RELATIONSHIPS BETWEEN PHOTOSYNTHESIS, RESPIRATION AND CARBOHYDRATE LEVELS IN WHEAT LEAVES

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by

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CORRIGENDA

Page	Line	Amended (corrected) version
3	2	Hopf
7	-	Fig. 1.3. Thick arrow from TP to P_i in the High P_i figure
11	25	protoplasts (instead of chloroplasts)
16	-	Fig. 1.4. The reaction (15) is not correct. It should be

Page	Line	Amended (corrected) version
19	-	In the footnote, include brackets : ½ (ADP)
25	23	Nyns
56	2	include "Section" between "See" and "2.2.4".
59	-	The free fructose + glucose level in row B is 9.2 ± 3.1
63	22	protoplasts (instead of chloroplasts)
73	4	23.5°C (instead of 21°C)
80	13	my (our)
82	20	my (our)
111	23/24	Lambers _et al_., 1983
122	14	0.3 mM KCN
132	11/12	Day and Lambers (1983) (instead of Lambers _et al_. 1983)
136	10	400-700 nm
142	11	include after "the Antimycin A-resistant rate" : "for the calculation of ρ".
142		In the "malate + succinate" line (at the end of the night), delete 0 in the "+ Antimycin A" column, and include a hyphen.
152	5	pyrophosphate
191		The equation A7 should be

$$g = \frac{E}{(w_i - w_o)} - \frac{E}{2}\,\frac{(w_i + w_o)}{(w_i - w_o)}$$

Page	Line	Amended (corrected) version
200		Include a new reference between the first and the second ones of this page : Day, D.A., H. Lambers, 1983. The regulation of glycolysis and electron transport in roots. Physiol. Plant. (in press)

STATEMENT

The work presented in this thesis is my own, and was performed in the Department of Environmental Biology, Research School of Biological Sciences, the Australian National University, Canberra, from February, 1980, to December, 1982. Specific contributions and co-operative work with others are referred to in the text. The following papers were written during the period of study:

1. Relationship Between Photosynthesis and Respiration. The Effect of Carbohydrate Status on the Rate of CO_2 production by Respiration in Darkened and Illuminated Wheat Leaves, by J. Azcón-Bieto and C.B. Osmond. *Plant Physiol.* (in press).

2. Cyanide-Resistant Respiration in Roots and Leaves. Measurements with Intact Tissues and Isolated Mitochondria, by H. Lambers, D.A. Day and J. Azcón-Bieto. *Physiol. Plant.* (in press).

3. The Effect of Photosynthesis and Carbohydrate Status on Respiratory Rates and the Involvement of the Alternative Pathway in Leaf Respiration, by J. Azcón-Bieto, H. Lambers and D.A. Day. *Plant Physiol.* (submitted).

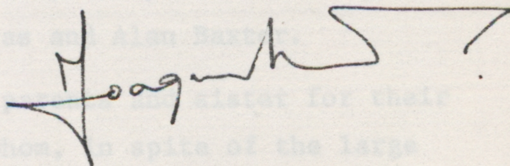
4. The Regulation of Respiration in the Dark in Wheat Leaf Slices, by J. Azcón-Bieto, D.A. Day and H. Lambers. *Plant Physiol.* (submitted).

Another two papers entitled:

5. Changes in Respiratory Pathways During Growth of *Phaseolus vulgaris* L. Leaves, by J. Azcón-Bieto, H. Lambers and D.A. Day.

6. Inhibition of Photosynthesis by Carbohydrates in Wheat Leaves, by J. Azcón-Bieto.

are in preparation.



J. AZCON-BIETO

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Many great hopes are born with this thesis. My son, Carles, also saw the light for the first time during the present year, and for this reason I would like to dedicate the thesis to him.

Science is the most intimate school of resignation and humility, for it teaches us to bow before the seemingly most insignificant of facts.

MIGUEL DE UNAMUNO

"The tragic sense of life"

ABSTRACT

1. Some relationships between the carbohydrate status and the rates of photosynthesis and respiration in the light and in the dark have been studied at the leaf level in wheat and other species. The physiological responses were analysed on the basis of the current biochemical knowledge of photosynthetic and respiratory pathways. On the other hand, some preliminary studies on the relationship between photorespiration and respiration at the mitochondrial level are also presented.

2. The rate of net CO_2 assimilation, A, of wheat leaves usually declined with time under constant environmental conditions, the rate of decline increasing with the CO_2 concentration. Stomatal conductance, g_s, also declined with time in some cases, but this was not the primary cause of the decrease in A. Treatments reducing the rate of translocation (e.g. lower temperatures, chilling the base of the leaf) produced a marked decline in A at atmospheric and high CO_2 concentrations. A period of photosynthesis at high CO_2 concentrations (but not at low CO_2) reduced the upper part of the curve of A vs intercellular CO_2 partial pressure, p_i. The initial slope of this curve, however, was not affected in either case. Photosynthetic rates in the upper part of this curve generally recovered after a short period of darkness. The stimulation of A by 2% O_2 (the so-called Warburg effect), and the apparent quantum yield, decreased after several hours in the light. Carbohydrate levels were also measured,

and the results suggest that end product inhibition of photosynthesis occurs in wheat leaves. Carbohydrate accumulation was also associated with a decrease in g_s and with an increased sensitivity of stomata to CO_2.

3. The rate of dark CO_2 efflux from mature wheat leaves at the end of the night was less than that found after a period of photosynthesis. After photosynthesis the dark CO_2 efflux showed a complex dependence on time and temperature. For about 30 min after darkening, CO_2 efflux included a component (15-20% of total) which was abolished by transferring illuminated leaves to 3% O_2 and 330 μbar CO_2 before darkening. After 30 min of darkness, a relatively steady-state CO_2 efflux was obtained. The temperature dependence of steady-state dark CO_2 efflux at the end of the night differed from that after a period of photosynthesis. The higher rate of dark CO_2 efflux following photosynthesis was correlated with accumulated net CO_2 assimilation and with an increase in the free glucose and fructose, sucrose, and starch levels in the leaf. It was also correlated with an increase in the CO_2 compensation point in 21% O_2, and an increase in the light compensation point. The interactions between CO_2 efflux from carbohydrate oxidation and photorespiration are discussed. It is concluded that the rate of CO_2 efflux by respiration is comparable in darkened and illuminated wheat leaves.

4. The rate of O_2 uptake by wheat leaves in the dark was much lower in leaves harvested at the end of the night than that in leaves harvested after a period of photosynthesis. O_2 uptake by both sets of leaves displayed substantial resistance to cyanide, but in the absence of inhibitors, the alternative pathway contributed to O_2 uptake only in the leaves harvested after a period of photosynthesis. Spinach

leaves showed similar trends in respiration in the dark, and in both spinach and wheat the level of free sugars and other carbohydrate fractions fell during the night. In pea leaves, free sugar levels remained high during the night and no change in either the rate of O_2 uptake or its inhibitor response was observed during the diurnal cycle. The respiratory properties of mitochondria isolated from matures leaves of wheat, spinach and peas did not vary significantly during the diurnal cycle.

Thin slices cut from wheat leaves harvested at the end of the night were insensitive to the alternative path inhibitor SHAM when it was applied in the absence of KCN. Adding sucrose to these slices stimulated O_2 uptake which was then sensitive to SHAM alone. In slices from leaves harvested after a light period, endogenous sugar levels and respiratory rates were higher, and this increase was due in part to the engagement of the alternative pathway. The uncoupler FCCP did not significantly stimulate O_2 uptake rates *per se* in slices from leaves harvested after a light period. However, it stimulated flux through the cytochrome path at the expense of that through the alternative path. When both FCCP and sucrose were added together to slices from leaves harvested after a light period, O_2 uptake was stimulated, and this stimulation was due to engagement of the alternative pathway .

These results suggest that wheat leaf respiration in the dark is regulated by both cell carbohydrate levels and by adenylate control of the mitochondrial respiratory chain. When leaf carbohydrate levels are substantial, the alternative path becomes engaged because the cytochrome chain is restricted. When leaf sugar levels are low, respiration is limited by substrate supply to the mitochondria, and

the alternative pathway is not expressed.

5. The rate of O_2 uptake in the dark in bean leaves and leaf slices decreased during development, showing a similar pattern to that of dark CO_2 efflux. The activity and capacity of the cytochrome path decreased but the capacity of the alternative path remained more or less constant with leaf expansion, and thus percentage cyanide resistance of respiration increased with leaf expansion. However, young leaves showed higher sensitivity to SHAM than mature leaves. Respiration of bean leaf slices was stimulated by the uncoupler FCCP at all ages, the stimulation being more pronounced in young leaves. Uncoupled leaf slice respiration was also sensitive to SHAM alone. The rate of overall respiration, the activities of the cytochrome and alternative pathways, and the extent to which FCCP stimulated respiration in bean leaf slices were positively correlated with endogenous free sugar levels, and negatively correlated with starch levels during aging. The results suggest that respiration of bean leaves during growth is regulated mainly by coarse control of the capacities of glycolysis and the cytochrome pathway, and these capacities decrease with leaf age. However, fine control of respiration by substrate and adenylate levels also occurs at all developmental stages within the limits imposed by the coarse control.

6. Mitochondria isolated from mature leaves of spinach, peas and wheat simultaneously oxidized glycine and TCA cycle substrates. The sensitivity of mitochondrial respiration to antimycin A and SHAM (or disulfiram) varied depending on whether glycine or TCA cycle substrates were used. NAD-linked substrates of the TCA cycle competed between them for access to the electron chain in pea mitochondria, but glycine, which is also NAD-linked, did not apparently compete with TCA

cycle substrates. The state 4 rate of O_2 uptake with malate or succinate was greatly stimulated by glycine, and *vice versa*, via increased alternative pathway. These results suggest that some of the electron transport of these preparations was specifically associated with glycine decarboxylase, including the alternative oxidase. This in turn suggests that mitochondrial electron transport functions in the light during photorespiration. The low degree of competition between glycine and TCA cycle substrates could be due either to intramitochondrial compartmentation of enzymes and cofactors, or to the existence of two distinct populations of mitochondria in leaves.

ABBREVIATIONS

GAS EXCHANGE PARAMETERS (units)

Abbreviation (units)	Definition
A ($\mu mol.m^{-2}.s^{-1}$)	Rate of net CO_2 assimilation
g ($mol.m^{-2}.s^{-1}$)	Total leaf conductance to water vapour diffusion
g_s ($mol.m^{-2}.s^{-1}$)	Stomatal conductance to water vapour diffusion
p_a (μbar)	External partial pressure of CO_2
p_i (μbar)	Intercellular partial pressure of CO_2
Γ (μbar)	CO_2 compensation point
γ (10^{-4} bar $CO_2.bar^{-1}$ O_2)	Oxygen dependence of the CO_2 compensation point
R_d ($\mu mol.m^{-2}.s^{-1}$)	Rate of CO_2 efflux by leaf respiration in the light
R_n ($\mu mol.m^{-2}.s^{-1}$)	Rate of CO_2 efflux by leaf respiration in the dark
R.Q. (ratio)	Respiratory quotient (R_n/V_t)
S_3,S_4 ($nmol.mg^{-1}$ mitoc. protein.min^{-1})	State 3 and state 4 rates of O_2 uptake by isolated mitochondria
v_{alt} (μmol O_2 $m^{-2}.s^{-1}$)	Rate of alternative pathway ($v_{alt} = \rho V_{alt}$)
V_{alt} (μmol O_2 $m^{-2}.s^{-1}$)	Capacity of the alternative pathway
v_{cyt} (μmol O_2 $m^{-2}.s^{-1}$)	Rate of cytochrome pathway
V_{res} (μmol O_2 $m^{-2}.s^{-1}$)	Rate of residual respiration
V_t (or V_T) (μmol O_2 $m^{-2}.s^{-1}$)	Rate of O_2 uptake by leaf respiration in the dark ($V_t = v_{cyt} + v_{alt} + V_{res}$)
ρ (ratio)	The extent of engagement of the alternative pathway ($\rho = v_{alt}/V_{alt}$); $\rho = 1$ indicates full engagement; $\rho = 0$ indicates no engagement.

BIOCHEMICAL SYMBOLS

ABA	Abscisic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumen
cyt	cytochrome
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
Disulfiram	Tetraethylthiuram disulfide
E_a	Activation energy
EDTA	Ethylene diaminetetra-acetate
FCCP	Carbonyl cyanide p-trifluoromethoxy phenyl hydrazone
F_p	Flavoprotein
HEPES	(N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid)
K_m	Michaelis constant
MDH	Malate dehydrogenase
ME	NAD-malic enzyme
MES	(2[N-morpholino]ethanesulfonic acid)
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
OAA	Oxalacetate
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
3-PGA	3-Phosphoglycerate
P_i	Inorganic phosphate
PK	Pyruvate kinase
pmf	Proton motive force
RCR	Respiratory control ratio
RuBP	Ribulose bisphosphate
SHAM	Salicylhydroxamic acid
TCA	Tricarboxylic acid
TES	(N-Tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid)
TP	Triose phosphate
TPP	Thiamine pyrophosphate

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CHAPTER 1

INTRODUCTION

1.1 THE FATE OF CARBOHYDRATES IN LEAVES: INTRODUCTORY HYPOTHESES AND FRAMEWORK

Carbohydrates are the end products of photosynthesis and their synthesis in higher plants takes place mainly in the leaves. Carbohydrates are subsequently exported to other organs and growing leaves, but a substantial fraction may remain in leaves, being either temporarily stored or used to satisfy their synthetic and energetic needs. The balance between carbohydrate production, utilization in metabolism, and export greatly depends on the developmental status of the leaf (Geiger, 1979), but other factors can be also important, especially in mature leaves. It has been hypothesized that excess carbohydrate accumulation in leaves can regulate the rate of photosynthesis by a feedback mechanism, although the evidence obtained with intact leaves is contradictory (Herold, 1980). On the other hand, carbohydrate is the main substrate for respiration under most conditions (ap Rees, 1980a), and increased substrate availability could increase the rate of this process. There is some evidence that plants which have lower respiratory rates in their mature leaves may give significantly higher dry matter yields (Heichel, 1971b; Wilson, 1975). It therefore appears that excess carbohydrate accumulation in leaves could influence several physiological processes (see Fig. 1.1) resulting in possible deleterious consequences for the plant carbon

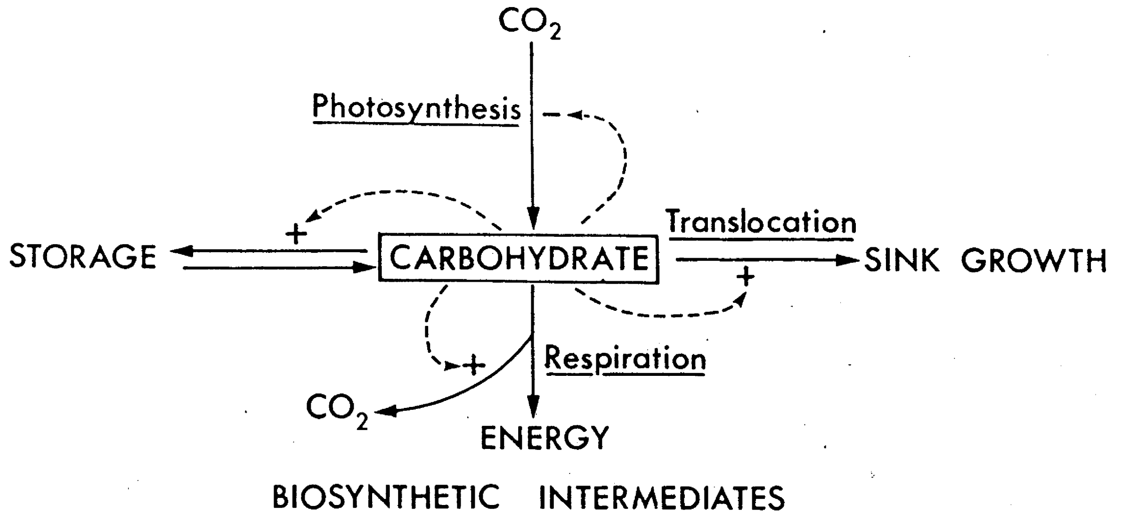

Fig. 1.1: Principal physiological processes involved in carbohydrate production and utilization in leaves. The dotted arrows indicate possible regulatory feedback interactions on the rate of these processes.

economy, since leaves constitute a large fraction of total plant biomass.

The basic purpose of this thesis is to study the extent to which the carbohydrate status affects the rates of photosynthesis and respiration in the light and in the dark in intact mature wheat leaves. The physiological responses observed will be analysed on the basis of the current knowledge of the photosynthetic and respiratory carbohydrate metabolism, which will be reviewed in this Introduction.

1.2 CARBOHYDRATE ACCUMULATION IN LEAVES DURING PHOTOSYNTHESIS

Carbon dioxide is mostly fixed by photosynthesis into two end carbohydrate fractions: starch, a chloroplastic storage polysaccharide, and sucrose, a transport sugar (ap Rees, 1980b). In some species other types of storage polysaccharides (e.g. fructosans,

Kandler and Hopf, 1980), and transport sugars (e.g. raffinose, Kandler and Hope, 1980; Giaquinta, 1980), also occur in leaves.

The rate of accumulation of carbon in the leaf in the form of starch, sucrose, and other carbohydrates increases with light intensity (Gordon *et al.*, 1980a) and especially with CO_2 concentration (Platt *et al.*, 1977; Guinn and Mauney, 1980; Kramer, 1981), as a result of increased rates of photosynthesis. Leaf carbohydrate levels usually increase during the light period (Chatterton, 1973; Upmeyer and Koller, 1973; Challa, 1976), and decrease during the night (Challa, 1976; Gordon *et al.*, 1980a,b). Low temperature promotes starch and sugar accumulation (Neales and Incoll, 1968; Levitt, 1980a), especially in leaves of cool-intolerant C_4 plants (Hilliard and West, 1970; Lush and Evans, 1974; Ku *et al.*, 1978). Some interactions seem to exist between temperature and CO_2 concentration in this respect (Hofstra and Hesketh, 1975). Carbohydrate accumulation is often observed under water stress (Levitt, 1980b). Levitt (1980a,b) concluded that the accumulation of carbohydrates in leaves under water and low temperature stresses is due to the fact that the rate of utilization of carbohydrates for growth is relatively more affected by the cited stresses than their rate of synthesis. The decline in the rate of translocation observed under these stresses (Wardlaw, 1968; Geiger and Sovonik, 1975) may also contribute to affect leaf carbohydrate levels.

