Plant Physiol. (1989) 91, 905–910
0032-0889/89/91/0905/09/$01.00/0

Received for publication March 10, 1989
and in revised form May 18, 1989

Influence of Low Temperature on Respiration and Contents of Phosphorylated Intermediates in Darkened Barley Leaves[1]

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ABSTRACT

The aim of this work was to examine the effect of exposure of leaves to low temperatures (5°C) upon the contents of phosphorylated intermediates and respiration in darkened barley (*Hordeum vulgare* L.) plants which differed in their carbohydrate status. In leaves that had previously been illuminated for 24 hours, there was a large increase in amounts of phosphorylated metabolites at 5°C during the first 3 hours of darkness, compared with control plants kept at 30°C. Hexose phosphates accounted for about two-thirds of this increase, which reached a peak after about 3 hours. At higher temperatures, there was a peak in the amount of fructose 2,6-bisphosphate and the rate of respiration which accompanied the transient increase in phosphorylated intermediates. At 5°C the increase in phosphorylated intermediates was not accompanied by appreciable changes in fructose 2,6-bisphosphate, and there was a rapid decline in the rate of respiration. Leaves that had previously been darkened for 24 hours and that were low in carbohydrate failed to accumulate phosphorylated intermediates when exposed to low temperatures. The results are discussed with respect to the acclimation of carbohydrate metabolism to low temperatures. The results suggest that respiratory carbohydrate metabolism is strictly controlled even when the carbohydrate supply and glycolytic intermediates are abundant. The possibility that accumulation of hexose phosphates may be involved in acclimation of metabolism to low temperature is discussed.

Low temperatures will influence carbohydrate metabolism in leaves in a number of different ways. In the whole plant, low temperatures will both diminish sink activity and export of carbohydrate from leaves, disrupting the balance between supply of, and demand for, assimilated carbon. Such temperature-dependent decreases in the utilization of photosynthate have been shown to lead to end-product inhibition of photosynthesis (1, 2, 4). Increased amounts of carbohydrate within the leaf could then lead to an increase in the availability of respiratory substrates and thereby increase the rate of respiration (10, 16). In the longer term, increased ratios of source to sink activity that occur at low temperatures will also influence the partitioning of carbohydrate, resulting in an increase in the formation of alternative carbohydrate reserves such as fructans in grasses (14). Exposure to low temperatures will also have consequences which occur purely at the metabolic level. For example, during photosynthesis the balance between sucrose synthesis and carbon fixation is perturbed when leaves are suddenly transferred to low temperatures because sucrose synthesis runs too slowly at lower temperatures (9, 12). A number of temperature-dependent metabolic factors may act to bring about this imbalance between the capacity to fix CO_2 and to synthesize sucrose. First, low temperatures raise thresholds for the metabolism of triose-P to sucrose because of large decreases in the affinity of the cytosolic Fru 1,6-P_2ase[3] for its substrate as the temperature is decreased (21). There is also the possibility that inactivation of the cytosolic Fru 1,6-P_2ase occurs *in vivo* at low temperatures (23). The consequence of either change would be that more phosphorylated intermediates would be required in the cytosol in order to drive sucrose synthesis. Second, it is likely that more orthophosphate is required in the cytosol at low temperatures (11, 12, 21) to allow export of triose-P from the chloroplast, since Pi feeding stimulates photosynthesis at lower temperatures (9, 12).

The aim of this work was to identify how low temperature (5°C) and varying carbohydrate reserves interact to influence changes in metabolite pools and respiration in darkened leaves of barley (*Hordeum vulgare* L.) plants.

MATERIALS AND METHODS

Materials

Barley (*Hordeum vulgare* L. cv Sonja) was grown in compost in 3.5-inch square pots for 10 d in a greenhouse, as described previously (9), under a maximum photosynthetic photon flux density of 500 to 600 μmol quanta·m^{-2}·s^{-1} at midday. Air temperature was kept at an average of 30°C during the day and 18°C at night. Pots of plants were either kept for 24 h at a photon flux density of 500 μmol quanta·m^{-2}·s^{-1} or in the dark for 24 h following a normal photoper-

[1] Supported by the Agricultural and Food Research Council, U.K. (Research grant PG50/67) and by the Science and Engineering Research Council, U.K. C. A. L. was supported by the British Council, by an Overseas Research Student Award, and by CNPq Brazil.

