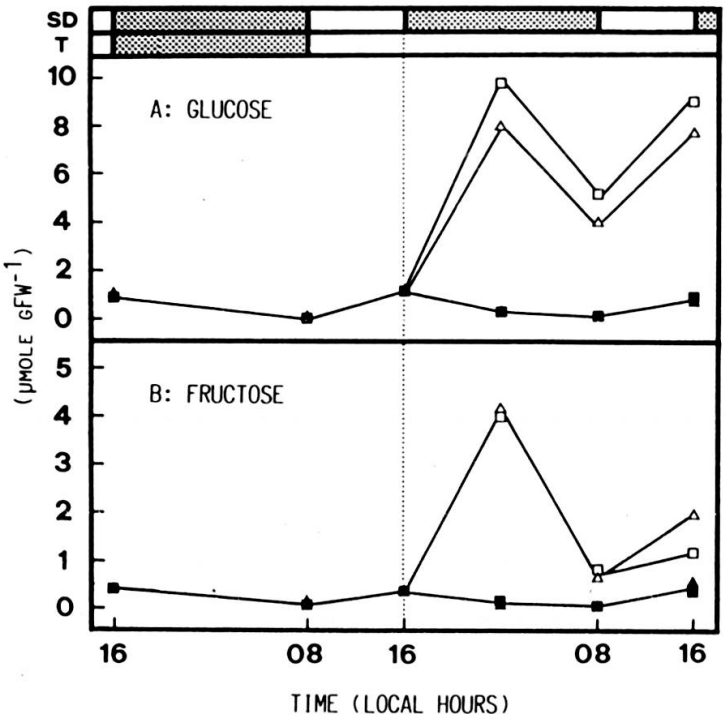

FIGURE 2.

Time course of the glucose and fructose content in spinach petioles (△, ▲) and leaves (□, ■) during the short day (▲, ■) and the transfer to continuous light (△, □). Equal ▲ and ■ values are represented by ■.

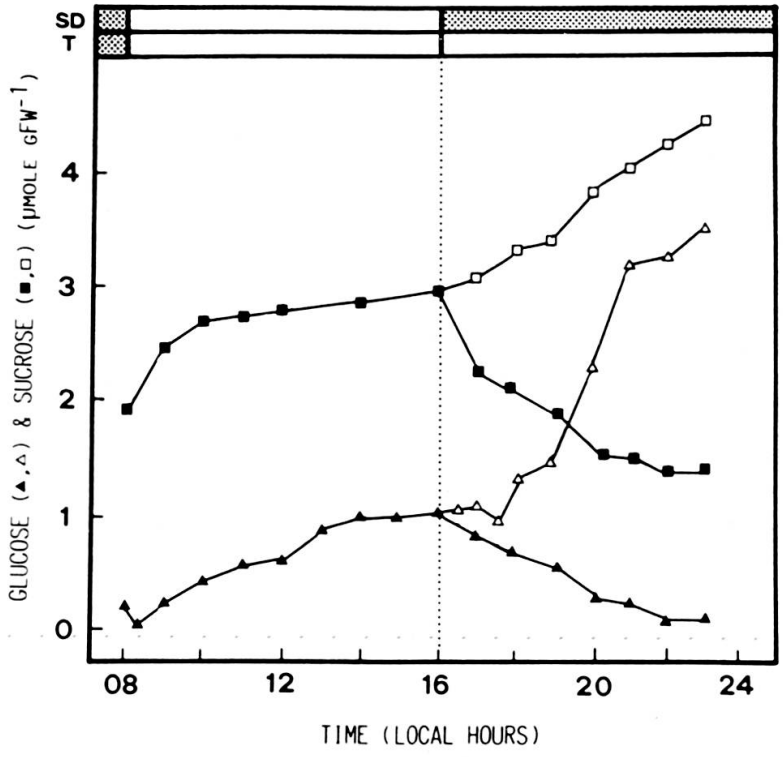

FIGURE 3.

Time course of the glucose and sucrose content in spinach petioles during the short day (▲, ■) and after transfer to continuous light (△, □).