Carbohydrate build-up in leaves also increases with low sink demand, e.g. by defruiting, depodding, ear removal, petiole girdling, etc. (King *et al.*, 1967; Neales and Incoll, 1968; Tanaka, 1977; Ho, 1979; Avery *et al.*, 1979; Setter *et al.*, 1980; Clough *et al.*, 1981).

1.2.1 Sucrose and Starch Synthesis in Leaves

The interaction between starch and sucrose formation appears to be very dynamic. The degree to which carbon is partitioned between starch and sucrose varies greatly among species (Huber, 1981a,b; leaves of CAM plants accumulate mainly starch, Osmond and Holtum, 1981), and with many different internal and external factors for a particular species. The capacity to synthesize sucrose and other transport sugars is very small in young leaves which are net importers of carbohydrates. However, this capacity increases sharply coincidentally with the leaf import to export transition (Giaquinta, 1980). In contrast to sucrose, young leaves can synthesize significant amounts of starch (Silvius *et al.*, 1978). The rate of starch synthesis is inversely related with the length of the daily photosynthetic period (Huber and Israel, 1982; Sicher *et al.*, 1982), and increases with low sink demand (Ho, 1979; Huber and Israel, 1982).

The metabolic pathways involved in the synthesis of starch and sucrose in leaves have been recently reviewed by Preiss (1982). It is well established that starch is synthesized within the chloroplast and sucrose in the cytosol (see Fig. 1.2). Triose-phosphates (TP) synthesized in the Calvin cycle are the common precursors of both starch and sucrose molecules. Triose phosphates, mainly dihydroxyacetonephosphate, are transported to the cytosol in counterexchange for orthophosphate (P_i) via the phosphate translocator of the inner envelope membrane (Heber and Heldt, 1981). The phosphate translocator can transport also 3-phosphoglyceraldehyde and 3-phosphoglycerate (3-PGA). However, this carrier only transports these compounds as divalent anions; 3-PGA is predominantly a trivalent anion at pH 8.0-8.5, the physiological pH of the chloroplast stroma in the light, and

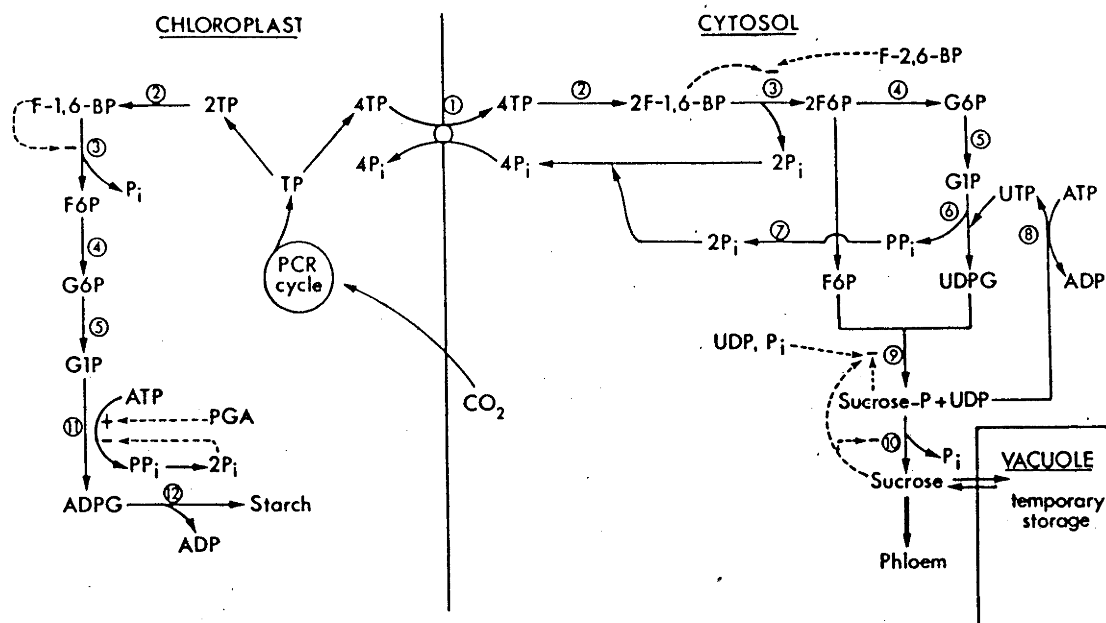

Fig. 1.2: Metabolic pathways involved in the synthesis of starch and sucrose in photosynthetic cells, showing important points of metabolic control (dotted arrows). Abbreviations of metabolites: TP, triose-phosphate; P_i, inorganic orthophosphate; F-1(2),6-BP, fructose-1(2),6-bisphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate: UDPG, uridinediphosphate glucose; PP_i, pyrophosphate; ADPG, adenosinediphosphate glucose; PGA, 3-phosphoglycerate.

Enzymes and metabolic sequences involved: PCR cycle, photosynthetic carbon reduction cycle; (1) phosphate translocator; (2) fructose-1,6-bisphosphate aldolase; (3) fructose-1,6-bisphosphatase; (4) phosphohexose isomerase; (5) phosphoglucomutase; (6) UDP-glucose pyrophosphorylase; (7) pyrophosphatase; (8) nucleoside phosphate kinase; (9) sucrose-phosphate synthetase; (10) sucrose phosphate phosphatase; (11) ADP-glucose pyrophosphorylase; (12) starch synthetase.

Reactions are shown in the direction of sucrose and starch synthesis, but in fact all enzymes other than 3, 7, and 10 catalyse physiologically reversible reactions. (After Kelly and Latzko, 1980; Herold, 1980; Preiss, 1982).

it is not significantly exported from the chloroplast during photosynthesis. The availability of cytosolic P_i is undoubtedly the most important factor regulating the activity of the phosphate translocator (Heber and Heldt, 1981). Low P_i concentrations in the cytosol inhibit TP export from cloroplasts, and high P_i concentrations increase excessively the rate of TP export and the Calvin cycle becomes depleted of intermediates; in both cases, the rate of photosynthesis of isolated chloroplasts results inhibited (Usuda and Edwards, 1982). In the intact cell, the vacuole stores large amounts of P_i which are in continuous exchange with cytosolic P_i (Kelly and Latzko, 1980). This system could be useful for buffering cytosolic P_i concentration in order to maintain an optimal adjustment between the rate of photosynthesis and the rate of TP export from the chloroplasts (Kelly and Latzko, 1980). Similarly, sucrose can also be temporarily stored in the vacuole of leaf cells (Herold, 1980; Kaiser *et al.*, 1982).

Herold (1980) and Robinson and Walker (1981) have reviewed the regulation of sucrose and starch synthesis (see Figs. 1.2 and 1.3). Sucrose-phosphate synthetase and fructose-1,6-bisphosphatase seem to be the most important regulatory enzymes of sucrose synthesis, since their activities are similar to the observed rates of sucrose formation *in vivo*. Sucrose-phosphate synthetase and sucrose-phosphate phosphatase are inhibited by sucrose-phosphate and sucrose, the products of their respective reactions (Hawker, 1967; Amir and Preiss, 1982). Sucrose phosphate synthetase from spinach leaves is also inhibited by P_i, especially at low fructose-6-phosphate concentrations (Amir and Preiss, 1982), and in some species is inhibited by sucrose (Huber 1981b). Fructose-1,6-bisphosphatase is inhibited by fructose-1,6-bisphosphate and by fructose-2,6-bisphosphate, a recently

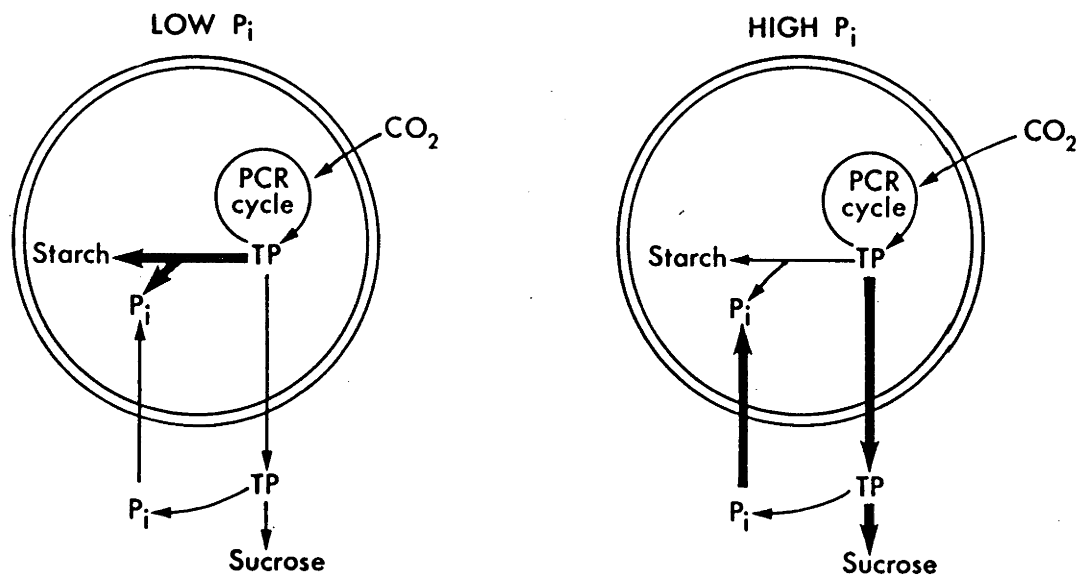

Fig. 1.3: The regulation of photosynthetic carbon partitioning between sucrose and starch in green cells by cytosolic P_i levels. The thick arrows indicate the pathway stimulated under different conditions. Abbreviations: PCR cycle, photosynthetic carbon reduction cycle; TP, triose-phosphate; P_i, inorganic orthophosphate. (After Robinson and Walker, 1981.)

discovered new metabolite in green cells, the importance of which is still unknown (Cséke *et al.*, 1982a).

Sucrose accumulation in the cytosol is likely to produce an increased concentration of triose-phosphate in the chloroplasts, and sugar phosphates in the chloroplasts and cytosol [sugar phosphates do not appear to be transferred to the vacuoles (Kaiser *et al.*, 1982)]. These effects can be due to a complicated chain of feedback inhibition of several enzymes, such as fructose-1,6-bisphosphatase, sucrose-phosphate synthetase, and sucrose-phosphate phosphatase (see above), or to a mass action effect (Herold, 1980). As a result, the P_i concentration can decrease in the cytosol, and TP export from the

chloroplasts is reduced. Under these conditions the 3-PGA/P_i ratio in the chloroplast rises and causes allosteric activation of ADP-glucose pyrophosphorylase, a key enzyme in the starch synthesis pathway (Robinson and Walker, 1981; Preis, 1982; see Figs. 1.2 and 1.3). The 3-PGA/P_i ratio in the chloroplasts of CAM plants is thought to be high under the conditions prevailing during deacidification, and this may explain the predominance of starch in these plants (Osmond and Holtum, 1981). Starch accumulation, unlike sucrose, does not result in P_i sequestration (Fig. 1.2). Recent studies suggest that starch is simultaneously synthesized and degraded in the light, the balance, which is very dependent on P_i levels, being normally in favour of synthesis (Stitt and Heldt, 1981). In the dark starch is degraded in the chloroplasts presumably due to a decrease in the ratio of 3-PGA to P_i. However, some interactions between starch degradation and sucrose metabolism can still occur in the dark. Gordon *et al.* (1980b) reported that massive starch degradation in barley leaves starts when sucrose levels decline to a low critical value, usually after several hours in the dark depending on the initial sucrose concentration.

Another mechanism of regulation of sucrose and starch partitioning could be the levels of enzyme activities that are present in the starch and sucrose biosynthetic pathways in leaves. Huber (1981b) reported that species such as wheat, barley, and spinach that accumulate more sucrose than starch in mesophyll cells, had higher sucrose-phosphate synthetase activity than those species accumulating mainly starch (tobacco, peanuts, beans). Variations in the activities of sucrose-phosphate synthetase, ADP-glucose pyrophosphorylase and other enzymes have also been related to changes in the carbon partitioning patterns between sucrose and starch observed under

different photoperiod length (Robinson *et al.*, 1981; Huber and Israel, 1982), under different sink demand of nodulated and non-nodulated soybean plants (Huber and Israel, 1982), and throughout leaf development (Giaquinta, 1980).

The physiological consequences for the plant of starch or sucrose accumulation in leaves are not obvious. The biochemical mechanism of regulation of the synthesis of these two products suggests that starch is a buffer of sucrose metabolism. However, a high sucrose concentration relative to starch could be advantageous in terms of agricultural productivity. Huber (1981a) reported that isolated protoplasts from high yielding wheat varieties partitioned less carbon into starch and more into sucrose than protoplasts isolated from low yielding varieties. High sucrose content relative to starch may favour the rate of sugar translocation to metabolic sinks because starch is not directly available for extracellular transport, and on the other hand the concentration of sucrose in the leaf is an important factor governing the rate of translocation (Troughton *et al.*, 1977; Giaquinta, 1980).

The role of large daily accumulation of starch in leaves, most of which is either respired or translocated during the following dark period, is not obvious. Some advantage may result from an available carbohydrate source for growth in the dark when leaf water potential is often more favourable for growth than in the light. Another possible effect of differences in the carbon partitioning into sucrose and starch relates to the end product control of photosynthesis (see next section).

1.2.2 Mechanisms of End Product Control of Photosynthesis

Accumulation of carbohydrates in leaves during photosynthesis is a common phenomenon which can be enhanced by several means including high photosynthesis rates, low translocation rates, or low sink demand, as discussed in Section 1.2. It has been long hypothesized that "the accumulation of assimilates in an illuminated leaf may be responsible for a reduction in the net photosynthesis rate of that leaf" (Boussingault, 1868; see Neales and Incoll, 1968). Literature on this topic is very abundant and it has been recently reviewed several times (Neales and Incoll, 1968; Geiger, 1976; Guinn and Mauney, 1980; Herold, 1980). At present, there is still no good evidence of the occurrence of end product inhibition of photosynthesis. The conclusions are often complicated by the fact that hormonal interactions may occur following defoliation and sink manipulation, especially in long term experiments. However, it seems that high CO_2 concentrations may have a deleterious effect on the rate of photosynthesis and growth, which can be accentuated when the sink demand and temperature are low (Hofstra and Hesketh, 1975; Guinn and Mauney, 1980; Clough *et al.*, 1981). The elimination of this effect, which could be due to the massive amounts of carbohydrates accumulated, might result in increased plant yields in a program of CO_2 fertilization. It may also be relevant to responses of plant production to elevated atmospheric CO_2 concentration.

Various types of end product (or feedback) control of the rate of CO_2 fixation are possible depending on the nature of the carbohydrate fraction accumulated, and this aspect will be examined in this section on the basis of the current knowledge of the regulation of starch and sucrose synthesis pathways. Sucrose accumulation in the cytosol is

likely to produce a decrease in the P_i concentration in the cytosol, as discussed in the previous section. Two main types of feedback control of photosynthesis can then occur. Sufficiently low P_i levels in the cytosol will inhibit the rate of TP export from the chloroplasts due to the properties of the phosphate translocator. Under these conditions the 3-PGA/P_i ratio in the chloroplast increases causing activation of the enzyme ADP-glucose pyrophosphorylase, and promoting starch synthesis. Excessive starch accumulation has been suggested to inhibit the rate of net photosynthesis via several mechanisms, including physical damage to the chloroplast (Cave *et al.*, 1981), interference with the pathways of light to the thylakoids and CO_2 to the fixations site, and binding of Mg^{2+} ions (see reviews by Neales and Incoll, 1968; Guinn and Mauney, 1980; and Herold, 1980). There is no definite proof to support these effects of starch, but it seems feasible that extremely high starch levels can lead to mechanical chloroplast damage.

Alternatively, very low P_i levels inside the chloroplast can restrict the rates of photophosphorylation and electron transport, probably via a decreased ATP/ADP ratio, which in turn, would limit the rate of ribulose-bisphosphate regeneration by the Calvin cycle (Robinson and Walker, 1981). Additionally, high sugar-phosphate levels which are present at low P_i concentrations, can compete with ribulose-bisphosphate for binding on RuBP-carboxylase (Badger and Lorimer, 1981). The decline in photosynthetic rates observed in isolated chloroplasts and in intact leaf tissues treated with mannose, a sequester of P_i (Herold, 1980; Robinson and Walker, 1981) suggests that photosynthesis *in vivo* could be inhibited by low P_i levels.

Leaves photosynthesizing at ambient CO_2 and O_2 levels (330 μbar,

21%) export most of the carbon from the chloroplasts as TP and glycolate, resulting from the carboxylation and oxygenation of RuBP. Oxygenation does not result in consumption of phosphate (Usuda and Edwards, 1982). However, the levels of TP and phosphorylated sugars increase very much in conditions where the oxygenation reaction is suppressed (e.g. high CO_2 and/or low O_2 levels) (Badger *et al.*, 1983), leading to a lower P_i availability in relation to ambient CO_2 and O_2 conditions. Therefore, the rate of photosynthesis could decline faster with time when the oxygenase reaction is inhibited due to the reduction in the RuBP regeneration capacity. Several observations are consistent with this hypothesis (Canvin, 1978; von Caemmerer and Farquhar, 1981). Further support is given by the fact that feeding spinach leaf discs with mannose produces a progressive decrease in the capacity of 2% O_2 to stimulate photosynthesis, which is finally lost (Harris *et al.*, 1981).

The extent by which end product control of photosynthesis is exerted by starch or sucrose accumulation may depend on differences in the capacities of their respective synthetic pathways, which are known to vary greatly among species (Huber, 1981a,b). The possible occurrence of end product control of photosynthesis in intact leaves of wheat, a species known to partition more photosynthetically fixed carbon into sucrose relative to starch (Huber, 1981a,b), will be experimentally examined in the next chapter.

1.3 CARBOHYDRATE AS SUBSTRATE FOR RESPIRATION

Respiration is a fundamental process for the performance of plants as living organisms. Early in the history of plant physiology, respiration was mainly regarded as "a path of energy degradation"

(James, 1953). Beevers (1960, 1970, 1974) integrated a more comprehensive knowledge on the biochemistry of respiration and linked this process to the rest of the plant cell metabolism. Beevers (1970) concluded that "respiration is the source of intermediates used in the synthesis of permanent cellular constituents as well as the source of ATP and reduced nucleotides, the driving force of these syntheses". He also argued that respiratory losses by a tissue were most likely a reflection of the demand of that tissue for ATP and reduced pyridine nucleotides, rather than the capacity of the tissue to catalyse respiratory reactions. In normal tissue the regeneration of these limiting metabolites is most probably linked to essential biosynthetic and maintenance activities of the cells. The concept thus emerged of a respiratory machinery governed by the pace of those reactions which consume its products.