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[3] Abbreviations: Fru 1,6-P_2, fructose 1,6-bisphosphate; Fru 6-P, fructose 6-phosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; Glc 6-P, glucose 6-P; Glc 1-P, glucose 1-P; PEP, phospho*enol*pyruvate; PGA, glycerate 3-P; UDPGlc, UDP-glucose.

iod. The plants were darkened for 10 min (time zero in the figures and tables) and then maintained at 5°C or at 25 to 30°C. Changes in metabolite pools during darkness were similar at 25 and 30°C. Potato PPi-dependent Fru 6-P kinase was obtained from the Sigma Chemical Co.

Metabolite Measurements

The darkened leaves were immersed in liquid N_2 in the dark and ground in liquid N_2 in a precooled pestle and mortar. Leaf samples were stored in envelopes of aluminum foil in liquid N_2 and then denatured and extracted in $HClO_4$ as described previously (12). Following neutralisation, sufficient charcoal was added (as a 100 mg·mL^{-1} suspension in water) to render the extract completely colorless. The extract was centrifuged at 2000*g* for 2 min. The supernatant was either immediately used or stored in liquid N_2 for the determination of metabolites by the methods of Lowry and Passonneau (13) in a Hitachi 557 dual-wavelength spectrophotometer (340–400 nm). For the measurement of Fru 2,6-P_2, leaves were extracted according to the method of Stitt *et al.* (18) A 0.2 g portion of leaf material was mixed in a pestle and mortar (cooled with liquid N_2) with 4.2 mL extraction medium (1.2 mL $CHCl_3$, 2.4 mL CH_3OH, 0.6 mL buffer containing 50 mM NaF, 10 mM EGTA, 50 mM Hepes [pH 8.5]) and then held at 4°C. Four mL H_2O was added, and the mixture was shaken vigorously and centrifuged. The supernatant was taken to dryness at 35°C, redissolved in 1 mL water, and stored in liquid N_2. Fru 2,6-P_2 was assayed as described (22). Internal standards were used, and the mean recovery of added Fru 2,6-P_2 was 102%. Soluble sugars were assayed spectrophotometrically (8), following extraction of leaves in $HClO_4$ or in $CHCl_3/CH_3OH$, as described above. For estimation of starch, the insoluble fraction was washed with 0.5 M Mes (pH 4.5), resuspended in 0.2 mL of the same buffer, and treated with 4 units of amylase and 14 units of amyloglucosidase for 2 h at 55°C. After centrifugation the supernatant was assayed for glucose as in Jones *et al.* (8).

Measurement of O_2 Uptake by Leaves

O_2 uptake in the dark was measured in air in an LD-2 leaf disc electrode (Hansatech Ltd, Kings Lynn, UK), containing matting moistened with air-saturated water. Illumination of the leaf pieces (10 cm^2) was continued until the leaf chamber was closed.

RESULTS AND DISCUSSION

Changes in Metabolite Pools during Darkness

Barley plants were exposed to 24 h continuous light or 24 h continuous darkness in order to manipulate the endogenous content of carbohydrate. After 24 h light, the mean content of starch plus sucrose and hexose was 21.5 μmol hexose equivalents·mg^{-1} Chl (Table I), whereas the corresponding content for leaves of plants kept for 24 h in the dark was 3.5 μmol hexose equivalents·mg^{-1} Chl. After this pretreatment, plants were transferred to darkness at 5 or 30°C for periods of up to 10 h. After a period of 24 h in the light, more carbohydrate was mobilized in the leaves kept in the dark at 30°C than in those kept at 5°C. In this instance, the principal difference was in the amount of starch mobilized (Table I). The changes in the total pool of hexose-P (Glc 6-P, Fru 6-P, and Glc 1-P) (Fig. 1) and the total change in esterified phosphate in all of the measured metabolites (including all those of glycolysis) (Fig. 2) that occurred at 30°C were similar to those reported by Stitt *et al.* (20) in showing an initial rapid fall within the first hour, followed by a rise, in turn followed by a gradual fall. At 5°C the pattern was the same, except that the transient fall in metabolites during the first hour was followed by a much larger peak. A comparison of Figure 1 with Figure 2, which illustrates the total change in esterified phosphate in all of the measured intermediates, shows that the majority of this increase at 5°C was accounted for by changes in the pools of hexose-P. Total esterified phosphate temporarily rose by about 500 nmol·mg^{-1} Chl in response to incubation at low temperature (Fig. 2). If hexose-P are largely cytoplasmic (6), and the cytoplasm has a volume of 25 μl·mg^{-1} Chl, then this total represents an increase in esterified phosphate of about 20 mM. In leaves of spinach plants that had previously been illuminated, there was also a comparably large increase in phosphorylated intermediates in the dark at low temperatures. Again, hexose-P accounted for the vast majority of the increase in esterified phosphate (data not shown). Accumulation of hexose-P was also observed in barley leaves following a normal photoperiod.