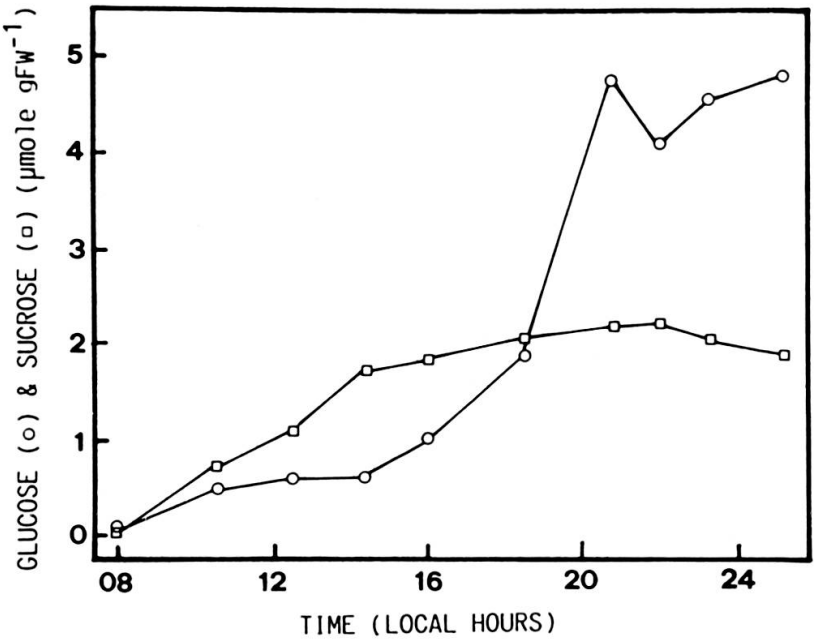

FIGURE 4.

Time course of glucose (○) and sucrose (□) content in spinach leaves during the light phase of the SD (08-16 h) and the transfer to continuous light.

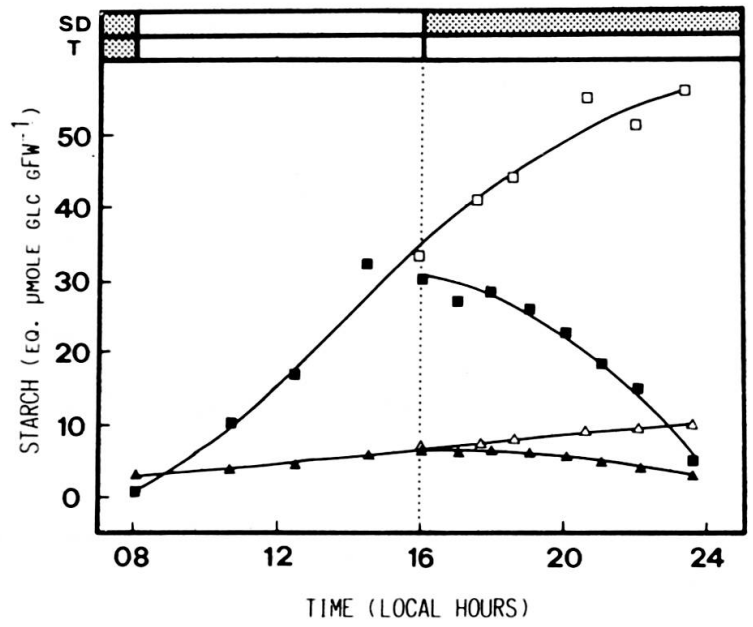

FIGURE 5.

Time course of starch content in spinach petioles (▲, △) and leaves (■, □) in short day (▲, ■) and during the transfer to continuous light (△, □).

The data in Table 1 indicate that when only the leaves are kept in darkness during the transfer to continuous illumination, there is no glucose increase in the petioles. Conversely, if only the petioles are kept in darkness, they are filled with the photosynthesis-derived products from the leaves as in the controls (T).

TABLE 1.

Effect of leaf photosynthesis on the glucose content of spinach petioles after a transfer (T) from the 28th day of short day conditions to continuous illumination. At the end of the SD light period (16 h), the darkening of petioles or leaves was performed by very delicately covering the respective plant parts with an aluminium foil. Mean ± standard error, n = 5.