McCree (1970) noted that a substantial portion of respiration of white clover plants was proportional to the daily net carbon gain by photosynthesis, while the remainder was proportional to existing plant biomass. This division of respiration led to the concept of a portion of respiration associated with growth (and directly linked to photosynthesis) and another portion linked to the maintenance of the life functions of the plant (Penning de Vries, 1972; Thornley, 1977). Penning de Vries *et al.* (1974) and Penning de Vries (1975) made a theoretical study linking the biochemistry of many known metabolic pathways with growth and maintenance respiration.

A variety of substrates can be respired, including carbohydrate, lipid, and protein, through pathways converging on the tricarboxylic acid cycle and thence on to respiratory chain phosphorylation. The contribution of these substrates to plant respiration has been

recently assessed by ap Rees (1980a) who concluded that most plant cells for most of their life use carbohydrate as their major respiratory substrate. Leaves are not an exception in this respect. He ascribed this dominance of carbohydrate as respiratory substrate in plants, which is more marked than in animals or micro-organisms, to the fact that carbohydrate is the principal product of photosynthesis and the main form in which carbon is moved about the plant. The pathways of carbohydrate utilization in respiration shall be outlined in the next sections. Although some of the information presented on these pathways has been obtained from non-photosynthetic organs, it is assumed that it also applies for leaves (unless otherwise stated).

Leaf respiration constitutes a very important fraction of total plant respiration, given the large contribution of leaves to plant biomass. There are some indications suggesting that a reduction in the rate of mature leaf respiration is associated with significant increases in dry matter yields in maize and perennial ryegrass (Heichel, 1971b; Wilson, 1975). That is, it appears that a fraction of leaf respiration appears to be *wasteful*. However, the nature of this wasteful respiration is not known. Little is known about the regulation of respiration in intact leaves and the possible influence of photosynthesis in this respect. It is also not known to what extent proposed biochemical mechanisms of regulation operate and interact with other mechanisms (e.g. carbohydrate supply) in leaves *in vivo*. It is obviously important, particularly in view of Wilson's and Heichel's results, to gain such information, since it may have a large impact on the manipulation of plant growth.

1.3.1 Pathways of Carbohydrate Respiration in Leaves

1.3.1.1 Glycolysis, oxidative pentose phosphate (OPP) pathway and tricarboxylic acid (TCA) cycle in the dark

Sucrose and starch, the major respiratory substrates, are converted to fructose-6-phosphate and glucose-6-phosphate which are oxidized via glycolysis and OPP pathway; the products are metabolized via the TCA cycle, and CO_2 is produced (Fig. 1.4). The principal locations of these pathways in the photosynthetic cell are the cytosol (glycolysis and OPP pathway) and the mitochondrion (TCA cycle). The chloroplast is also capable of a complete OPP pathway and a partial glycolysis as far as 3-PGA [the enzyme phosphoglycerate mutase is absent in pea chloroplasts (ap Rees, 1980b) but this question still is in dispute (see Dennis and Miernyk, 1982)]. However, glycolysis can still proceed in chloroplasts if 2-PGA is supplied externally, because enolase, pyruvate kinase, and pyruvate dehydrogenase are present in these organelles (ap Rees, 1980b; Dennis and Miernyk, 1982).

Glucose is mainly oxidized under aerobic conditions through glycolysis and TCA cycle in leaf tissues (Stitt and ap Rees, 1978; ap Rees, 1980a,b); the contribution of total OPP pathway to glucose metabolism has been estimated to be well below 30%, and may only be 10-15% of the total CO_2 released (ap Rees, 1980a,b). An estimate of no more than 30% was reported for wheat leaves, although some interference could result from CO_2 released during pentan synthesis from hexose (e.g. xylose, arabinose, which are constituents of cell walls), which is significant in wheat leaves (Stitt and ap Rees, 1978). Chloroplasts are capable of considerable rates of OPP pathway during starch breakdown. The function of the OPP pathway is probably to provide NADPH for reductive biosyntheses in the first instance, and secondarily to supply pentose-phosphate and erythrose-phosphate for

Fig. 1.4: Pathways of carbohydrate oxidation in aerobic conditions in leaf cells, showing important points of metabolic control (dotted arrows).

Abbreviations of metabolites: Glu, glucose; Fru, fructose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F-1(2),6-BP, fructose-1(2),6-bisphosphate; 3-PGAL, 3-phosphoglyceraldehyde; DHAP, dihydroxyacetonephosphate; 1,3-DiPGA, 1,3-diphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxalacetate; ASP, aspartate; Mal, malate; AcCoA, acetylcoenzyme A; α-KG, α-ketoglutarate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate.

Enzymes and metabolic sequences involved: (1) starch phosphorylase; (2) amylases and other hydrolytic enzymes; (3) invertase; (4) glucokinase; (5) fructokinase; (6) phosphoglucomutase; (7) phosphohexose isomerase; (8) phosphofructokinase; (9) fructose-1,6-bisphosphate aldolase; (10) triosephosphate isomerase; (11) NAD-specific glyceraldehydephosphate dehydrogenase; (12) phosphoglycerate kinase; (13) phosphoglyceromutase; (14) enolase; (15) pyruvate kinase; (16) phosphoenolpyruvate carboxylase; (17) malate dehydrogenase; (18) transaminase; (19) NAD-specific malic enzyme; (20) pyruvate dehydrogenase; (21) acetyl-CoA hydrolase; (22) citrate synthetase; (23) aconitase; (24) isocitrate dehydrogenase; (25) α-ketoglutarate dehydrogenase; (26) succinyl-CoA synthetase; (27) succinate dehydrogenase; (28) fumarase; (29) glucose-6-phosphate dehydrogenase; (30) 6-phosphogluconate dehydrogenase; (31) ribosephosphate isomerase; (32) ribulose-phosphate-3-epimerase; (33) transketolase; (34) transaldolase. (Modified after ap Rees, 1980a,b; Turner and Turner, 1980; Wiskich, 1980.)

CYTOSOL

OPP Pathway

Sucrose
H_2O
Starch
P_i
(1)
(2)
(3)
G1P
(6)
NADP
NADPH
(29)
NADP
NADPH
(30)
Hexose
Glu
(4)
G6P
6-P-gluconate
Ru5P
ATP
ADP
(7)
CO_2
(32)
(31)
Fru
F6P
(33)
Xu5P
(5)
E4P
R5P
ATP
AMP
Citrate
PEP
3PGA
6-P-gluconate
ATP
−
+
ADP
(8)
3-PGAL
F-1,6-BP
(34)
(33)
S7P
(9)
DHAP
(10)
3-PGAL
P_i
F-2,6-BP
NH_4^+
NAD
P_i
(11)
NADH
1,3-DiPGA
ADP
(12)
ATP
ASP
3-PGA
(13)
Glycolysis
NH_2
(18)
P_i
H_2O
2-PGA
(14)
(16)
H_2O
OAA
PEP
NADH
ATP
+
NH_4^+
(17)
CO_2
(15)
−
ATP
Citrate
NAD
ADP
Mal
PYR

MITOCHONDRION

CO_2
(19)
Mal
PYR
NAD
NADH
NAD
CoA
(20)
NADH
CO_2
CoA
(21)
AcCoA
Acetate
Fatty acids
(22)
CoA
OAA
citrate
NADH
(23)
(17)
isocitrate
NAD
NAD
(24)
Mal
CO_2
TCA
Cycle
NADH
(28)
a-KG
Amino acids
NAD
CoA
fumarate
(25)
FpH_2
NADH
CO_2
(27)
succinyl-CoA
Fp
succinate
ATP
ADP+P_i
(26)
CoA

synthetic reactions (e.g. nucleic acids, phenilpropanoid compounds) (ap Rees, 1980b). Some NADPH can be directly oxidized by the respiratory chain, resulting in ATP formation (see later).

The integration of glycolysis in the cytosol and the TCA cycle in the mitochondrion is achieved by the existence of several transport systems in the inner mitochondrial membrane (Wiskich, 1977, 1980). Pyruvate transport appears to be insufficient to sustain the maximum rates of TCA cycle activity observed *in vitro* and Day and Hanson (1977) proposed that malate, which is very rapidly transported by the dicarboxylate carrier, supplements the cycle with extra pyruvate through its oxidative decarboxylation by NAD-dependent malic enzyme, a unique enzyme of plant mitochondria. The operation of PEP-carboxylase and malate dehydrogenase can continuously supply oxalacetate and malate in the cytosol at significant rates. This system would permit the diversion or drainage of TCA cycle intermediates (e.g. oxalacetate, acetate, α-ketoglutarate) for biosynthetic or anaplerotic reactions without affecting the rate of regeneration of citrate. Another interesting feature of this scheme is that it results in a bypass of an important regulatory glycolytic enzyme, pyruvate kinase (Fig. 1.4).

(i) Regulation

The regulation of glycolysis, OPP pathway and TCA cycle in the dark is very complex and involves many factors (Turner and Turner, 1980; Wiskich, 1980; ap Rees, 1980b). The main regulatory enzymes of these pathways are hexose kinases, glucose-6-phosphate dehydrogenase, phosphofructokinase (PFK) and pyruvate kinase (PK), which catalyse non-equilibrium reactions under *in vivo* conditions and are

strategically located at initial points of metabolic pathways (Fig. 1.4). Other possible regulatory steps are pyruvate dehydrogenase, citrate synthetase, and other TCA cycle dehydrogenases. Glucose-6-phosphate dehydrogenase is inhibited by NADPH, and it is probably controlled *in vivo* by the ratio NADP to NADPH (ap Rees, 1980b). The application of the cross-over theorem suggests that PFK and PK are the main enzymes regulating glycolytic flux (Turner and Turner, 1980). Both enzymes are inhibited by ATP and citrate, and are activated by NH_4^+ salts. Phosphofructokinase is additionally inhibited by ADP, AMP, some glycolytic intermediates (especially phospho-enolpyruvate, PEP), and by 6-phosphogluconate, and is stimulated by P_i. Buchanan's group recently reported that fructose-2,6-bisphosphate stimulates PFK activity in leaves (Csêke *et al.*, 1982a), but the factors controlling the synthesis of this metabolite in plants are unknown at present. The fact that PK can be bypassed (see above) and that PFK is affected by a wider range of potential regulators suggest that this latter enzyme can affect most decisively the rate of carbohydrate utilization in glycolysis.

The integrated control of glycolysis and TCA cycle in plant tissues and particularly in leaves *in vivo*, is still not very well understood. The rate of respiration in leaves has been found to be correlated with the rate of prior photosynthesis, and it is also frequently stimulated upon addition of exogenous sugars, especially in starved leaves (see Section 1.3.2.1). These facts suggest that glycolysis could be regulated by the substrate supply. On the other hand, it has been suggested that carbohydrate oxidation is mainly regulated by the *energy charge**, or in other words, by the energy

* The energy charge, a concept developed by Atkinson (1977), is a

demand (Beevers, 1974), as in animals cells (Atkinson, 1977). However, the control of PFK in plants and animals appears fundamentally different, in the sense that AMP stimulates the animal enzyme but inhibits the plant enzyme (Turner and Turner, 1980). The activation of PFK from mammalian tissues and yeast by AMP was one of the factors supporting the control of glycolysis and TCA cycle by energy charge. The stronger inhibition of plant PFK by the phosphorylated intermediates of glycolysis, especially PEP, and by the initial metabolites of the OPP pathway (6-phosphogluconate) and the TCA cycle (citrate) suggests that the activity of this enzyme may be regulated *in vivo* not only by the rate at which ATP is used, but also by the demand of biosynthetic intermediates. The regulation of glycolysis by substrate supply would be consistent with this suggestion. In this sense, the stimulation of glycolytic flux by NH_4^+ may reflect an increased demand of carbon skeletons for amino acid synthesis (Bassham *et al.*, 1981). Consistently, the enzyme AMP deaminase, which produces NH_4^+ from AMP, has been found to activate PFK and PK in yeast (Yoshino and Murakami, 1982). Interestingly, this enzyme is also present in plants (see Yoshino and Murakami, 1982).

1.3.1.2 Mitochondrial Electron Transport and Oxidative Phosphorylation

TCA cycle intermediates are oxidized in the mitochondrial matrix,

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measure of the metabolic energy stored in the adenine nucleotide system, and is given by the ratio

$$\frac{(ATP) + \frac{1}{2}ADP}{(ATP) + (ADP) + (AMP)} .$$

The system is fully charged when all the adenylate present is converted to ATP (energy charge = 1), and fully discharged when only AMP is present (energy charge = 0).

and the electrons are subsequently transferred via NADH or FADH to the mitochondrial respiratory chain. The structure of the respiratory chain of plant mitochondria is more complex than its mammalian counterpart (Fig. 1.5). In addition to the traditional sequence of electron carriers from substrate to cytochrome oxidase, which is inhibited by rotenone, antimycin A and KCN, and is associated to three energy transducing sites, plant mitochondria possess sequences of redox components resistant to several inhibitors. These are the rotenone-resistant NADH dehydrogenase (see next section) and the cyanide and antimycin A-resistant, alternative terminal oxidase (see Section 1.3.1.2.2); both pathways bypass energy transducing sites. Plant mitochondria can oxidize malate via NAD-linked malic enzyme in addition to malate dehydrogenase, and leaf mitochondria oxidatively decarboxylate glycine via an NAD-linked glycine decarboxylase; in both cases the NADH formed can be oxidized by the respiratory chain with the formation of three molecules of ATP (Hanson and Day, 1980) (see also Section 1.3.2.2.1). Plant mitochondria also characteristically oxidize external NAD(P)H (see next section).

This complex respiratory chain makes it possible to transfer electrons to oxygen without the synthesis of ATP (e.g. by electron transport through the rotenone and cyanide-resistant pathways, Fig. 1.5). The organization of the respiratory chain has been recently reviewed by Storey (1980), Day *et al.* (1980) and Palmer and Møller (1982).

The respiratory chain is located in the inner membrane of mitocondria, and during electron transport to oxygen through the cytochrome chain protons are translocated out of the matrix to create a proton motive force (pmf) across the inner membrane (which is

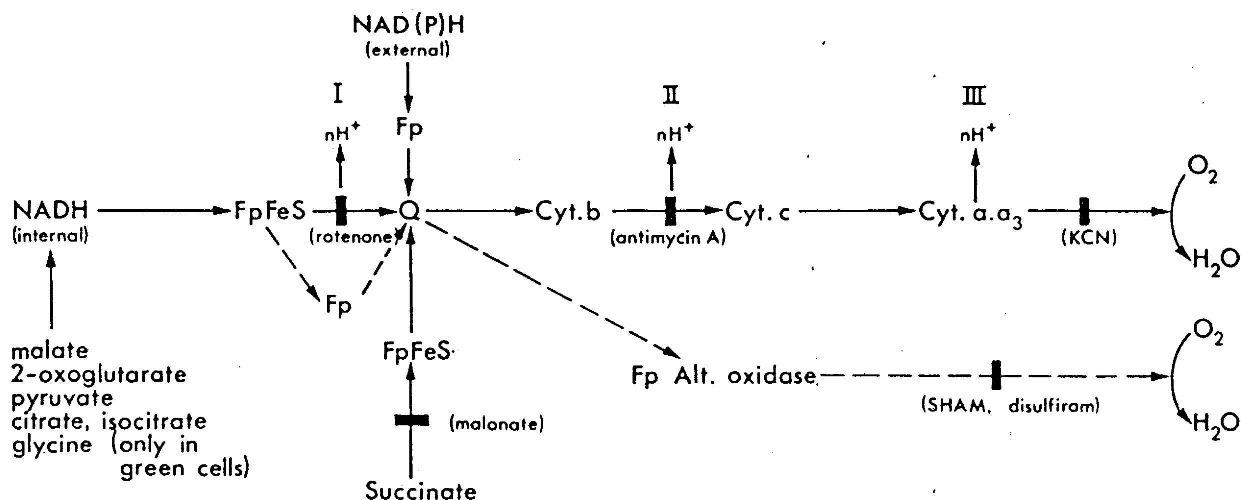

Fig. 1.5: Schematic diagram of the respiratory chain in the inner membrane of plant mitochondria, showing the electron transport carriers, the proton translocation sites, and the sites of inhibition (inhibitors are shown in brackets). Abbreviations: Fp, flavoproteins; FeS, iron sulfur proteins; Q, ubiquinone; Cyt, cytochromes; SHAM, salicyl hydroxamic acid; disulfiram, tetraethylthiuram disulfide (modified after Storey, 1980; Day *et al.*, 1980; and Hanson and Day, 1980).

relatively impermeable to H^+). According to the chemiosmotic hypothesis (Mitchel, 1966) this pmf is used to drive phosphorylation of ADP; H^+ move back across the membrane through the ATPase complex catalysing the synthesis of ATP from ADP to P_i. Electron flow that is coupled to H^+ translocation is subject to control by the pmf. In the presence of ADP and P_i, the pmf is dissipated and electron flow is rapid; in the absence of ADP, the pmf is high and exerts a "back pressure" on electron transport, inhibiting it. This constitutes the familiar respiratory control seen with isolated mitochondria (see Appendix III). A high extramitochondrial ATP/ADP ratio will also inhibit oxidative phosphorylation and hence electron transport.

Adenine nucleotides communicate with the matrix (and therefore the catalytic site of the ATPase complex, which is localized in the matrix side of the inner membrane) via a specific carrier which catalyses $ADP^{3-}_{in}/ATP^{4-}_{out}$ transport (Klingenberg, 1980). Due to the electrogenic nature of this transport, this exchange of ADP for ATP is essentially unidirectional in energized mitochondria, resulting in a higher ATP/ADP ratio in the cytosol than in the mitochondria (Klingenberg, 1980). However, when external ATP is very high, and ADP low, uptake of the latter is inhibited, oxidative phosphorylation is also inhibited and hence electron transport is restricted. Thus cytosolic ATP/ADP (+P_i — the phosphate potential) may determine, at least in part, respiratory O_2 consumption *in vivo*.