Table II shows in detail the changes in metabolites which occurred during darkness in the barley plants which had been exposed to 24 h continuous light. At 30°C the pools of all metabolites, except PGA and PEP, fell overall during a period of 3 h darkness, and only PEP was maintained at high levels after 10 h darkness, indicating that the metabolism of PEP is being controlled in these leaves. The ratio pyruvate/PEP also

Table I. *Contents of Sucrose and Starch in Leaves of Darkened Barley Plants at Different Temperatures, Following upon a Light Pretreatment*

Plants were illuminated for 24 h and then darkened at 5 and 25°C. Starch and sucrose were determined as described in "Materials and Methods." Data are means ± SE of three determinations.

Carbohydrate	Carbohydrate Content				
	0 h	5°C		25°C	
		3 h	10 h	3 h	10 h
	μmol hexose equivalents·mg^{-1} Chl				
Sucrose	11.5 ± 1	10.4 ± 1	9.3 ± 0.5	10.1 ± 1	9.0 ± 1
Starch	10.0 ± 1	7.2 ± 2	5.1 ± 1	6.9 ± 2	2.5 ± 1

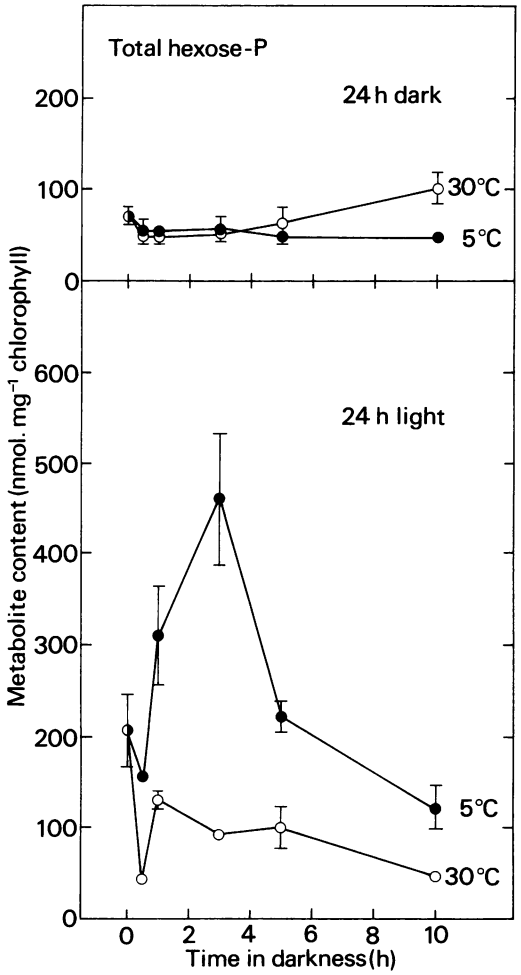

Figure 1. Changes in hexose-P in leaves of barley plants that were either illuminated or darkened for 24 h and then transferred to darkness at 5 or 30°C. The data are means ± SE of three determinations.