	Petioles-Glucose (μmole g FW^{-1})		
Local time	16 h 00	20 h 00	08 h 00 *
Short day control (SD)	0.9 ± 0.3	0.2 ± 0.2	0.1 ± 0.1
Transfer (T)	—	4.8 ± 0.9	8.8 ± 1.2
T (petiole darkened)	—	4.2 ± 1.2	8.8 ± 1.2
T (leaf darkened)	—	0.4 ± 0.3	0.6 ± 0.3

* Day after the treatment (29th).

To better characterize the specific role of light in bringing about the sharp increase in glucose content in the petioles, the effect of light irradiance was examined. Below a threshold of about 15 Wm^{-2}, there is no increase in glucose content detectable at any time after the transfer to continuous light (Fig. 6A). Above this threshold, there is no linear relationship between the extent of the glucose increase and light irradiance which has no or little effect on the moment at which this phenomenon begins (Fig. 6B). On the other hand an increase in malate is almost directly proportional to the light irradiance (results not shown). Indeed, when glucose and malate accumulation are examined as a function of the product of light irradiance × duration of the light treatment it is clear that the patterns of glucose and malate accumulation are different (Fig. 7).

In an other experiment, the light phase of the SD, which was normally given from 8 h to 16 h, was shifted and given from 16 h to 24 h by extending the night period (DSD) (see Fig. 1). This treatment brought about a large and sharp increase in glucose content in the petioles without any lengthening of the light phase or increase in light irradiance (Fig. 8). However, the increase was similar to the one after transfer to continuous light (T).

The influence of the light off signal given at the end of the normal SD light was tested by terminating the SD night at different times. Fig. 9 shows that the

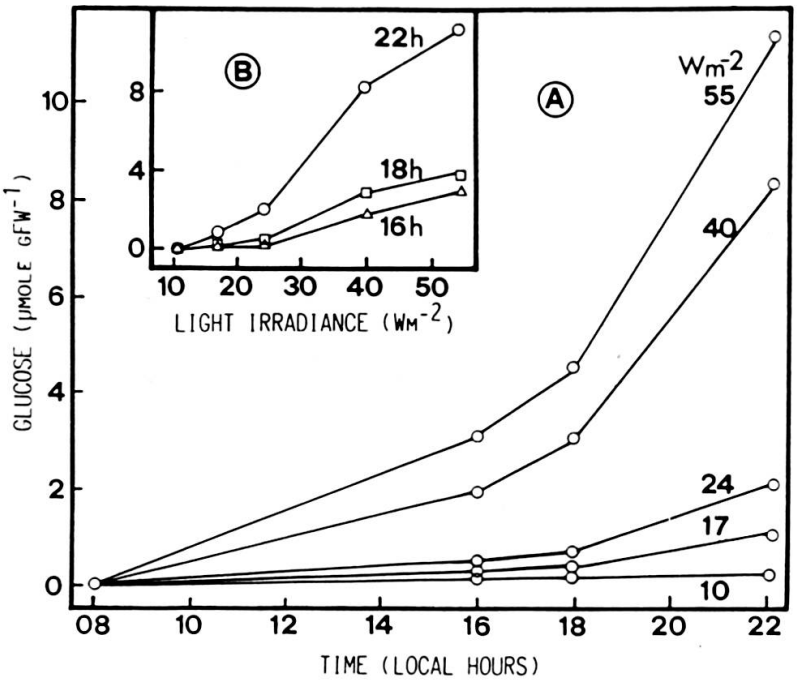

FIGURE 6.

Glucose content in spinach petioles at different times and light irradiances. A: glucose content as a function of local time. B: glucose content as function of light irradiance for plants sampled at the end of the SD and two and six h after the transfer to continuous illumination.