There is some evidence that extramitochondrial ATP/ADP ratio controls the rate of electron transport to oxygen in isolated plant mitochondria, but only when this ratio exceeds approximately 20 (Dry and Wiskich, 1982). However, the sensitivity of respiration to the ATP/ADP ratio increases when the ADP concentration is very low, and therefore, it may depend more on total adenylate concentration than relative concentration (Dry and Wiskich, 1982). Cytosolic ATP/ADP ratios measured *in vivo* are much lower than 20, and usually less than 10 (Heber, 1974; Hampp *et al.*, 1982; Stitt *et al.*, 1982), but still respiration seems controlled by adenylate turnover as shown by the effect of uncouplers of oxidative phosphorylation (e.g. FCCP) in intact tissues (Beevers, 1974; Day *et al.*, 1980; Wiskich, 1980). However, the technical problems of directly measuring the ATP/ADP ratio in the cells *in vivo*, which arise because the adenylate system has a very fast turnover, may have been the reason for these contrasting results.

Other factors can affect electron transport rates. For example, the availability of respiratory substrate from glycolysis (which in turn would depend on the carbohydrate status), the rate of substrate transport through several mitochondrial carriers (e.g. pyruvate, dycarboxylate) (Day and Hanson, 1977; Wiskich, 1977, 1980), and the capacity of the various respiratory linked dehydrogenases. These latter enzymes appear to have lower activities than the capacity of the cytochrome chain, as shown by the observation that the oxidation of substrate alone does not saturate the cytochrome pathway, nor necessarily two substrates simultaneously oxidized (Day and Wiskich, 1977). Likewise the operation of non-phosphorylating electron transport pathways in plant mitochondria (see below) is not restricted by adenylate turnover, and may principally depend on the availability of reducing equivalents from the TCA cycle. Obviously, the control of electron transport in plant mitochondria is a very complex phenomenon, which still is not fully understood *in vivo*.

(i) NAD(P)H oxidation pathways

NAD(P)H oxidation by plant mitochondria is a complex process involving at least three distinct dehydrogenases (Palmer and Møller, 1982). Exogenous NAD(P)H, which can be produced in glycolysis and OPP pathway, can be oxidized by a dehydrogenase system, which may involve specific dehydrogenases for NADH and NADPH, located on the outer surface of the inner membrane. This system donates electrons to ubiquinone with the formation of 2 molecules of ATP upon subsequent transfer to cytochrome oxidase. The physiological significance of the external NAD(P)H oxidation is not known, but Palmer and Møller (1982) suggested that it could be a mechanism to regulate the cytosolic

NAD(P)H/NAD(P) ratio.

Endogenous NADH is preferably oxidized by a rotenone-sensitive NADH-dehydrogenase coupled to three energy transducing sites, as in mammalian mitochondria. However, endogenous NADH can also be oxidized by a rotenone-resistant dehydrogenase which bypasses the first energy transducing site (Fig. 1.5). The nature of this latter enzyme is not known, but its affinity for NADH (K_m, 80 μM) is much lower than that of the rotenone-sensitive oxidase (K_m, 8 μM) (Møller and Palmer, 1982). This property suggests that the rotenone-resistant NADH dehydrogenase can be significantly engaged in respiration only when NADH levels are very high, which can occur when cytosolic malate concentration increases or when the rate of electron flow through the normal respiratory chain is suppressed by the phosphorylation potential. Palmer and Møller (1982) suggested that the operation of this dehydrogenase could permit TCA cycle turnover for production of biosynthetic intermediates in the presence of a high NADH/NAD ratio.

(ii) The alternative pathway

A striking feature of plant mitochondria is the presence of a pathway resistant to inhibitors of the cytochrome path (e.g. antimycin A, cyanide). The alternative path has recently been extensively reviewed (Day *et al.*, 1980; Laties, 1982), but the precise nature of the components of this path is still unclear. A flavoprotein containing copper and a quinol-oxidoreductase (perhaps the terminal oxidase?) have been implicated (Rich, 1978; Huq and Palmer, 1978). It is well known that the alternative path branches from the conventional electron transport chain at the ubiquinone level (Storey, 1976); the alternative oxidase has a lower affinity for oxygen (K_m, 10-30 μM)

than does the cytochrome oxidase (K_m, <1 μM) (Solomos, 1977; Kano and Kageyama, 1977), and it is not coupled to ATP synthesis, i.e. it leads mainly to production of heat.

The regulation of the electron flow through the alternative path is a controversial issue. The most accepted model (Bahr and Bonner, 1973) suggests that the alternative pathway becomes engaged only when the level of ubiquinone reduction is very high, and this can occur when the cytochrome path is either saturated (state 3 — see Appendix III — or uncoupled respiration in the presence of adequate substrate levels) or restricted (e.g. by oxidative phosphorylation). A considerable experimental and theoretical body of evidence supports the validity of this model (Laties, 1982).

The contribution of the alternative pathway to respiration can be estimated by using inhibitors of the cytochrome and alternative paths, in titration experiments (Day *et al.*, 1980). Inhibitors of the alternative pathway are hydroxamic acids, propyl gallate and disulfiram (Schonbaum *et al.*, 1971; Grover and Laties, 1981; Laties, 1982); hydroxamic acids in particular have been widely used for studies with isolated mitochondria, cells, and intact tissues (Day *et al.*, 1980; Laties, 1982, Lambers, 1982). These techniques are discussed in depth in Chapter 5 and in Appendix IV.

The presence of the alternative path in most plant tissues, including leaves, has been clearly established (Henry and Nyms, 1975; Laties, 1982; Lambers, 1982). However, there is more uncertainty about the operation of this path in normal respiration especially in intact tissues, and about its possible function. It is known that the rate of respiration of mature leaves is little inhibited or sometimes is stimulated by cyanide (James, 1953; Ducet and Rosenberg, 1962), and

that a combination of SHAM and KCN almost fully inhibits leaf respiration (Lambers *et al.*, 1979; Kinraide and Marek, 1980). However, these latter authors reported that the sensitivity of shoots of *Senecio aquaticus* and leaves of *Bryophyllum tubiflorum* to SHAM alone was very low, suggesting that the alternative pathway, although present, is not engaged in normal respiration in these leaves.

The carbohydrate status seems to affect the degree of engagement of the alternative pathway in roots and in *Chlorella* cells (see Lambers, 1982), but there is no information for leaves. This aspect will be studied in this thesis.

Apart from its role in thermogenesis in the male reproductive organs of the *Araceae* (Meeuse, 1975), the function(s) of the alternative pathway remains a mystery. Several hypotheses have been proposed, but the experimental evidence is not conclusive (Laties, 1982). It has been suggested that the alternative path serves to remove excess carbohydrate from the cell in an energy overflow mechanism (Lambers, 1982). The results of Yoshida and Tagawa (1979) suggest that the alternative path may be involved in the resistance of plant tissues to chilling temperatures (see also Laties, 1982). This pathway could also play a role in seed germination and in defense against infections by other organisms (Laties, 1982). Nothing is known about the possible function of the alternative path in leaf respiration.

1.3.2 Relationship between Photosynthesis and Respiration in Leaves

1.3.2.1 Substrate supply as a factor in leaf respiration in the dark

The rate of respiration of leaves in the dark is usually higher

after a light period than after a dark period (James, 1953; Stoy, 1965; Heichel, 1971a). The carbohydrate level in the leaves constantly decline during a dark period, and complete starvation may occur if the dark period is sufficiently long (James, 1953). Ludwig *et al.* (1975) and Challa (1976) measured the CO_2 output of tomato and cucumber leaves, respectively, during the whole night after a light period in which the rate of photosynthesis was varied by changing the light level or CO_2 concentration. They found that the integrated rate of respiration during the night increased proportionally to the integrated rate of net photosynthesis in the preceding light period. In contrast, Heichel (1970) reported that the rate of CO_2 released into a stream of CO_2-free air by attached maize leaves after a period of illumination reached a maximum 20 min after darkening. The maximal rate of respiration was quantitatively related to the light intensity during the previous light period, but appeared to be independent of the rate of net CO_2 fixation.

However, a similar relationship between photosynthesis and respiration than that observed in cucumber and tomato leaves (see above) has also been repeatedly demonstrated by several workers in whole plants (Ludwig *et al.*, 1965; McCree and Troughton, 1966; McCree, 1970, 1974; Penning de Vries, 1972, etc.). It is remarkable that the relationship between photosynthesis and respiration is maintained during water stress. Ferrar (1980) subjected two woody species from South African savannas to slow water stress and found that the rate of dark CO_2 efflux of whole shoots (including leaves) measured at the end of the night declined with leaf water potential. Plotting respiration at different water potentials against average photosynthesis of the previous day resulted in a linear relationship except at the highest

photosynthesis rates. This relationship differed depending on whether the plants had experienced water stress or not. Pre-stressed plants showed lower respiration rates for any given rate of photosynthesis than non-stressed plants, and the ratio of respiration to photosynthesis was also much lower in pre-stressed plants.

McCree (1970) fitted an empirical equation in which the rate of dark CO_2 output of white clover plants is proportional to photosynthesis and dry weight of living material on the plant. This information served to develop the theoretical concepts of growth and maintenance respiration (see Section 1.3). Considerable experimental evidence agrees with the conclusions of this model of respiration (Hunt and Loomis, 1979) but some results are contradictory, as in the case of *Lolium perenne* plants grown in the field (Jones *et al.*, 1978) or in barley plants (Winzeler *et al.*, 1976; Farrar, 1980). From a biochemical point of view, there is still no clear distinction between growth and maintenance respiration, and it is also not clear what the relationships between them and the non-phosphorylating pathways of electron transport in mitochondria (see above) are. These pathways consume carbohydrate without producing energy. However, growth and maintenance respiration seem to differ in their response to temperature. Whereas the fraction of total plant respiration proportional to the daily net photosynthetic carbon input (i.e. growth regulation) is relatively insensitive to temperature changes in the physiological range, the maintenance component increases dramatically with temperature (McCree, 1974; Ryle *et al.*, 1976; Breeze and Elston, 1978).

The application of this model of respiration to leaves is not very successful. In contrast to the explanation given for whole and

rapidly growing plants, the large enhancement in the rate of leaf respiration associated with photosynthesis (see above) cannot be primarily related to growth requirements because mature leaves were used in these studies. An alternative explanation is that respiration is used for synthesis of compounds (e.g. amino acids) in the leaf which can be utilized for growth in other plant parts, and/or that respiration provides energy for transport of assimilates (Ho and Thornley, 1978), whose concentration increases after a period of photosynthesis. However, these requirements cannot be the primary mechanism regulating the rate of leaf respiration since experiments in which the rate of leaf export was decreased by lowering temperature and sink demand (e.g. by defruiting, depodding) have shown that the rate of mature leaf respiration increased in relation to control plants which were not manipulated (Rook, 1969; Tanaka, 1977; Ho, 1979; Avery *et al.*, 1979). Similar results have been reported for wheat plants after ear removal (Birecka, 1968; Birecka *et al.*, 1969). An increase in leaf carbohydrate levels was found in these conditions. A similar correlation between leaf respiration and carbohydrate concentration was obtained even in conditions where the sink demand was not altered (Challa, 1976; Cunningham and Syvertsen, 1976; Coggeshall and Hodges, 1980; Moser *et al.*, 1982). Similar relationships between carbohydrates and respiration are found in other plant organs (James, 1953; Saglio and Pradet, 1980; Moser *et al.*, 1982), and in whole plants (Alberda, 1968; Breeze and Elston, 1978; Penning de Vries *et al.*, 1979).

Respiration of leaves is stimulated upon addition of several sugar solutions (James, 1953; Tetley and Thimann, 1974; Goldthwaite, 1974), especially in starved leaves (James, 1953). Similar

observations have been made with other plant organs, e.g. roots, coleoptiles, meristems, carrot slices, embryos, etc. (James, 1953; Saglio and Pradet, 1980).

The evidence presented here suggests that carbohydrate availability may play an important role in regulating the rate of leaf respiration, which could account for the proportionality between the rates of photosynthesis and respiration observed in leaves, and perhaps in whole plants. However, it is not known which is the biochemical basis of the increase in respiration due to carbohydrate accumulation. There is little or no information about the existence of other possible regulatory mechanisms, such as the adenylate system, in leaves *in vivo*, nor on the possible interactions between these mechanisms and the regulation by substrate supply. The stimulatory effects of uncouplers of oxidative phosphorylation (e.g. 2,4-dinitrophenol) on plant respiration, including leaves (Beevers, 1953; Porter and Runeckles, 1956) suggests that respiration can also be regulated by adenylate turnover (Beevers, 1970, 1974). However, it seems clear that the regulation of respiration is not simply due to the growth and maintenance requirements, at least in mature leaves.

1.3.2.2 Leaf respiratory metabolism during photosynthesis

The central problem in the study of relationships between photosynthesis and respiration is the question of whether or to what extent the respiratory pathways function in illuminated leaves. This topic has been recently reviewed in length by Graham (1980), but it seems that the question is far from being solved. Classically, it has been considered that respiration is inhibited in the light by the increased cytosolic phosphorylation potential (Heber, 1974; Heber and Heldt,

1981), but Graham (1980) argued that the TCA cycle is operative in the light. Evidently, respiration is a very complex phenomenon and control by a single mechanism seems unlikely (see above).

The evidence suggests that partial glycolysis and OPP pathway in the chloroplasts (see Section 1.3.1.1) are inhibited in the light; otherwise the reactions would interfere with the starch synthesis and the Calvin cycle (see ap Rees, 1980b; Buchanan, 1980; Dennis and Miernyk, 1982). Mechanisms of inhibition include the inactivation of the enzymes phosphofructokinase and glucose-6-phosphate dehydrogenase by the increased NADPH/NADP ratio, and a light modulation of the latter enzyme (Buchanan, 1980; Dennis and Miernyk, 1982; Cséke *et al.*, 1982b). These and other chloroplastic enzymes differ in their regulatory properties from their cytosolic counterparts (Dennis and Miernyk, 1982).

The situation is more complex in the rest of the cell. Complete suppression of glycolysis and TCA cycle is unlikely because these pathways are the unique net source in the cell of several compounds (e.g. acetate, α-ketoacids) which are required for numerous biosynthetic reactions known to occur very rapidly in the light. These include amino acid and lipid synthesis. Chloroplasts are the sites of formation of many amino acids (Miflin and Lea, 1977) and most fatty acids (Stumpf, 1980). However, these organelles are unable to synthesize α-ketoacids (e.g. pyruvate, oxalacetate, α-ketoglutarate), which must be supplied by other cell compartments (Miflin and Lea, 1977; Larsson, 1979) due to the absence of phosphoglyceromutase, PEP-carboxylase, citrate synthetase and other enzymes of the TCA cycle (ap Rees, 1980b; Randall and Givan, 1981). Liedvogel and Stumpf (1982) concluded that acetate utilized in fatty acid synthesis in spinach

chloroplasts is mainly provided by the mitochondrion.

The biochemical evidence suggests that glycolysis and the TCA cycle can operate in the light, although some modifications probably occur in relation to the dark pattern (Graham, 1980). Studies in which leaves were fed with radioactive carbon compounds (e.g. CO_2, TCA cycle intermediates) are consistent with the view that the TCA cycle has a marked anaplerotic function in the light, as proposed by Kent (1979) and Woo and Canvin (1980), since radioactivity traversing TCA cycle intermediates (e.g. citrate) accumulates in amino acids and other compounds to a greater extent in the light than in the dark (Bidwell, 1963; Graham, 1980). The anaplerotic flow through the TCA cycle also increases with CO_2 concentration (Platt *et al.*, 1977).

A mechanism for replenishing the carbon compounds of glycolysis and TCA cycle used in synthetic reactions probably involves the cooperation of the enzymes PEP carboxylase and malate dehydrogenase in the cytosol, and NAD-malic enzyme in the mitochondrion (see Fig. 1.4). The higher reducing conditions of the cell during photosynthesis, which are reflected by the increased malate to aspartate ratio (Graham, 1980), would favour the operation of cytosolic malate dehydrogenase towards malate formation, and hence it would increase the availability of this compound for mitochondria.

The origin of PEP in the light is uncertain. In this sense, leaves metabolize exogenous glucose through glycolysis and TCA cycle in the dark, but do not do so significantly in the light; in contrast, exogenous malate is equally well metabolized in the light and in the dark (Graham, 1980). These results suggest that glucose catabolism is blocked in the light, probably at the phosphofructokinase step. This enzyme is controlled by adenylate turnover and many other factors and

it is thought that it is the main enzyme regulating glycolysis (see above). The inhibition of PFK during photosynthesis would avoid the existence of a futile cycle between fructose-6-P and fructose-1,6-bisphosphate, involving the enzymes fructose-1,6-bisphosphatase, a key enzyme in the sucrose synthesis pathway (see Fig. 1.2), and PFK. Interestingly, a recently discovered metabolite, fructose-2,6-bisphosphate, inhibits the enzyme fructose-1,6-bisphosphatase, and stimulates PFK (Cséke *et al.*, 1982a). The importance of this metabolite for regulating these enzymes in the light is unknown. Pyruvate kinase, which is also controlled by the adenylate system, can be bypassed by the mechanism involving PEP carboxylase and malic enzyme. Therefore, it is probable that PEP used in anaplerotic reactions is mainly generated from triose-phosphate from current photosynthesis rather than from stored sugars. However, Bassham *et al.* (1981) suggested that NH_4^+ can increase the anaplerotic flow for amino acid synthesis through activation of phosphofructokinase and pyruvate kinase.

The operation of the anaplerotic reactions of TCA cycle results in net CO_2 production (e.g. 1 molecule of CO_2 per molecule of glutamine formed from two molecules of PEP). However, it is not known how significant the rate of this CO_2 production in intact leaves in the light may be, compared to that in the dark. Several authors support the view that respiratory CO_2 production is very small in the light (Mangat *et al.*, 1974; Canvin *et al.*, 1976; Peisker and Apel, 1980), but others suggest that it may be significant (Graham, 1980; Azcón-Bieto *et al.*, 1981). This aspect will be studied in detail in wheat leaves in this thesis.