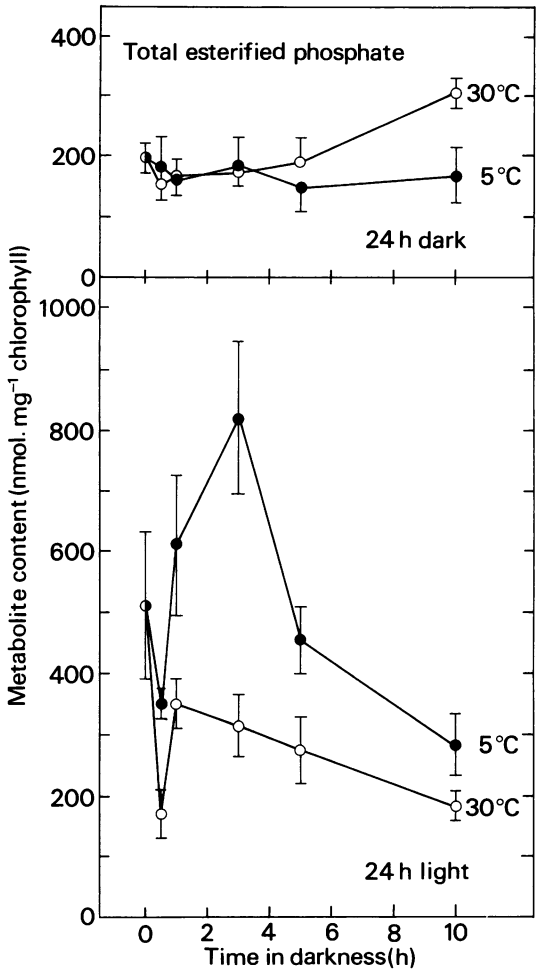

Figure 2. Changes in total esterified phosphate in leaves of barley plants that were either illuminated or darkened for 24 h and then transferred to darkness at 5 or 30°C. The data are means ± SE of three determinations. For a complete list of metabolites measured, see Table II.

fell during darkness, while the ratio of PGA to triose-P rose markedly. At 5°C the total amount of metabolites rose appreciably during the first 3 h of darkness and then declined slowly over the next 7 h, parallel to the changes in hexose-P. Although the majority of this change in intermediates was accounted for by changes in the pools of hexose-P, as noted above, it is important to note that pools of all of the glycolytic intermediates (except UDP-Glc) increased, including pools of triose-P and Fru 1,6-P_2, both of which usually fall rapidly to very low levels in the dark at 30°C (20). The ratio of pyruvate to PEP fell during darkness, as at 30°C. After 3 h, PGA/triose-P ratios reached values (7.2) at 5°C which approached those commonly observed in illuminated leaves or in darkened protoplasts (ratios in these are typically between 5 and 10) (20), rather than the much larger values (12.2–65) commonly observed in darkened leaves at higher temperatures or at lower temperatures after 24 h darkness (Table III; ref. 20). Ratios of Glc 6-P to Fru 6-P were high in both temperature treatments in the dark, indicating a cytosolic location for these compounds (6), and were further increased at 5°C.

Table III and Figures 1 and 2 summarize the changes that occurred in leaves of barley plants that were exposed to 24 h continuous darkness prior to transfer to high and low temperatures. At 30°C contents of phosphorylated metabolites showed a small gradual increase. The ratio of Glc 6-P to Fru 6-P decreased after 10 h indicating a shift toward chloroplastic compartmentation of hexose-P. At 5°C there was very little change in the amounts of metabolites during 10 h of darkness, only hexose-P fell slightly (Fig. 1) and the amount of PEP rose while the amount of pyruvate remained constant, leading to a decrease in the pyruvate/PEP ratio.

Changes in Amounts of Fru 2,6-P_2 during Darkness

Figure 3 shows the changes that occurred in the pool of Fru 2,6-P_2 during darkness at high and low temperature in leaves that had previously been illuminated for 24 h. Recovery experiments showed that our procedures resulted in no sig-

Table II. *Metabolite Contents of Leaves of Darkened Barley Plants at Different Temperatures, Following upon a Light Pretreatment*

Plants were illuminated for 24 h and then darkened at 5 and 30°C. Metabolites were determined as described in "Materials and Methods." Data are means ± SE of three determinations.