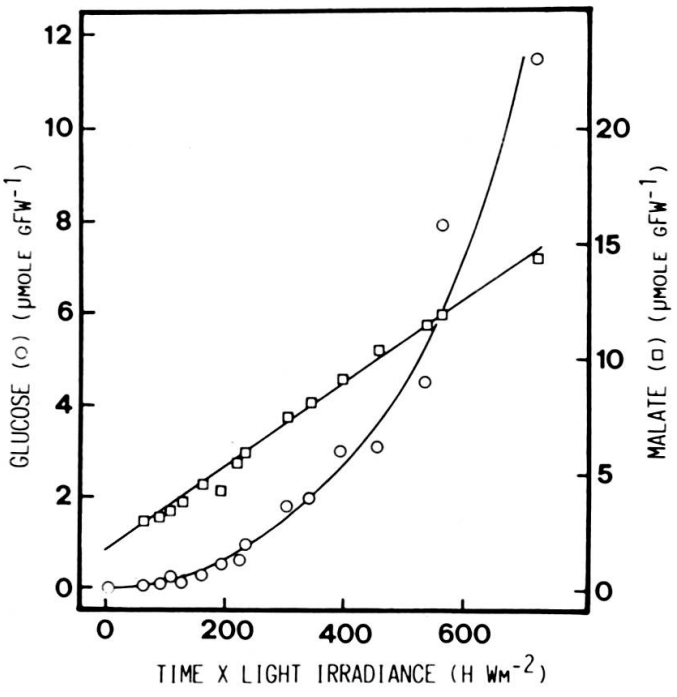

FIGURE 7.

Relationship between the glucose (○) and malate (□) content in spinach petioles during the light phase of the short day and the transfer to continuous light as a function of the product irradiance × duration of light.

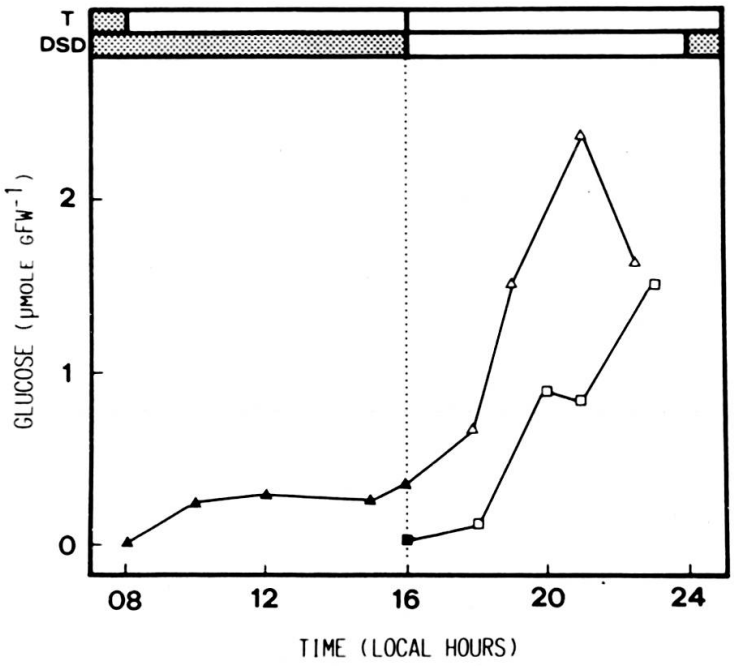

FIGURE 8.

Time course of the glucose content in spinach petioles during the SD (▲) and transfer to continuous light (△), and the displaced short day (DSD) treatment (□).