NADH generated in the mitochondrion can be transported to other

cell compartments through several metabolic shuttle systems, e.g. malate/oxalacetate (Woo *et al.*, 1980; Day and Wiskich, 1981, Fig. 1.6) and malate/aspartate (Journet *et al.*, 1981). However, it is not known if the TCA cycle operates beyond succinate oxidation in the light because this reaction requires the operation of the mitochondrial electron chain, a more uncertain aspect of the problem. The most widely accepted view is that electron transport coupled to oxidative phosphorylation is inhibited in the light by the increased cytosolic ATP/ADP ratio (Heber, 1974; Hampp *et al.*, 1982). However, Stitt *et al.* (1982) measured the levels of ATP, ADP and AMP in the chloroplasts, cytosol and mitochondria of wheat protoplasts using a technique which permitted to kill the protoplasts in about 0.1 sec, and found that the cytosolic ATP/ADP ratio was lower in the light than in the dark, but that the mitochondria appeared to be de-energized. They concluded that oxidative phosphorylation was restricted by a mechanism other than the ATP/ADP ratio. Similarly, Dry and Wiskich (1982) have found that the ATP/ADP ratio restricts respiration in isolated mitochondria only when its value exceeds approximately 20, which is much higher than the values commonly found *in vivo* (see above). They suggested that the absolute concentration of ADP may be more important in regulating respiration. The technical problems in measuring correctly the ATP/ADP ratio *in vivo* (see Section 1.3.1.2) may have been the reason for these contrasting results.

The possibility that uncoupled electron transport to oxygen (e.g. by increased membrane permeability to protons or by the operation of the rotenone and cyanide resistant pathways — see Section 1.3.1.2) occurs in the light cannot be ruled out. This would permit the reoxidation of NADH produced in the TCA cycle without affecting the energy status of the cell.

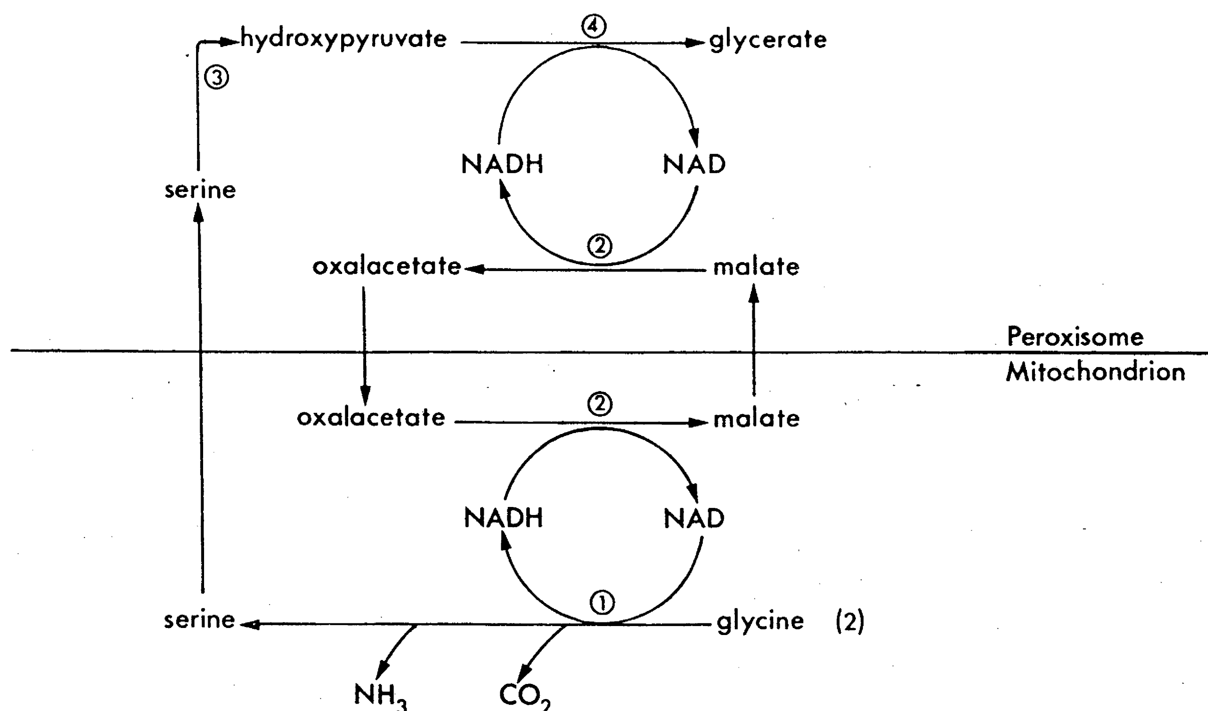

Fig. 1.6: Scheme of the malate/oxalacetate shuttle for transferring reducing equivalents generated in the glycine decarboxylation reaction from the mitochondrion to the peroxisome. Enzymes: (1) glycine decarboxylase complex; (2) malate dehydrogenase; (3) transaminase; (4) hydroxypyruvate reductase (after Day and Wiskich, 1981).

(i) Relationships between respiration and photorespiration

Photorespiration, defined as the process associated with the metabolism of glycolic acid, differs greatly from the process of respiration as defined in this Introduction (Tolbert, 1980; Lorimer and Andrews, 1981). However, several connections can occur between both processes.

The principal reaction contributing to CO_2 evolution during photorespiration in leaves is the oxidative decarboxylation of glycine in mitochondria (Osmond, 1981; Lorimer and Andrews, 1981). This reaction involves an inner membrane-bound glycine-decarboxylase linked

to serine hydroxymethyltransferase in the matrix space, which can donate electrons to the respiratory electron chain with formation of 3 molecules of ATP (Woo and Osmond, 1976; Moore *et al.*, 1977; Keys, 1980). However, reoxidation of the NADH generated in the glycine decarboxylation can also occur via malate dehydrogenase when oxalacetate is present (Woo and Osmond, 1976; Moore *et al.*, 1977; Day and Wiskich, 1981). On this basis, it has been suggested that a shuttle system (e.g. malate/oxalacetate, malate-glutamate/aspartate-α-ketoglutarate) could operate for transferring reducing equivalents from the mitochondria to the peroxisomes (Day and Wiskich, 1981; Journet *et al.*, 1981, Fig. 1.6). The operation of the shuttle could inhibit electron transport since it has been shown that addition of oxalacetate to mitochondria oxidizing glycine causes a severe, although not complete, inhibition of oxygen consumption in the presence of ADP (Moore *et al.*, 1977; Day and Wiskich, 1981), and it also de-energizes the mitochondria (Moore, A.L., unpublished).

However, the mechanism by which NADH generated in the glycine decarboxylation reaction is reoxidized *in vivo* is not known. *Whole cell* estimates of the rates of glycine oxidation linked to the electron chain or to the shuttle system in isolated pea leaf mitochondria are only just sufficient to account for the most conservative estimates of leaf photorespiration rates (Day and Wiskich, 1981). If oxidative phosphorylation is inhibited in the light, which is possible (see above), the shuttle system could be functional. Reoxidation of NADH by non-phosphorylating electron transport pathways, which are independent of the phosphate potential, is unlikely to be a major mechanism, since the capacity of these pathways in leaf mitochondria is much lower than that of the phosphorylating pathway (Day and

Wiskich, 1981). However, they may supplement a shuttle mechanism.

Photorespiratory glycine decarboxylation could also interact with TCA cycle oxidations through a competition for available NAD. Little is known about this important question, but oxidative glycine decarboxylation was not inhibited by concurrent oxidation of malate, succinate and α-ketoglutarate in pea leaf mitochondria (Day and Wiskich, 1981).

1.4 PROPOSAL OF STUDY

The evidence reviewed in this Introduction indicates that we know little about the possible interactions between carbohydrates, photosynthesis, and respiration, especially at the intact leaf level. There is no definite experimental evidence, for instance, that photosynthesis is regulated by the end product *in vivo*, although several biochemical mechanisms have been advanced. Likewise, the regulation of respiration in the dark and in the light in intact leaves by the products of photosynthesis, particularly carbohydrates, and the extent to what other proposed biochemical mechanisms of regulation (e.g. adenylate control) operate and interact *in vivo* with the former are problems still not very well understood. The indication that some leaf respiration appears to be *wasteful* and that its elimination could result in improved plant yields (Heichel, 1971b; Wilson, 1975) justifies the study of such questions. Particularly important in this context is the possibility that respiratory carbon losses, other than those caused by photorespiration, occur in illuminated leaves. The answer to this question is of critical importance for the calculation of the daily carbon balances in plants, since the carbon losses might be very important during the day given that the availability of

respiratory substrate and temperatures are normally higher than during the night.

The participation of the alternative pathway, which apparently consumes carbohydrate wastefully, at least in terms of energy conservation, in leaf respiration has been very little studied, and absolutely nothing is known about the regulation (e.g. by carbohydrate levels) and function of this pathway in leaves.

The basic purpose of the present thesis is to try to answer these questions by designing gas exchange experiments using intact leaves (mainly wheat), and to establish appropriate links with mechanisms proposed at the molecular and cellular levels. Some preliminary studies on the relationship between the oxidation of respiratory and photorespiratory substrates at the mitochondrial level are also included.

CHAPTER 2

INHIBITION OF PHOTOSYNTHESIS BY CARBOHYDRATES IN WHEAT LEAVES

2.1 INTRODUCTION

As discussed in the previous chapter, manipulation of the sink for photosynthate can cause accumulation of carbohydrates in leaves and result in lower rates of photosynthesis (Neales and Incoll, 1968; Guinn and Mauney, 1980; Herold, 1980). Evidence for end product inhibition of photosynthesis in wheat leaves is equivocal. Birecka and Dakič-Wlodkowska (1963) and King *et al.* (1967) were able to inhibit flag leaf photosynthesis by removing the sink (removal of the ear) and stimulate flag leaf photosynthesis by spraying the ear with DCMU (which inhibited photosynthesis in the ear, thereby increasing the sink). However, Austin and Edrich (1975) were unable to confirm the ear removal response. Nevertheless, King *et al.* (1967) showed that ear removal caused an accumulation of carbohydrates in the flag leaf. They were able to simulate this response by keeping plants in continuous light for a week. Leaf carbohydrates built up and photosynthesis was inhibited.

Although these results suggest the occurrence of end product inhibition of photosynthesis in wheat, the treatments and the measurement methods used do not exclude other factors. Hormonal and stomatal responses could be involved. Thus, in this chapter I describe experiments which are designed to avoid these difficulties and to

determine if the photosynthetic rate is related to the carbohydrate status in wheat leaves. Carbohydrate status was increased by increasing the rate of photosynthesis under otherwise comparable conditions, by increasing CO_2 concentration or reducing O_2 concentration. In other experiments a portion of the leaf, below the region in which photosynthesis was monitored, was chilled to reduce transport of photosynthate (Wardlaw, 1968; Geiger and Sovonik, 1975). All these treatments produced the expected increase in soluble sugars in photosynthetic tissues. Starch does not normally accumulate in wheat leaves (Evans *et al.*, 1975).

2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Triticum aestivum (cv. Gabo) plants were grown from seed in a controlled environment cabinet in pots of soil. They were watered twice a day, and were fertilized every other day with nitrate-type Hewitt's solution containing: KNO_3, 4 mM; $Ca(NO_3)_2$, 4 mM; $MgSO_4$, 1.5 mM; NaH_2PO_4, 1.33 mM; EDTA FeNa, 60 μM; $MnSO_4$, 10 μM; $ZnSO_4$, 1 μM; $CuSO_4$, 1 μM; H_3BO_3, 50 μM; Na_2MoO_4, 0.5 μM; NaCl, 0.1 mM; $Co(NO_3)_2$, 0.2 μM. The pH of the solution was 6.5. Quantum flux (400-700 nm) was about 600-700 $\mu E.m^{-2}.s^{-1}$. The day/night temperature regime was 25/20 °C with a daylength of 13 h. Relative humidity was between 60 and 80%.

2.2.2 General Experimental Conditions

Wheat plants were selected from the growth cabinet near the end of the night period (10 a.m.). One or two attached recently mature leaves were enclosed in a photosynthetic chamber, which received an

incident quantum flux of 1000 $\mu E.m^{-2}.s^{-1}$. The rest of the plant, which was kept intact, was also illuminated. CO_2 and water exchanges were measured in leaves using an open system gas analysis apparatus described in Appendix I. Calculations of gas exchange parameters were made as described in Appendix II. Water vapour pressure deficits were maintained at about 8-12 mbar at 20-23 °C and at 20-23 mbar at 30 °C in most experiments.

2.2.3 Light Responses of Photosynthesis

Net CO_2 assimilation (A) was measured in wheat leaves for 1 h at a quantum flux of 1000 $\mu E.m^{-2}.s^{-1}$ to open stomata. Incident quantum flux was measured with a quantum sensor (Lambda Instruments, model LI-190 SR, Lincoln, Nebraska, U.S.A.). Quantum flux was then decreased by interposing copper screens, and A was measured at every step. Finally, dark CO_2 efflux was measured. Leaf temperature was kept constant during these procedures. These measurements were repeated after a period of 4 h at a quantum flux of 1000 $\mu E.m^{-2}.s^{-1}$. The leaf temperature and CO_2 pressure were varied as indicated in the text. Each experiment was replicated three times.

2.2.4 CO_2 Responses of Photosynthesis

Net CO_2 assimilation (A) and transpiration rates were first measured in flag wheat leaves at ambient CO_2 and O_2 pressures for about 1 h until steady-state rates were reached. Measurements were then repeated at several CO_2 concentrations below ambient, and finally at CO_2 pressures higher than ambient. After the determination of the first curve of A versus intercellular partial pressure of CO_2 (p_i), which took 2½-3 h from the start of the light period, leaves were

allowed to photosynthesize for 5 h at external partial pressures of CO_2 of either 800 μbar (Expt A) or 50-60 μbar (Expt B). The curve of A versus p_i was then determined again over the next 1½-2 h. At the end of this time, a piece of the enclosed leaf was taken for carbohydrate analysis. This piece was obtained by carefully sectioning the leaf with a sharp razor blade. In the experiments where the rate of photosynthesis declined with time (Expt A), the remaining leaf fragment was kept in darkness for 3 h. At the end of this period, the curve of A versus p_i was determined again for studying recovery. The rest of the leaf was then used for carbohydrate analysis. Expt A was performed three times and Expt B two times. Leaf temperatures during the light and dark periods were 20 and 18 °C, respectively.

2.2.5 Leaf Base Chilling Methods

A small portion (about 2 cm^2) of the base of a wheat leaf, whose upper part was enclosed in the photosynthetic chamber, was chilled by circulating icy water from a bath through a sandwich-type brass jacket in close contact with both sides of the leaf. The surface of both sides of the jacket formed a square of 4 cm^2. Temperature of the leaf region in contact with the jacket was 0-2 °C. These procedures did not produce alterations in the temperature of the enclosed portion of the leaf, which was kept at 21 °C. Two experimental procedures were adopted:

(1) A leaf was allowed to photosynthesize for 4 h at external CO_2 pressures of either 350 μbar or 700 μbar. Then the chilling treatment was applied as described above and the gas exchanges of the enclosed part of the leaf were monitored for a subsequent period of 4 h. At the end of this period, the cooling jacket was removed, and the leaf

gas exchanges were measured for about another hour.

(2) In other experiments, leaves were subjected to the chilling treatment from the start of the light period, and the gas exchanges were monitored for 5 h. CO_2 pressures inside the chamber were either 310 μbar or 800 μbar. The CO_2 compensation point, which was measured using a closed system (see Section 4.2.2), and the rate of dark CO_2 efflux in air 30 min after the light was turned off, were determined consecutively before and after the chilling period.

All experiments were duplicated.

2.2.6 Carbohydrate Determination

Leaf segments were killed in liquid nitrogen and freeze-dried. Sugars were extracted in boiling water for 15 min and analysed using enzymatic methods. Free glucose plus fructose were measured from the leaf extract using a glucose specific assay (Calbiochem-Behring Glucose s.v.r. No. 870104), after converting fructose to glucose with phosphoglucoisomerase (Sigma P-5381). Glucose was converted to glucose-6-phosphate in the presence of hexokinase, and then oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase, reducing a molar equivalent of NADP. The change in absorbance at 340 nm is proportional to the glucose concentration in the range from 0 to 10 $ng.ml^{-1}$, and was measured with a Varian 634 spectrophotometer. The assay was performed at room temperature and was initiated by adding an aliquot of sample. Sucrose was hydrolysed by incubating the leaf extract at 37 °C for 2 h in a water bath with invertase (Sigma I-5875) in 0.1 N acetate buffer (pH 4.6). Since invertase also has been reported to hydrolyse some small fructosans (Tetley and Thimann, 1974) the sugars resulting from the action of this enzyme are referred to as

the invertase fraction. This fraction was obtained by subtracting the amount of free glucose plus fructose from the total glucose assayed. Starch content was obtained by incubating the leaf extract at 37 °C for 48 h in a water bath with 0.5% "Clarase 900" (Miles Laboratories) and 0.2% amyloglucosidase (Sigma A-7255) in 0.1 N acetate buffer (pH 4.6). "Clarase 900" is a mixture of several digestive enzymes which hydrolyse starch and sucrose to hexoses. Starch was estimated by subtracting the glucose in glucose plus fructose and invertase fractions from total glucose assayed in the Clarase digest.