Metabolite	Metabolite Content				
	0 h	5°C		30°C	
		3 h	10 h	3 h	10 h
	nmol·mg⁻¹ Chl				
Glc 6-P	142 ± 29	350 ± 54	89 ± 20	65 ± 5	30 ± 2
Fru 6-P	48 ± 8	77 ± 13	22 ± 4	17 ± 1	9 ± 1
Glc 1-P	16 ± 3	34 ± 5	13 ± 1	11 ± 2	9 ± 2
UDPGlc	32 ± 8	33 ± 3	4 ± 1	4 ± 2	1 ± 1
Fru 1,6-P_2	8 ± 2	12 ± 4	3 ± 1	2 ± 1	2 ± 1
Triose-P	16 ± 3	21 ± 2	7 ± 1	4 ± 1	3 ± 1
PGA	102 ± 32	152 ± 20	92 ± 14	132 ± 26	72 ± 3
Glycerate 2-P	6 ± 1	9 ± 1	4 ± 1	6 ± 1	4 ± 1
PEP	19 ± 2	25 ± 6	22 ± 7	26 ± 1	22 ± 5
Pyruvate	81 ± 20	61 ± 10	23 ± 12	42 ± 7	31 ± 7
Metabolite ratio (mol/mol)					
PGA/triose-P	6.4	7.2	13.1	33.0	24.0
Glc 6-P/Fru 6-P	3.0	4.6	4.1	3.8	3.3
Pyruvate/PEP	4.3	2.4	1.0	1.6	1.4

Table III. *Metabolite Contents of Leaves of Darkened Barley Plants at Different Temperatures, Following upon a Dark Pretreatment*

Plants were darkened for 24 h and then transferred to darkness at 5 or 30°C. Metabolites were determined as described in "Materials and Methods." Data are means ± SE of three determinations. Glycerate 2-P in these leaves was present at below the levels at which it could be accurately detected.

Metabolite	Metabolite Content				
	0 h	5°C		30°C	
		3 h	10 h	3 h	10 h
	nmol·mg⁻¹ Chl				
Glc 6-P	41 ± 7	35 ± 7	29 ± 2	28 ± 5	62 ± 12
Fru 6-P	13 ± 2	9 ± 2	8 ± 1	9 ± 1	23 ± 2
Glc 1-P	16 ± 1	13 ± 4	11 ± 1	14 ± 2	18 ± 2
UDPGlc	6 ± 1	8 ± 5	2 ± 1	4 ± 1	6 ± 4
Fru 1,6-P_2	2 ± 1	2 ± 1	2 ± 1	1 ± 1	2 ± 1
Triose-P	5 ± 2	5 ± 2	5 ± 1	4 ± 1	7 ± 2
PGA	68 ± 3	69 ± 15	61 ± 4	56 ± 3	118 ± 7
PEP	9 ± 3	7 ± 1	22 ± 11	13 ± 5	30 ± 8
Pyruvate	28 ± 4	28 ± 4	26 ± 1	35 ± 1	34 ± 3
Metabolite ratio (mol/mol)					
PGA/triose-P	13.6	13.8	12.2	14.0	16.9
Glc 6-P/Fru 6-P	3.2	3.9	3.6	3.1	2.7
Pyruvate/PEP	3.1	4.0	1.2	2.3	1.1

nificant losses of Fru 2,6-P_2 during extraction and analysis. There was a modest peak in the pool of Fru 2,6-P_2 at 25°C, but a slight fall in the amount of Fru 2,6-P_2, followed by gradual increase, at 5°C (a slight shift in the pattern between experiments increased standard errors, but the changes were consistent). Both the amounts of Fru 2,6-P_2 and the changes were much lower than previously reported (17) following transfer of barley leaves to darkness. Sicher *et al.* (17) and Baysdorfer *et al.* (5) both noted a large rise in Fru 2,6-P_2 in barley leaves during the first 30 min of darkness. This rise was enhanced if carbohydrate accumulation was increased by prolonging the preceding photoperiod or by lowering the temperature during illumination. However, their observations contrast with those of Stitt *et al.* (18), who observed a fall in the amount of Fru 2,6-P_2 in spinach leaves following darkening.

Changes in Respiration Rate at High and Low Temperatures

Increased leaf carbohydrate reserves are apparently accompanied by increased rates of respiration (2) in accord with the 'energy-overflow' hypothesis, which proposes a stimulation of