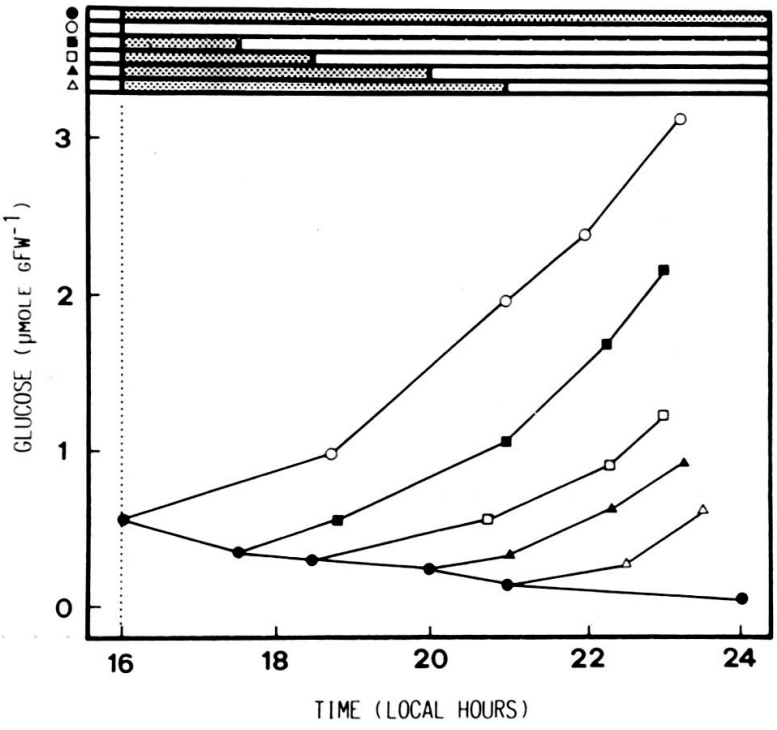

FIGURE 9.

Time course of the glucose content in spinach petioles subjected to different night lengths after a normal SD light period: 1 h 30 (■), 2 h 30 (□), 4 h (▲) and 5 h (△) darkness. Control experiments are shown for comparison: normal SD-night (●) and transfer to continuous illumination (○).

potential for the glucose increase is not switched off at least during the first five hours of the SD night period.

During the transfer to continuous illumination, the glucose increase is paralleled by that of fructose (Fig. 2). It was thus interesting to test for the invertase activity which is responsible for the transformation of sucrose into glucose and fructose. The invertase extracted from 4-week old spinach petioles can be shown to be a fully soluble enzyme (no activity in the pellet, see material and methods) having an optimum activity at pH 5.2. It is thus a soluble and acidic invertase whose affinity constant for sucrose (Km) is 2.0 ± 0.3 mM. Its potential activity, whether based on the fresh weight or on the protein content of the extracts, is the same in SD and after the transfer to continuous illumination, as shown in Table 2. There is also no variation in its pH optimum nor in its solubility (results not shown).

TABLE 2.

Invertase activity of spinach petioles at the end of the SD light period (SD) (16 h) and after 10 h of supplementary light (T). The assay was performed with 2 concentrations of sucrose (2 mM and 100 mM), and the results expressed on a fresh weight and total protein basis. The % changes in activity of T with respect to SD are given in parenthesis. Mean ± standard error, n = 5.

Invertase activity	Sucrose (mM)	
	2	100
µmole glc min^{-1} mg $protein^{-1}$		
SD	37.5 ± 3.9	96.6 ± 8.6
T	31.9 ± 4.1 (−15%)	88.1 ± 8.3 (−9%)
µmole glc min^{-1} g FW^{-1}		
SD	165 ± 17	425 ± 38
T	180 ± 23 (+9%)	498 ± 47 (+17%)

DISCUSSION

Changes in hexose content and biological rhythms

In SD, the glucose, fructose, sucrose and starch content in the primary leaves and petioles of spinach plants increases in the light phase and decreases in the dark phase. After the transfer to continuous illumination, the starch content (Fig. 5), and malate content (Fig. 7) in the petioles increase almost linearly with the lengthening

of the light period, showing thus a direct response to day length. In contrast, the same condition results in a specific modification of sugar contents: the glucose and fructose levels which are in some kind of dynamic equilibrium between 15 and 17 h in SD, increase sharply at about 16 or 19 h after the transfer to continuous illumination. The sucrose content also follows the same time-dependent changes, but to a lesser extent. Thus, the sharp increase in hexoses seems to correspond to a specific action of the photoperiod on sugar metabolism (Figs 8, 9).

Light irradiance modulates the glucose content of the petioles above a certain threshold, but does not affect the moment at which it increases (Figs 6A and B). The quantity of light as well as its total duration are therefore not the critical factor determining this phenomenon. The data in Figs 8 and 9 demonstrate that the determinant factor is in fact the coincidence between the presence of light (external factor) and a particular state in the organization of sugar metabolism (internal factor). Although these experiments were not designed to study biological rhythms, the results strongly suggest the existence of an endogenous rhythm in sugar metabolism which is synchronized by the light on and off signals in SDs. It can thus be assumed that the increase in the two hexoses can only be generated if light is given during a particular phase of this rhythm in sugar metabolism (Figs 8, 9).