2.3 RESULTS

2.3.1 Changes in the Rate of Net CO_2 Assimilation Throughout the Day

The rate of net CO_2 assimilation (A) of wheat leaves measured at ambient CO_2 and O_2 levels reached a maximal value within the first hours of the light period and then slowly declined in the next hours. This pattern was frequently observed in leaves photosynthesizing at temperatures lower than 25 °C, especially when the initial rate of photosynthesis was high, but was not normally seen at higher temperatures. Typical examples are shown in Fig. 2.1. Leaf conductance to diffusion of water vapour, however, remained constant or slightly increased. The intercellular CO_2 partial pressure, p_i, therefore increased with time especially in leaves photosynthesizing at 22 °C (Fig. 2.1). In this latter case, p_i typically varied from about 260 μbar to 280 μbar during the examined period. When the CO_2 partial pressure during the photosynthetic period was about twice the ambient level, A declined more rapidly (Fig. 2.2). In contrast, A remained constant during 6 h when CO_2 pressure inside the chamber was low (Fig. 2.2). Stomatal conductance to water vapour (g_s) increased

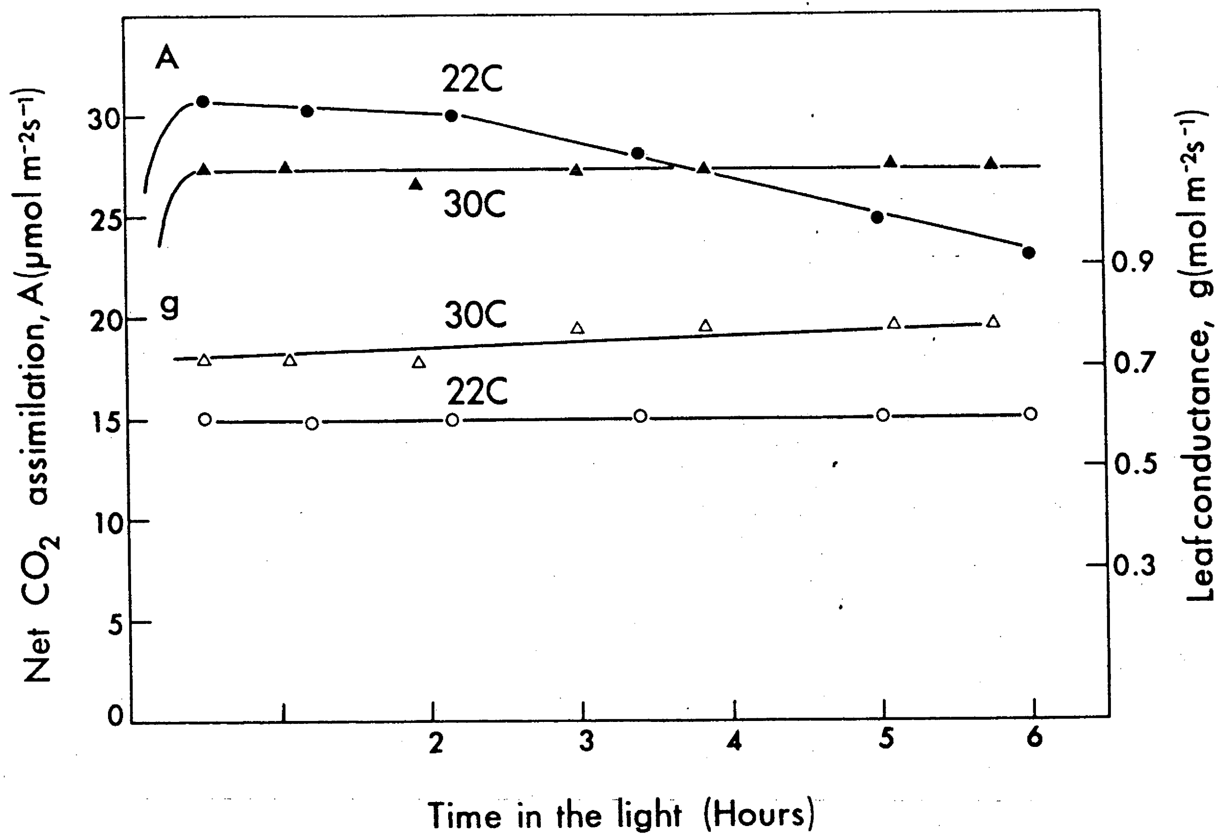

Fig. 2.1: Time-course of net CO_2 assimilation (●,▲) and total leaf conductance to diffusion of water vapour (○,△) in wheat leaves at two temperatures. External CO_2 pressure was 340 μbar and O_2 concentration was 21%.

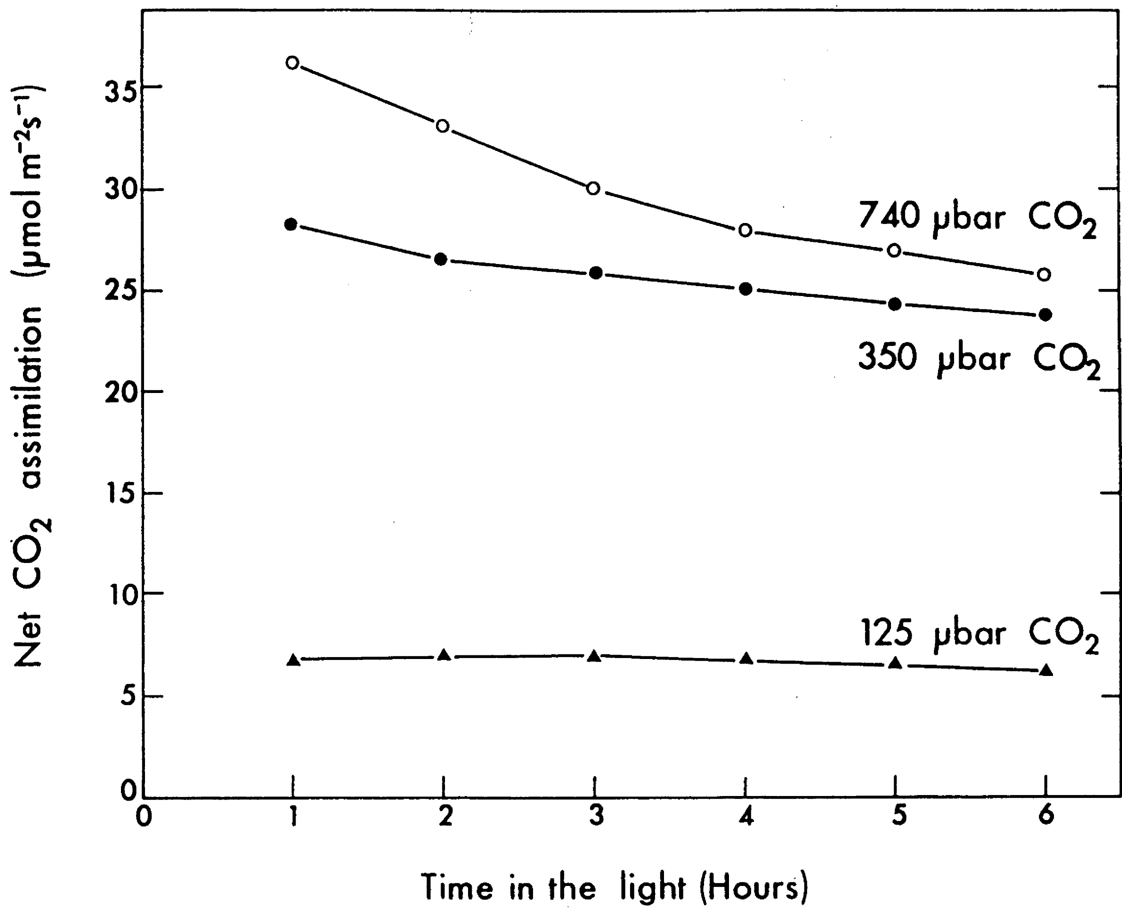

Fig. 2.2: Time-course of net CO_2 assimilation in wheat leaves at several external CO_2 pressures. Leaf temperature was 22 °C, and O_2 concentration was 21%.

with time at low CO_2 pressures, but it slightly decreased at high CO_2 pressures (not shown). In this latter case, p_i values increased from 580 μbar in the first hour to 625 μbar in the sixth hour, suggesting that stomatal closure was not responsible for the assimilation decline.

Leaf carbohydrate concentration was very low at the end of the night and increased during the light period more or less proportionally to the integrated net CO_2 assimilation (Fig. 2.3). Invertase sugars (mostly sucrose) were the main carbohydrate fraction accumulated. Sucrose accumulated more rapidly in the leaf for a given increase in the integrated carbon assimilation when the internal level of sucrose was low. This suggests that sucrose translocation is more efficient when sucrose levels are high, as has been proposed by Troughton *et al.* (1977).

The effects of high CO_2 levels on the time-courses of A and g_s were studied in some detail at two temperatures. Assimilation declined considerably with time in wheat leaves at both 20 and 30 °C (Table 2.1). Stomatal conductance declined in parallel with A during the first 4 h of the light period, so p_i remained constant. However, g_s declined relatively more rapidly over the next 3 h, and therefore p_i values slightly decreased at the end of the 7th hour in the light, but not enough to explain the observed changes in A. Leaf carbohydrate levels increased with time, being very high after 7 h in the light. Carbohydrate accumulated significantly less in leaves photosynthesizing at 30 °C than at 20 °C, in spite of the much higher assimilation rates observed at 30 °C (however, free sugars were an exception in this respect) (Table 2.1). This suggests that translocation of recently accumulated assimilates is more efficient at 30 °C. Similar effects of temperature on translocation have been

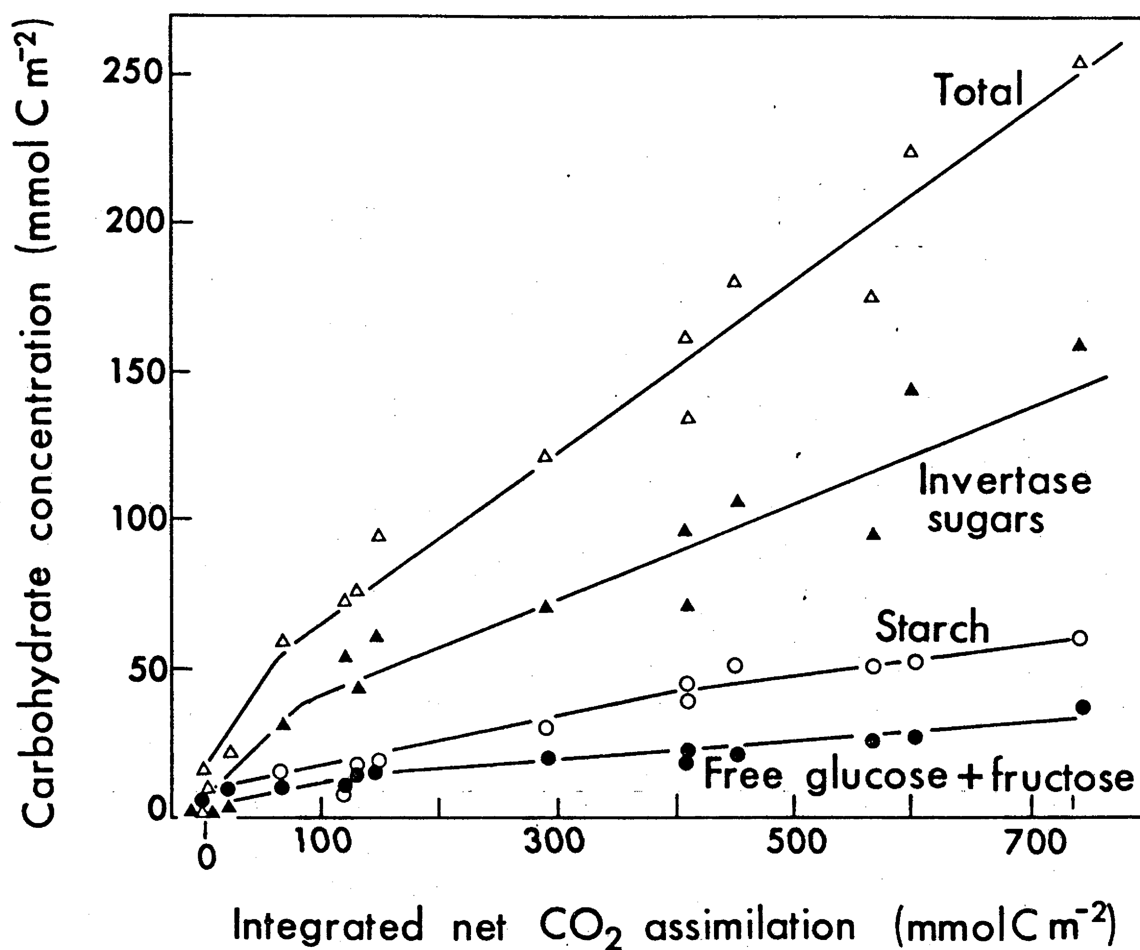

Fig. 2.3: Relationship between several carbohydrate fractions and integrated net CO_2 assimilation in wheat leaves obtained by varying the length of the light period and the CO_2 concentration. Leaf temperature was 23.5 °C.

Table 2.1

Time-course of net CO_2 assimilation (A), stomatal conductance to water vapour (g_s), intercellular partial pressure of CO_2 (p_i) and several carbohydrate fractions in wheat leaves at high external CO_2 pressures (825 μbar). Carbohydrate concentration was measured in leaf fragments sampled from the leaf enclosed in the photosynthetic chamber after 4 h and 7 h in the light, respectively, and after an additional period of 2 h in darkness. The values shown are means ± S.E. of three experiments (except for the values shown in Section D which correspond to a single experiment).

After a Period of	Temp	Gas Exchange Parameters			Carbohydrate Concentration			
		A	g_s	p_i	Free Fructose + Glucose	Invertase Sugars	Starch	Total
	°C	μmol CO_2 $m^{-2}.s^{-1}$	$mol.m^{-2}.s^{-1}$	μbar CO_2	mmol C m^{-2}			
A. 1 h in the light	20	43.0 ± 1.1	0.67 ± 0.1	713 ± 24	— not measured —			
	30	51.0 ± 0.5	0.45 ± 0.01	588 ± 2				
B. 4 h in the light	20	37.4 ± 1.2	0.54 ± 0.08	709 ± 22	75.7 ± 0.3	159 ± 26	86.5 ± 14.5	321.2 ± 11.5
	30	47.7 ± 1.1	0.41 ± 0.01	583 ± 11	96.8 ± 15.5	114 ± 15	50.6 ± 14	261.4 ± 21.5
C. 7 h in the light	20	34.6 ± 0.2	0.45 ± 0.06	690 ± 21	114 ± 18	233.6 ± 1	178.5 ± 21.5	526 ± 39.5
	30	43.3 ± 1.1	0.28 ± 0.02	524 ± 15.5	140 ± 15.5	194.3 ± 22.8	129 ± 15.3	463.3 ± 22.6
D. 7 h in the light + 2 h in the dark	30	49.4	0.30	495	95	143	87	325

reported earlier (Geiger and Sovonik, 1975). A period of only 2 h in darkness was sufficient to produce substantial recovery of A (Table 2.1). Leaf carbohydrate levels decreased substantially during the dark period, presumably due to translocation and respiration of assimilates.

If wheat leaves were given 740 μbar CO_2 and 2% O_2 at 20 °C the initially high rates of photosynthesis (29 μmol CO_2 $m^{-2}.s^{-1}$) declined dramatically (to 19 μmol CO_2 $m^{-2}.s^{-1}$ after 9 h). In this experiment, carbohydrates accumulated to very high levels (total 582 mmol $C.m^{-2}$ = sucrose 398 mmol $C.m^{-2}$ + free glucose and fructose 51 mmol $C.m^{-2}$ + starch 133 mmol $C.m^{-2}$).

The rate of decline of photosynthesis with time in a wheat leaf was apparently increased by chilling the base of that leaf (Fig. 2.4A). This effect was more marked when the leaf had been pre-illuminated for several hours at high CO_2 concentration. The chilling treatment of the leaf base did not initially affect the value of p_i, indicating that A and g_s were declining in parallel. However, in the experiments performed at high CO_2, g_s declined relatively more rapidly about 1.5 h after the start of the chilling treatment, and therefore p_i values decreased (Fig. 2.4A). When the chilling treatment was halted, neither A nor g_s showed signs of recovery, at least within the first hour (Fig. 2.4A). Very high carbohydrate levels were measured in these leaves at the end of the experiments, ranging from 400 to 600 mmol $C.m^{-2}$. When the leaf base was chilled without a pre-illumination period, A also declined with time (Fig. 2.4B). Assimilation declined more rapidly with time at high CO_2 pressures, and p_i and g_s showed similar patterns to those observed in the previous experiment. The CO_2 compensation point and the rate of dark CO_2 efflux increased

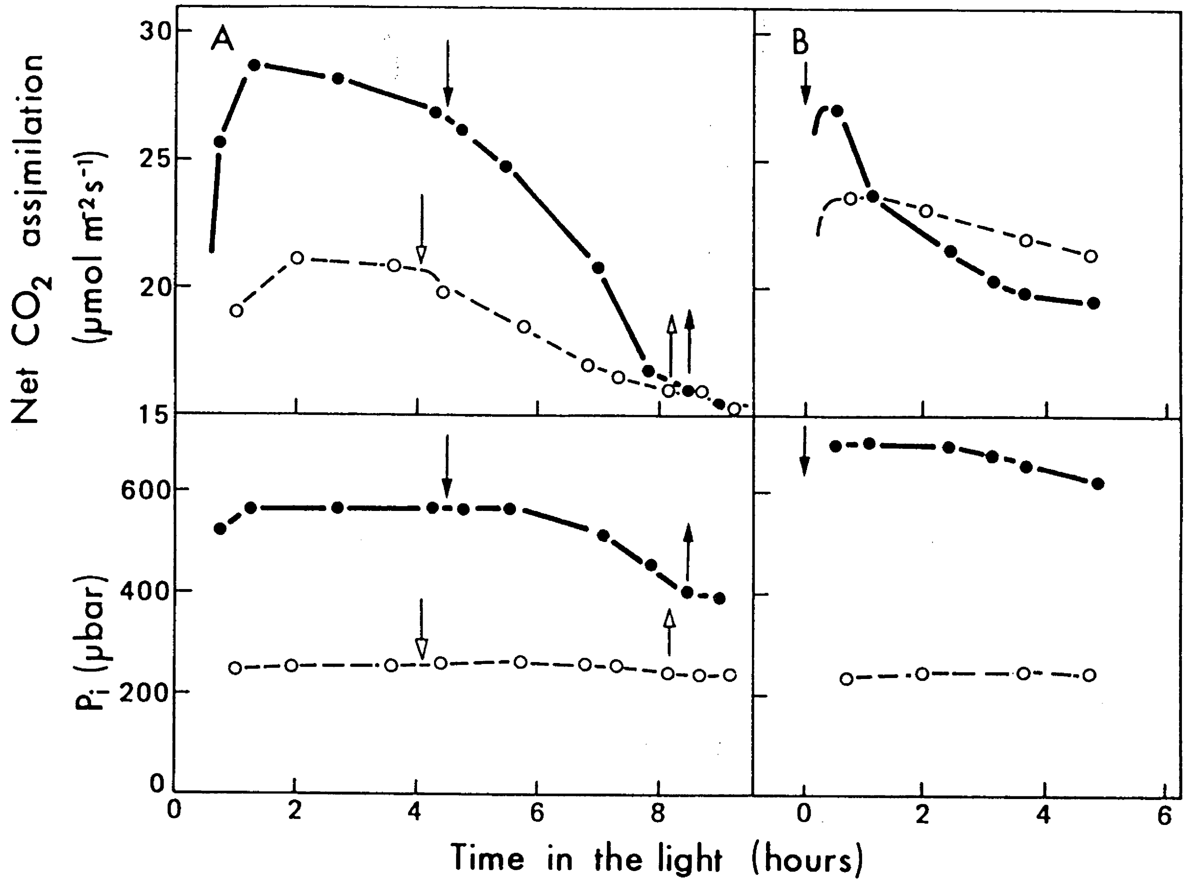

Fig. 2.4: Effect of chilling the base of a wheat leaf on the rate of net CO_2 assimilation and p_i in the rest of that leaf. The chilling treatment was applied (as indicated by the arrows) either after 4 h of photosynthesis (A) or at the beginning of the photosynthetic period (B). These experiments were performed either at high (●) or ambient (○) CO_2 concentrations. Typical experiments at each CO_2 concentration are shown. For further details see Section 2.2.5.