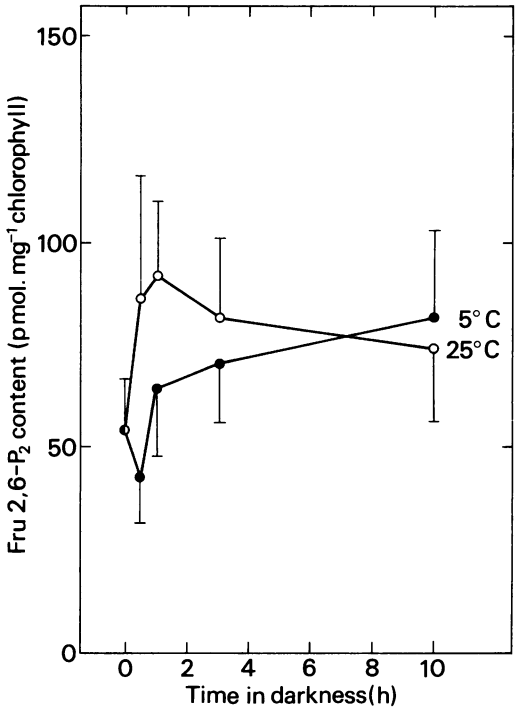

Figure 3. Changes in the amount of Fru 2,6-P_2 in leaves of barley plants that were illuminated for 24 h and then transferred to darkness at 5 or 25°C. The data are means ± SE of three determinations.

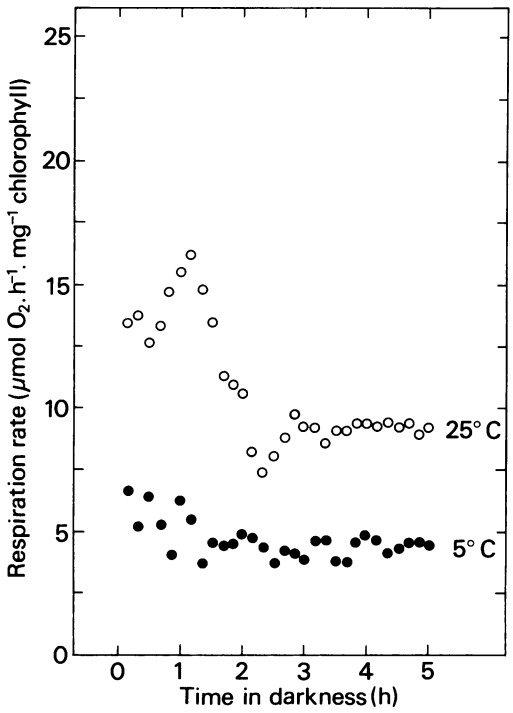

Figure 4. Effect of temperature on respiratory O_2 uptake by darkened barley leaves. Leaves were illuminated for 24 h and then transferred to darkness at 5 or 25°C. The data are means of three determinations. Standard errors were less than 15% of the means.

respiration and engagement of the alternate (cyanide-insensitive) pathway under conditions of 'excess' carbohydrate (3, 10). We took advantage of the difference in the behavior of metabolite pools in leaves that had been previously illuminated and then darkened at 5 and 25°C to test the hypothesis that increased pools of respiratory intermediates would give rise to increased rates of respiration. Figure 4 shows changes in the respiration rates of barley leaves following darkening of leaves that had previously been kept for 24 h in the light. At 25°C, a peak occurred in the respiration rate after about 1 h and was followed by a decline and then a slight increase to a steady state value. In contrast, there was a gradual fall in the respiration rate at 5°C over the first 1 to 2 h of darkness, followed by a steady rate of respiration over the next 3 h.

Azcon-Bieto and Osmond (2) measured changes in the rate of dark CO_2 efflux in darkened wheat leaves following a period of carbohydrate accumulation and showed both that the rate of dark CO_2 efflux was stimulated by preillumination and that the effect of photosynthetic activity on dark CO_2 efflux was more accentuated and lasted longer at lower temperatures. This was partly ascribed to the dissipation of photorespiratory intermediates during the first 30 min of darkness. If the concentration of O_2 was lowered to 3% in the preceding light period, then dark CO_2 efflux at 20 and 30°C rose to a peak within 20 to 30 min of darkness (2). Our estimates of respiration over several hours are therefore unlikely to be complicated by additional O_2 uptake processes which depend upon the oxidation of photorespiratory intermediates generated in the light.