Changes in hexose content and sink-source relationships

In petioles, it seems very unlikely that the hexose increase is due to a direct hydrolysis of their endogenous starch pool. The following observations support this view: (1) the rate of starch accumulation is almost constant in the light (Fig. 5), (2) complete starch hydrolysis could not even account for the glucose and fructose increase and, (3) the differential darkening of leaves and petioles clearly indicates that the source of hexoses can only be foliar photosynthesis (Table 1). This idea is reinforced by the fact that 4-week old spinach plants have their primary leaves about 80% expanded (Frosch *et al.,* 1986) and are thus exporting sucrose to sink tissues via the phloem (Fellows and Geiger, 1974; Gerhardt *et al.,* 1987; Giaquinta, 1978; Giersch *et al.,* 1980; Kaiser and Heber, 1984; Lucas and Madore, 1988; Servaites *et al.,* 1989; Thrower, 1962; Turgeon and Webb, 1976; Ziegler, 1975). As reducing saccharides are considered to be virtually absent from sieve tubes (Giaquinta, 1980), the photoperiodically controlled glucose and fructose increase in spinach petioles is thus likely to be derived from the circulating sucrose exported from the leaves. This suggests that (a part of) unloading of sucrose is under photoperiodic control and accompany the flowering process.

Invertase, compartmentation and possible molecular targets for photoperiodic control of the hexose content in spinach petioles

There are several possible pathways for sucrose unloading in sink regions (Giaquinta *et al.,* 1983). They may have different structural routes (symplastic via

plasmodesmata and/or apoplastic) together with different enzymes, namely invertase and sucrose synthase (Lucas and Madore, 1988). This latter enzyme is unlikely to function in a predominant manner in the present phenomenon since it would produce fructose and UDP-glucose instead of glucose and fructose. Moreover, it is shown in this study that we detected an invertase activity in spinach petioles that is fully soluble with an acidic pH optimum, both these properties being typical of vacuolar invertase (Ap Rees, 1984; Avigad, 1982; Sung *et al.*, 1988), where sucrose (Geiger *et al.*, 1983; Gerhardt and Heldt, 1984; Gerhardt *et al.*, 1987; Giaquinta, 1978; Fisher and Outlaw, 1979) together with hexoses (Asami *et al.*, 1985; Wagner, 1979) can also be found. This invertase activity was initially suspected to be specifically activated by the lengthening of the photoperiod but the results showed that this hypothesis was partly inappropriate since no activity change could be detected *in vitro*. Thus, the *in vivo* enhanced invertase activity can be brought about by a simple increase in substrate (sucrose) concentration or availability. In fact, the potential *in vitro* activity at the end of the SD light period is large enough to account for the observed increase in hexoses during the transfer to continuous illumination. This might be an indication that sucrose availability towards invertase could be under photoperiodic control. Theoretical (Degli Agosti and Greppin, 1987*b*) studies on compartmentation (Degli Agosti and Greppin, 1989) have also suggested that the vacuolar compartment is clearly involved in the hexose increase. In these conditions control could be tentatively situated at the level of the sucrose carriers present in the plasmalemma (Reinhold and Kaplan, 1984) and/or in the tonoplast (Guy *et al.*, 1979; Kaiser and Heber, 1984). If the unloading is symplastic, then besides the tonoplast permeability to sucrose, plasmodesmata could also be controlled, since they play a role in phloem unloading (Ding *et al.*, 1988). It is noteworthy to mention that changes in structural and compositional properties of membranes have been found in spinach leaves subjected to photoperiodic induction of flowering (Penel *et al.*, 1988). This phenomenon could directly or indirectly affect diffusional and/or carrier mediated sucrose transport.