Table 2.2

Effect of chilling the base of wheat leaves for 5 h in the light on the CO_2 compensation point (Γ) and dark CO_2 efflux (R_n). The experimental conditions are described in Section 2.2.5. The values shown are means ± S.E. of four experiments.

	Γ	R_n
	μbar CO_2	μmol CO_2 $m^{-2}.s^{-1}$
At the end of the night	34.5 ± 0.5	0.64 ± 0.01
After a chilling period of 5 h in the light	42 ± 1.5	1.10 ± 0.02

considerably after the chilling period (Table 2.2). These responses were consistent with an increase in the rate of CO_2 efflux by respiration in the light, as discussed in depth in Chapter 4.

2.3.2 Changes in the Properties of Photosynthesis Throughout the Day

The light dependence of photosynthesis was also affected by a period of light. The initial slope of the curve of A versus incident quantum flux (the apparent quantum yield) was significantly lower after a 4 h period of photosynthesis at ambient CO_2 levels (Fig. 2.5). This pattern was observed at 20 °C but not at 30 °C. The light-dependence of stomatal conductance was not affected by this treatment at either 20 or 30 °C (not shown). Similar reductions in the quantum yield were obtained by increasing CO_2 pressures to about double ambient levels (Fig. 2.6), even at 30 °C. In this latter case the quantum yield varied from 0.075 to 0.062 mol CO_2.Einstein^{-1} after 4 h in the light. Stomatal conductance declined after a high CO_2 treatment but p_i levels were not significantly decreased: they ranged from

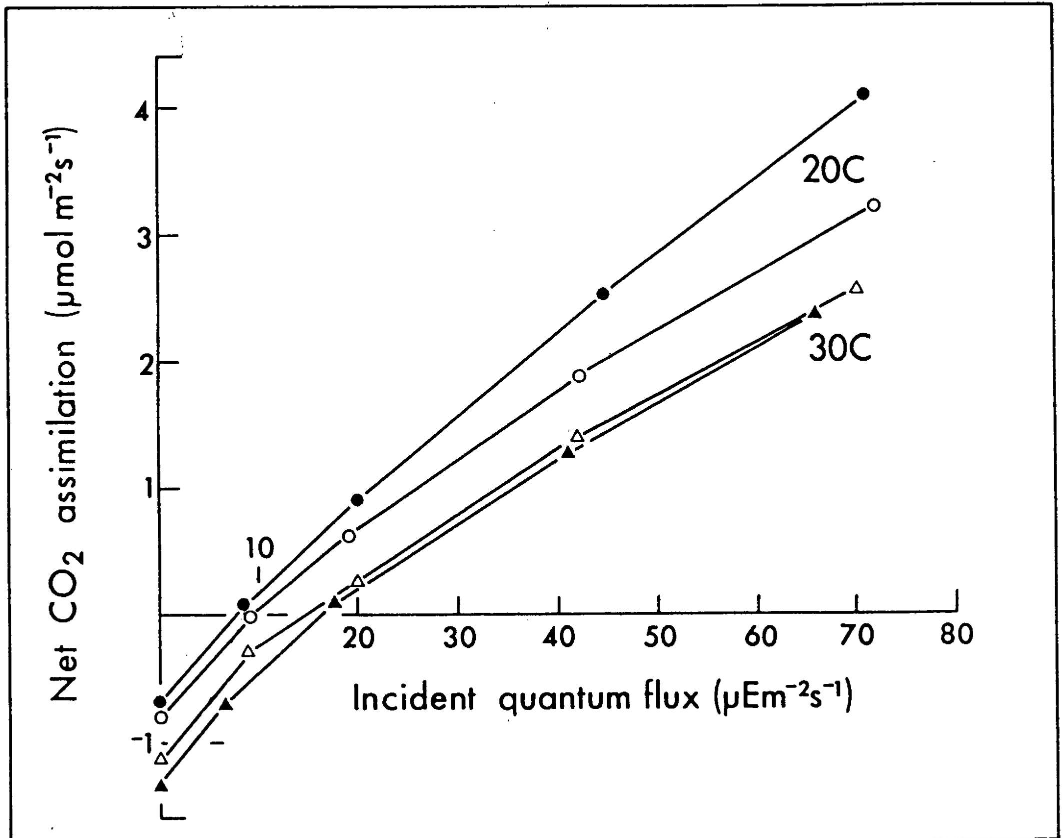

Fig. 2.5: Light response curves of net CO_2 assimilation in wheat leaves determined *before* (closed symbols) and *after* (open symbols) a period of photosynthesis of 4 h. External CO_2 partial pressures were 320-330 μbar. Two typical experiments performed at two temperatures are shown. The apparent quantum yield values (which were calculated without the rate of dark CO_2 efflux) were: At 20 °C: 0.064 (before;●) and 0.051 (after;○) mol CO_2.Einstein^{-1}. At 30 °C: 0.047 (before;▲) and 0.046 (after;△) mol CO_2. Einstein^{-1}. For further experimental details see Section 2.2.3.

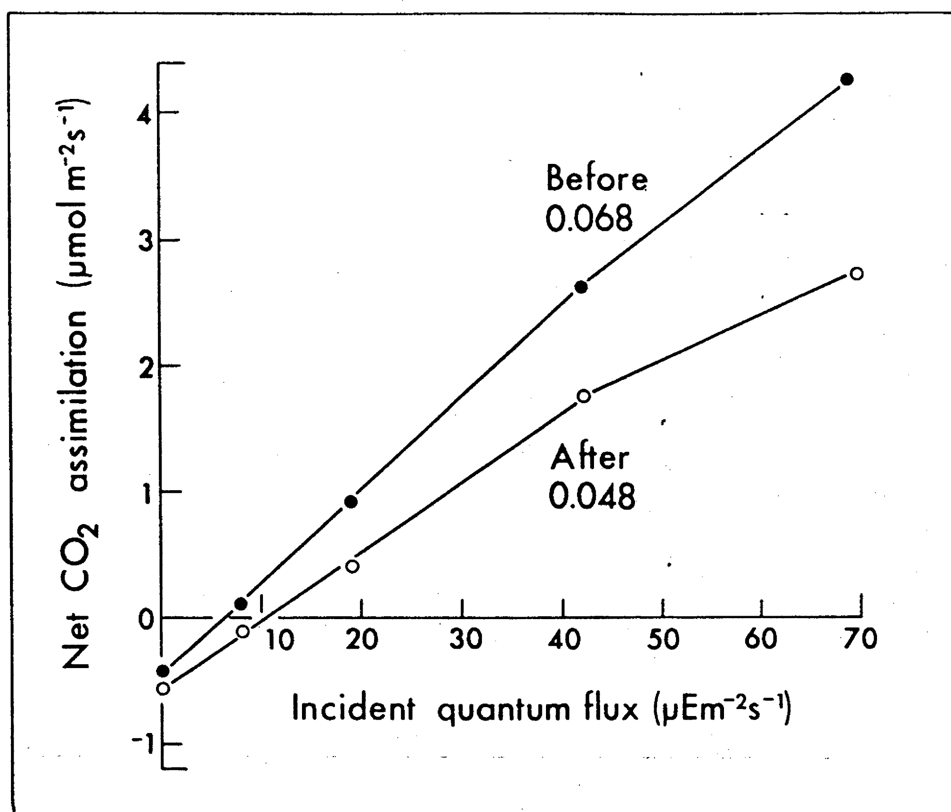

Fig. 2.6: Effect of a period of photosynthesis of 4 h at 640 µbar CO_2 on the light response curve of net CO_2 assimilation in wheat leaves. Temperature was 20 °C. For further details, see legend of Fig. 2.5. A typical experiment is shown.

610 to 620 μbar during the determination of the first curve of A vs quantum flux, and from 575 to 610 μbar during the determination of the second curve.

The increase in the rate of respiration observed after a period of photosynthesis (see Figs. 2.5 and 2.6) appears to be insufficient to account for the differences in A observed. Changes in the light transmission properties of the leaf during the day, which were studied by placing a silicon cell under the leaf, did not occur at any of the temperatures examined (20, 25 and 30 °C). Transmitted light in these leaves was only 6-7% of total incident light. Leaf absorbance could not be measured in the same conditions as the experiments were performed, but it is unlikely that changes in absorbance were large enough to account for the large variations observed in the apparent quantum yield. Once again, it is probable that the carbohydrate status is involved in these responses.

The CO_2 response curve of photosynthesis in flag wheat leaves was affected in a complex way after a 5 h period of photosynthesis at high CO_2 concentrations: the upper part of the curve of A vs p_i decreased substantially but the initial slope (up to about 150 μbar CO_2) was unaffected (Fig. 2.7A). However, the initial part of this curve was slightly displaced towards higher p_i values, presumably due to an increase in respiration (see Chapter 4), resulting in an increase in the CO_2 compensation point (Fig. 2.7A). The upper part of the curve of A vs p_i almost recovered to the level observed at the beginning of the day after a period of 3 h in darkness (Fig. 2.7A). In contrast, the curve of A vs p_i was not affected at all after a 5 h period of photosynthesis at CO_2 pressures near the compensation point (Fig. 2.7B). These results suggest that photoinhibition and photoperiodical

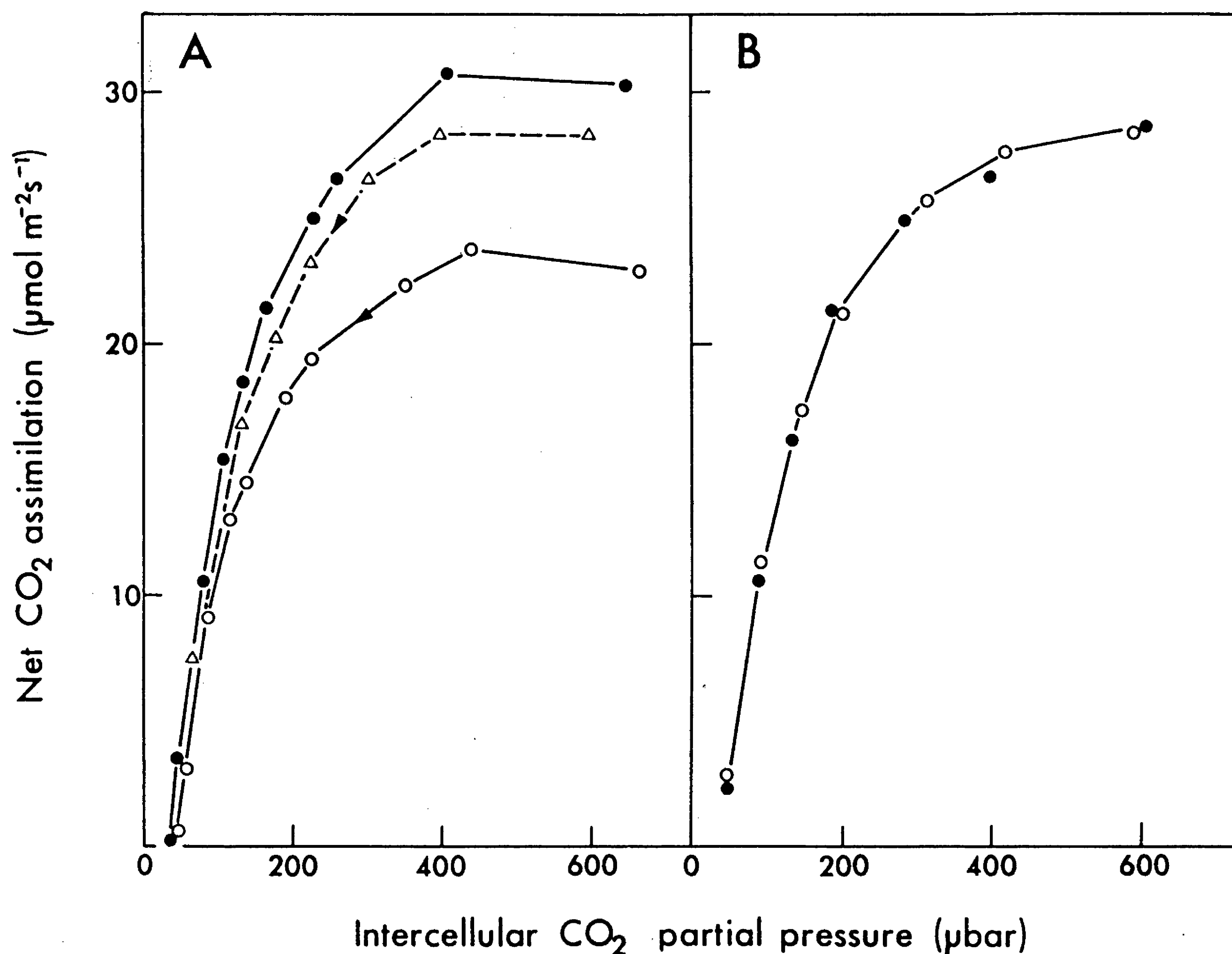

Fig. 2.7: Effect of a period of photosynthesis on the curve of A versus p_i in flag wheat leaves. See 2.2.4 for experimental details. (A): the curve A versus p_i was determined at the beginning of the light period (●), after 5 h in the light at 800 μbar CO_2 (o), and after a further 3 h in the dark (Δ). (B): the curve of A versus p_i was determined at the beginning of the light period (●) and after 5 h in the light at 50-60 μbar CO_2 (o). Two typical experiments are shown. The arrows indicate the direction in which the p_i level was changed (i.e. increased or decreased).

effects cannot be responsible for the decline in A, since light intensity and timing were the same in both cases.

Stomatal conductance to water vapour (g_s) was rather insensitive to p_i changes at the beginning of the light period, but it decreased and showed sensitivity to p_i after a high CO_2 light period (Fig. 2.8A). In contrast, the stomatal responses to p_i were not affected by a period of photosynthesis at low CO_2 pressures (Fig. 2.8B). Leaf carbohydrate levels were measured after the determinations of the curves of A vs p_i (see Section 2.2.4). Carbohydrate levels were much higher after a period of photosynthesis at high CO_2 pressures than at low CO_2 pressures, and they decreased considerably after a dark period (Table 2.3).

The O_2 sensitivity of CO_2 fixation (the Warburg effect) in wheat leaves also declined following an extended period of photosynthesis (Fig. 2.9). The reduction in the O_2 sensitivity of photosynthesis was less in leaves with lower photosynthetic rates (Table 2.4).

2.4 DISCUSSION

Several physiological processes were affected in wheat leaves when carbohydrate accumulated during short periods of photosynthesis, viz., net CO_2 assimilation (A) and stomatal conductance (g_s) significantly declined, while CO_2 efflux by respiration increased. The relationship between carbohydrate levels and respiration in the dark and in the light is analysed in detail in Chapters 3, 4 and 5.

A similar decline in A has been observed in leaves of many species: soybean (Upmeyer and Koller, 1973; Bhagsari *et al.*, 1977; but see Potter and Breen, 1980), alfalfa (Chatterton, 1973), cucumber (Hopkinson, 1964; Challa, 1976), *Mimulus* (Milner and Hiesey, 1964),

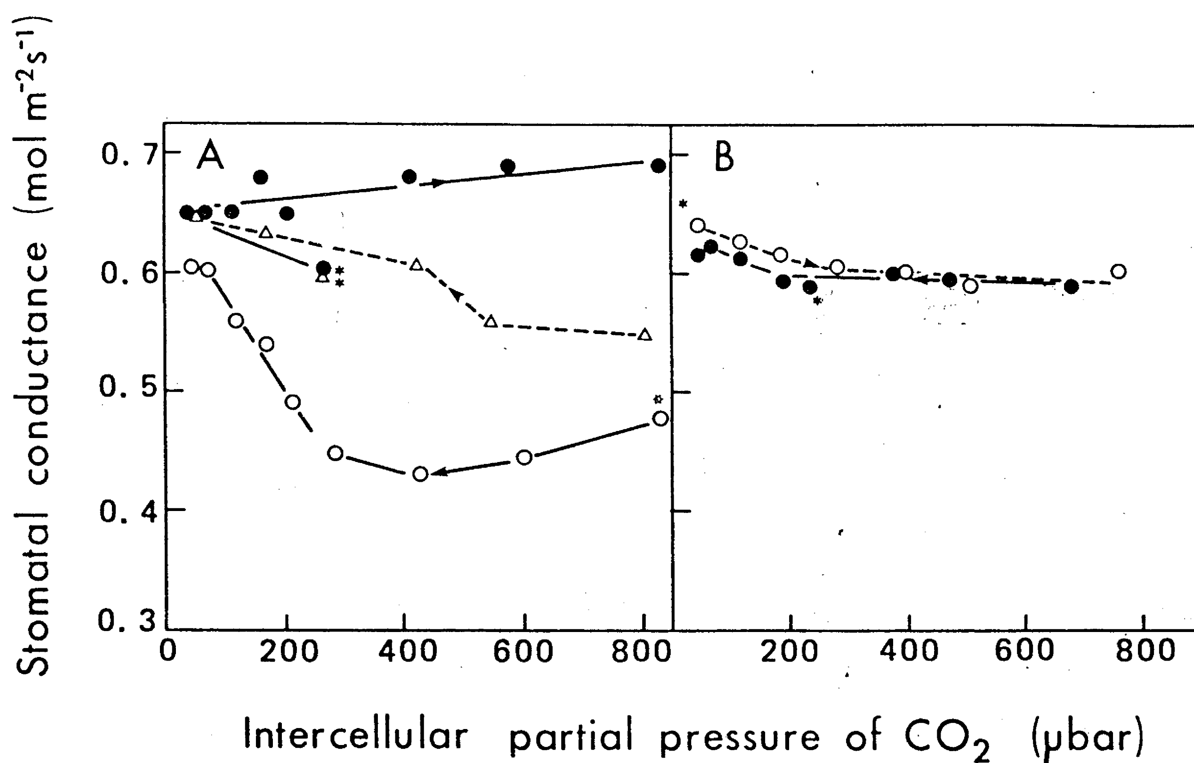

Fig. 2.8: Effect of a period of photosynthesis on the sensitivity of stomata to CO_2 in flag wheat leaves. The symbols and experimental conditions are the same as in Fig. 2.7. The horizontal arrows on the curves indicate the direction in which measurements proceeded. The asterisks indicate the first measurement in each curve. In two of these curves, the first measurement was made at ambient CO_2 levels and the next one was made at high CO_2 levels (see the curve symbolized by the triangles in A, and the curve symbolized by the closed circles in B).