At the higher temperature (25–30°C), in leaves which had been previously illuminated, the transient peak in the rate of respiration occurred at the same time as an increase in the content of Fru 2,6-P_2, which might indicate that it results from a Fru 2,6-P_2-mediated stimulation of the glycolytic flux. Although there were no large overall changes in the pools of phosphorylated metabolites, the increase in respiration also coincided with the appreciable transient increase in metabolite pools which occurred between 30 min and 1 h of darkness. Increases in respiration, metabolite pools, and Fru 2,6-P_2 therefore occurred more or less simultaneously. However, the rate of respiration declined dramatically between 1 and 2 h darkness without appreciable changes in Fru 2,6-P_2 (Fig. 3), metabolite pools (Figs. 1 and 2), or in soluble carbohydrate (Table I). The respiratory rate did not therefore appear to be controlled by the availability of substrate at this stage.

The results presented for changes at 5°C (Table II; Figs. 3 and 4) provide clearer evidence which directly contradicts the energy-overflow hypothesis. At 5°C, the following coincident features were observed after darkening the plants: (a) there were very large increases in pools of respiratory substrates, particularly of hexose-P, (b) the amount of Fru 2,6-P_2 declined slightly and then rose gradually between 1 and 10 h darkness, (c) the respiration rate decreased rapidly and remained constant, (d) the ratio of pyruvate to PEP fell, indicating a restriction on the conversion of PEP to pyruvate, (e) there was a high degree of energisation (provision of ATP and NAD(P)H), indicated by the low PGA/triose-P ratio (7), and (f) respiration rates followed a very different pattern at 5°C and 25°C despite virtually identical contents of soluble carbohydrate (Table I). There was, therefore, no obvious correlation between the respiratory rate and the availability of

respiratory substrate and there was no peak in the respiratory rate at 5°C which corresponded to an increase in glycolytic intermediates or of the content of Fru 2,6-P_2. Indeed, the content of Fru 2,6-P_2 rose while metabolites fell and the respiration rate remained constant.

The high degree of energisation at 5°C, indicated by the low PGA/triose-P ratio, may be responsible for restricting the respiration rate. However, it is under precisely these conditions of high energisation that the alternate pathway is most likely to be engaged (unless it is inactivated at low temperature) and yet the use of glycolytic substrates for respiration is evidently restricted. A comparison of respiratory fluxes with the pool size of respiratory substrates (apart from carbohydrates) emphasizes how effectively glycolysis is being controlled under these conditions. A respiration rate of 4 μmol $O_2 \cdot h^{-1} \cdot mg^{-1}$ Chl at 5°C (Fig. 4) would deplete hexoses at a rate of 670 $nmol \cdot h^{-1} \cdot mg^{-1}$ Chl. In other words, the pool of phosphorylated intermediates accumulated after 3 h at 5°C would be depleted after less than 30 min respiration. Stitt *et al.* (18) were also unable to find a correlation between the temporary increase in respiration in darkness and the behavior of metabolite pools as there were large decreases in available metabolites but only small changes in respiration rate during the period between 3 and 20 h in the dark in spinach leaves.

Significance of Metabolite Accumulation at Low Temperature

It is now well established that the accumulation of carbohydrate within leaves by treatments such as prolonged illumination or low temperature girdling of the petiole restricts photosynthesis (1). Stitt *et al.* (19) have shown that, in the light at 20°C, there are marked increases in the pools of UDP-Glc, hexose-P, and triose-P (which are predominantly cytosolic) under conditions in which carbohydrate has accumulated and its export is restricted. In darkened spinach leaves, there was also a positive correlation between the amount of starch and the context of hexose-P in the dark (20). The results presented in this paper further show that exposure to low temperatures of leaves that had been previously illuminated and that are rich in carbohydrate results in an accumulation of phosphorylated intermediates, hexose-P in particular. Although the rise in intermediates that occurs in darkened barley and spinach leaves at low temperatures may be a simple reflection of the accumulation of carbohydrate, the increase in metabolite pools may have an additional functional significance in that it could compensate for temperature-dependent changes in enzyme velocity and the temperature-dependent changes in the kinetic properties of the cytosolic FBPase (21, 23), as discussed in the introduction, and thus allow maintenance of sucrose synthesis from starch, via the cytosolic Fru 1,6-P_2ase, and maintenance of glycolysis in the dark at low temperatures. For similar reasons, higher contents of metabolites are required to maintain photosynthesis at lower temperatures (11, 21) and may, in part, be used to offset the limitation by phosphate status which occurs at low temperatures (9). The accumulation of hexose phosphates which has been observed during cold-induced sweetening in potato tubers (15) could also be a metabolically related phenomenon.

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