Changes in hexose content in leaves

In the leaves, the same considerations as for petioles draw attention to the vacuolar compartment. Although, the possibility that changes in hexose content may be localized in specialized tissue like the vascular or paraveinal mesophyll (Franceschi and Giaquinta, 1983) can not be ruled out. Indeed, invertase activity has been localized in leaves of *Gomphrena globosa* in vascular parenchyma only (Eschrich and Eschrich, 1987). Nevertheless, in leaves, the situation is more complex and some specific points need to be outlined. The apparent sucrose production rate is decreasing at the end of the SD and during the transfer to continuous light (Fig. 4). The decreasing rate of sucrose accumulation may contribute to the photoperiodic controlled increase of glucose by about 50% (see Fig. 4). Moreover, considering that

sucrose is exported to sink tissues like petioles and hypocotyls (Degli Agosti, 1985) where the hexose increase is also observed, then it is very likely that the sucrose turnover is increased in leaves. This would mean a higher rate of synthesis of this substance after the photoperiodic transfer.

The increase in sucrose synthesis may be brought about by enhancing the *in vivo* activity of sucrose-phosphate synthase (SPS), which is a key enzyme of this pathway, regulated by various effectors (Amir and Preiss, 1982; Doehlert and Huber, 1983; Kerr *et al.*, 1987; Sicher and Kremer, 1985). This view is strengthened by the fact that this enzyme can display an endogenous rhythm in its *in vitro* activity peaking about 10-15 h after the light-on signal in pea, bean, soybean, tobacco, and sugar beet (Huber *et al.*, 1984; Huber *et al.*, 1985; Rufty *et al.*, 1983; Vassey, 1989). Interestingly, cultivars which show no photoperiodic control of flowering lack this *in vitro* rhythm (Huber *et al.*, 1984). However, this simple relation may be irrelevant in spinach as Stitt *et al.*, (1988) and Servaites *et al.*, (1989) did not find an *in vitro* rhythmic activity of SPS in this plant. The present data suggest that in our experimental conditions the *in vivo* sucrose-phosphate synthase activity should necessarily be increased at some critical time during the photoperiod. Fluxes through cytoplasmic fructose-bisphosphatase, an other important control point in sucrose biosynthesis (Cseke *et al.*, 1984), should also be enhanced by a lowering of its main regulator fructose-2,6 bisphosphate (Huber, 1986; Stitt, 1987). Finally, at the chloroplast level the present data show that the starch accumulation rate is decreased during the transfer to continuous light (Fig. 5). Thus, partitioning of carbon is likely to be involved with its associated regulatory pathways (Preiss, 1984) and may at least partly contribute to the higher sucrose fluxes. In this respect, the present results support the view showing that partitioning of newly fixed carbon between soluble sugars and starch is influenced by day-length (Chatterton and Silvius, 1979, 1980*a*, 1980*b*; Robinson, 1984) and is photoperiodically controlled (Britz *et al.*, 1985*a*, 1985*b*), where starch accumulation rate has been observed to decrease in plants acclimating or acclimated to longer light periods.

Photoperiodic control of sugar metabolism

The glucose and fructose increase is not restricted to spinach plants. It has also been observed in *Coleus blumei* (Degli Agosti, 1985), *Sinapis alba,* both long-day plants, and with a mirror pattern in *Chenopodium rubrum,* a short-day plant (Degli Agosti *et al.*, 1989). Moreover, a similar and rapid increase in hexoses at the end of the daily photoperiod has also been clearly observed in *Glycine max* (see Fig. 7A of Kerr *et al.*, 1985*a* and Fig. 3B of Kerr *et al.*, 1985*b*). However, the physiological significance of this phenomenon was not discussed by the authors. Carbon export rates from sources leaves are controlled by sink and source tissue physiology (Geiger 1987). At the end of a normal day, the decrease in light intensity is rapidly

accompanied by a slowing down of the carbon exchange rate and hence sucrose and starch synthesis (Servaites *et al.*, 1989). One would expect that in these conditions the short term carbon export rate would diminish. However, this has clearly been shown not to be the case (Servaites *et al.*, 1989). Maintaining a constant export rate at the end of the day may thus be achieved by increasing sink demand. A photoperiodic control of this process would be rather useful for matching the complex internal network of carbohydrate metabolism to the periodic modifications of the light environment.

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