Table 2.3

Carbohydrate levels in flag wheat leaves corresponding to the experiments shown in Figs. 2.7 and 2.8. In B, C and D, carbohydrates were measured after the determination of the curve of A vs p_i. The values shown are means ± S.E. of two experiments.

Conditions	Carbohydrate Concentration			
	Free Fructose + Glucose	Invertase Sugars	Starch	Total
	mmol $C.m^{-2}$			
A. At the end of the night	5.7	6	0	11.7
B. After 5 h in the light at 50-60 μbar CO_2	18.6 ± 3.1	66.3 ± 8.3	23.8 ± 1	99.3 ± 10.3
C. After 5 h in the light at 800 μbar CO_2	19.1 ± 2.6	155.6 ± 18.1	85 ± 25	260 ± 40.4
D. After 5 h in the light at 800 μbar CO_2 plus 3 h in the dark	19.1 ± 1.5	126.5 ± 1.5	51.8 ± 14.5	197.4 ± 11.4

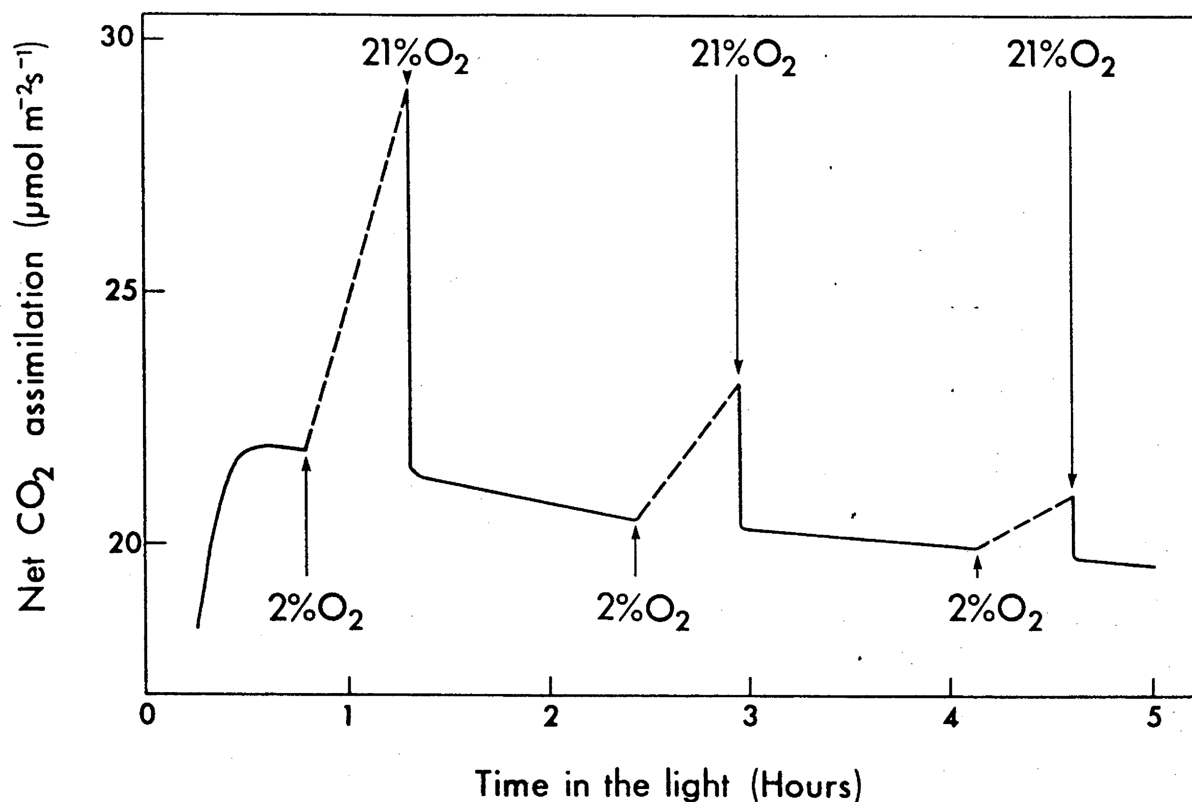

Fig. 2.9: Time-course of the O_2 sensitivity of photosynthesis in wheat leaves. The rate of net CO_2 assimilation (A) was normally measured at 21% O_2, but measurements of A at 2% O_2 were intercalated approximately every 1.5 h. The arrows indicate transfer to 2% O_2 or 21% O_2, and the dotted lines represent the periods at 2% O_2 during which measurements were not taken. External CO_2 pressure was 330 μbar and temperature was 21 °C. A typical experiment is shown.

Table 2.4

Effect of decreasing O_2 concentration from 21 to 2% on the rate of net CO_2 assimilation (A) at different times during the light period. Temperature was 21 °C. For other details see legend to Fig. 2.9.

Experiment	Time in the light, Hours					
	1½ - 2			4 - 5		
	A_{21}	A_2	Increase	A_{21}	A_2	Increase
	μmol CO_2 $m^{-2}.s^{-1}$		%	μmol CO_2 $m^{-2}.s^{-1}$		%
1	19.3	25.2	31	18.8	21	12
2	21.9	29.0	32	19.9	21	6
3	12.5	19.2	54	13.5	18	33

Panicum virgatum (Ku *et al.*, 1978), *Populus tremuloides* (Bate and Canvin, 1971), *Digitaria decumbens* (Chatterton *et al.*, 1972), etc. However, A remained more or less constant during the normal light period in leaves of beans (Geiger, 1976) and sunflower (Bate and Canvin, 1971; Potter and Breen, 1980).

Cooling a short portion of the translocation path to near 0 °C produces in most cases a severe inhibitory effect on the rate of sugar export, at least for a few hours (Wardlaw, 1968; Geiger and Sovonik, 1975). In this case carbohydrate will accumulate more rapidly in the leaf. The use of this technique for studying variations in A has provided contradictory results (see Neales and Incoll, 1968, for a review). Coulson *et al.* (1972) observed that A declined about 1.7% per hour after cooling a 4 cm zone of the petiole of a sugat beet leaf to 0.7-2.5 °C. Geiger (1976) chilled the primary leaf petiole and node of a bean plant but no effect on the rate of photosynthesis of that leaf was observed. In both cases the rate of translocation was

reduced. However, A was low in these studies (about 11 and 2 μmol $CO_2.m^{-2}.s^{-1}$, respectively) and a large carbohydrate build-up is not expected under these conditions.

My results are consistent with the occurrence of end product inhibition of photosynthesis in wheat leaves, as suggested by Birecka and Dakić-Wlodkowska (1963) and King *et al.* (1967), since A declined more rapidly under conditions favouring large carbohydrate build-up (e.g. high CO_2 and low O_2 pressures in the air, lower translocation rates) and the increase in respiration was not large enough to account for this decline. On the other hand, A substantially recovered after a short period of darkness in which carbohydrates were significantly removed from the leaf. Factors such as stomatal closure, photo-inhibition and timing effects were not significantly involved in the diurnal changes in A.

The mechanism of this inhibition is not clear but it is correlated with accumulation of soluble carbohydrates. Starch is a relatively small carbohydrate fraction in wheat leaves. This mechanism may differ from that occurring in species accumulating mainly starch (Guinn and Mauney, 1980; Herold, 1980). The possible effects of fructosans have not been investigated here. According to recent photosynthetic models (Farquhar *et al.*, 1980; Farquhar and von Caemmerer, 1982), photosynthesis can be divided into a ribulose-1,5-bisphosphate (RuBP)-limited region and a RuBP-saturated region. RuBP-limited photosynthesis occurs when the concentration of RuBP falls below the concentration of RuBP binding sites of the enzyme RuBP carboxylase-oxygenase (Rubisco). Such conditions occur at high p_i where carboxylation capacity exceeds the RuBP regeneration capacity of the electron transport system. Conversely, when p_i is low and the

capacity to regenerate RuBP exceeds carboxylation and oxygenation demands, RuBP will increase above the Rubisco site concentration and be saturating. Considerable experimental evidence supports this model (von Caemmerer and Farquhar, 1981; Farquhar and Sharkey, 1982; Badger *et al.*, 1983). Badger *et al.* (1983) have measured levels of photosynthetic intermediates in rapidly killed bean leaves and have observed that the levels of these intermediates are in agreement with the predictions of Farquhar *et al.* (1980). They have conclusively shown that the concentration of RuBP is saturating for photosynthesis at low CO_2 pressures and limiting at high CO_2 pressures.

My results suggest that carbohydrate accumulation in wheat leaves is associated with a reduction in the RuBP regeneration capacity, but does not affect the RuBP carboxylation (and oxygenation) capacity of the leaves (Fig. 2.7). The decline in the apparent quantum yield when carbohydrate accumulate (Figs. 2.5 and 2.6) is consistent with the reduction in RuBP regeneration capacity. It has been suggested that soluble sugar accumulation may reduce the rate of RuBP regeneration by decreasing available stromal inorganic phosphate, P_i (Herold, 1980; see Section 1.2.2). Very low P_i levels inside the chloroplast can restrict the rates of photophosphorylation and electron transport, probably via a decreased ATP/ADP ratio (Robinson and Walker, 1981). The use of mannose, which sequestrates P_i in isolated chloroplasts and in intact leaf tissues, produces a decline in A which is consistent with this hypothesis (Herold, 1980; Robinson and Walker, 1981).

The faster decline in A in conditions where photorespiration is suppressed, i.e. high CO_2 and low O_2 pressures (see also Milner and Hiesey, 1964) and the reduction in the magnitude of the Warburg effect when carbohydrate accumulated (Fig. 2.9) or when leaf discs were fed

with mannose (Harris *et al.*, 1981), are likely to reflect the limitation of photosynthesis by P_i availability. Carboxylation of RuBP results in a consumption of P_i (which is later released when starch or sucrose is synthesized), but oxygenation does not result in P_i consumption (Usuda and Edwards, 1982). An increase in the carboxylation rate relative to the oxygenation rate produces a rise in the level of phosphorylated compounds (Badger *et al.*, 1983), leading to lower stromal P_i concentration which in turn may result in reduction of the RuBP regeneration capacity, and therefore of the photosynthetic rate.

Carbohydrate accumulation also affected stomatal conductance, g_s, in wheat leaves, especially at high CO_2 pressures. Stomatal conductance initially declined with time more or less in parallel with A, so the intercellular partial pressure of CO_2, p_i, remained constant; however, this proportionality was lost when carbohydrate accumulation was very large (Fig. 2.4, Table 2.1). The decline in A was not always associated with that of g_s, especially when the CO_2 pressure in the air was kept at ambient levels (Fig. 2.1), suggesting that the decline in A preceded that of g_s. Stomatal conductance also decreased with time of the day in leaves of unaltered plants of soybean (Upmeyer and Koller, 1973) and *Panicum virgatum* (Ku *et al.*, 1978), but in both cases the decline in A also seemed to occur earlier. Plants with altered source-sink relations also show accumulation of carbohydrate in the leaves and a decrease in g_s, although the interpretation of this correlation is complicated by the possible variations induced by the artificial manipulations, e.g. depodding, girdling the petiole (Setter *et al.*, 1980).

The increase in the carbohydrate content in wheat leaves not only

decreased g_s but also sensitized stomata to CO_2. It is interesting that this phenomenon has also been observed under several stresses, e.g. water stress (*Xanthium strumarium*, Raschke, 1975; *Phaseolus vulgaris*, von Caemmerer, 1981), chilling (*Xanthium strumarium*, Drake and Raschke, 1974; Raschke *et al.*, 1976) and salinity (mangroves, Ball, 1981). Stomata was more sensitive to CO_2 in the presence of externally applied abscisic acid (ABA) (several species, Raschke, 1975; Dubbe *et al.*, 1978). Interestingly, these authors also observed reductions in the RuBP regeneration capacity of the leaf. Since leaf ABA levels increase in response to water stress, chilling and salinity (Raschke *et al.*, 1976; Itai and Benzioni, 1976; Walton, 1980), it is easy to explain why stomata are more sensitive to CO_2 under the mentioned stresses. As far as I am aware, the effects of *carbohydrate status* on ABA levels are not known. When translocation from soybean leaves is reduced by girdling the petiole or depodding, leaf carbohydrate and free ABA levels increased (Setter *et al.*, 1980). This correlation is interesting but may be casual since as the authors argue, the translocation-obstructing treatments may have produced an accumulation of ABA if the synthesis of this compound remains unaltered. Setter *et al.* (1980) do not support the view that increase in ABA levels are related to carbohydrate accumulation, although the experimental evidence they provide is inconclusive in my opinion. More experiments are needed in the absence of alterations in the source-sink relations.

Other explanations for the effect of carbohydrate build-up on g_s may be possible. The initially parallel declines on A and g_s, due to carbohydrate accumulation, which has been observed in many other situations (Wong *et al.*, 1979) may suggest that stomata respond

directly to the photosynthetic activity (i.e. accumulation of photosynthetic metabolites, perhaps sugars). In this sense, it has been shown that a variety of compounds (including sugars) can be transported from the mesophyll cells to the epidermal cells (Dittrich and Raschke, 1977; Thorpe, 1980). Osmotical effects caused by sugar increases may also affect stomatal aperture (Raschke, 1970).

In summary, decline in A and g_s in wheat leaves under constant environmental conditions appears to be associated with an increase in the leaf carbohydrate level. My results are consistent with a decrease in stromal phosphate availability being responsible for the decline in A. The mechanism(s) for the decline in g_s are even more speculative at this stage although they might involve signals from the mesophyll cells to the stomata (e.g. movements of ABA, sugars, etc.).

Information about the intracellular and intercellular distribution of sugars and phosphate, and about hormonal changes (e.g. ABA, cytokinins) produced by carbohydrate accumulation is necessary to determine the nature of the complex relationships between carbohydrate build-up and photosynthesis and stomatal responses.

CHAPTER 3

THE EFFECT OF CARBOHYDRATE STATUS AND TEMPERATURE ON THE RATE OF DARK CO_2 EFFLUX IN WHEAT LEAVES

3.1 INTRODUCTION

The rate of CO_2 efflux by respiration from single leaves and whole plants in the dark is linearly related to the rate of previous photosynthesis when the latter is varied by changing the light level or CO_2 concentration (McCree, 1970, 1974; Ludwig *et al.*, 1975; Challa, 1976). McCree (1970) fitted an empirical equation in which the rate of dark CO_2 efflux is proportional to photosynthesis and dry weight of living material on the plant. This information served to develop the theoretical concepts of growth and maintenance respiration (Penning de Vries, 1972; Penning de Vries *et al.*, 1974; Thornley, 1977). Both of these components of respiration are thought to involve, principally, carbohydrate oxidation through glycolysis, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle. Growth respiration appears to be less sensitive to temperature than maintenance respiration (McCree, 1974; Ryle *et al.*, 1976; Breeze and Elston, 1978). Explanations of the complex interaction between photosynthesis, temperature and dark respiration are uncertain, although it is probable that the interaction may be mediated by the carbohydrate level (Alberda, 1968; Challa, 1976; Breeze and Elston, 1978).

The experiments described in this chapter investigate the relationship between photosynthesis, its products (particularly

carbohydrates), temperature, and CO_2 efflux by respiration in the dark in mature wheat leaves.

3.2 MATERIALS AND METHODS

3.2.1 General Experimental Conditions

Mature leaves from wheat plants grown in the conditions described in Section 2.2.1 were used. The open gas exchange system used in the experiments reported in this chapter has been described in Appendix I. Several carbohydrate fractions (free glucose plus fructose, sucrose and starch) were measured as described in Section 2.2.6.

3.2.2 Effect of a Period of Photosynthesis on the Rate of CO_2 Efflux in the Dark

A pair of mature wheat leaves from the same plant was enclosed in the photosynthetic chamber and the rate of dark CO_2 efflux in ambient air was monitored for 2 h at the end of the night and after a period of photosynthesis of 6.25 h at ambient CO_2 and O_2 levels. Leaf temperatures were 13.5, 20, 24, 27, and 30 °C in darkness. Leaf temperatures during the light period were 2-4 °C higher than in the dark period. This experiment was repeated three times at every leaf temperature, using a different plant each time.

In a similar experiment, the rate of dark CO_2 efflux was monitored for 1 h at the end of the night and after a period of photosynthesis of 6.25 h during which the oxygen content of the air in the last 20 min was 3%. In this experiment temperature was kept constant in the light and in the dark. In the experiments performed at 30 °C, the O_2 concentration in the dark period was 21 or 3%. Three replicates were done at every O_2 concentration in the dark, but no difference was found in the time course of dark CO_2 efflux after the

light period, and the data were mixed. This experiment was also performed at 20 °C in leaves selected from six plants, but the O_2 concentration in the dark period was 21%.

3.2.3 Relationship Between Dark CO_2 Efflux and Leaf Carbohydrate Status

Mature wheat leaves were allowed to photosynthesize for variable periods of time up to 7 h, at ambient and high (800 μbar) external CO_2 partial pressures; the rate of dark CO_2 efflux was then measured 30 min after the termination of the photosynthetic period. Leaves were immediately killed in liquid nitrogen and stored frozen for carbohydrate determination. Leaf temperature was 21 °C in darkness and 23.5 °C in the light.

3.2.4 Study of the Temperature Dependence of Dark CO_2 Efflux

The rate of dark CO_2 efflux of mature wheat leaves selected at the end of the night was measured at different temperatures up to 40 °C. The first measurement was made at 11 °C. Other leaves were allowed to photosynthesize for 6.25 h at 22 °C, at ambient CO_2 and O_2 levels. Then dark CO_2 efflux was measured at 20 °C, 30 min after the light was switched off; leaf temperature was increased in steps to 42 °C in some experiments, or decreased to 8 °C in other experiments.

3.3 RESULTS

3.3.1 Properties of Dark CO_2 Efflux

Dark CO_2 efflux measured after a period of photosynthesis was much higher than at the end of the preceding night period (Figure 3.1). The curves representing the time-course of dark CO_2 efflux (see also Figure 3.2) are averages of three or six individual curves. The

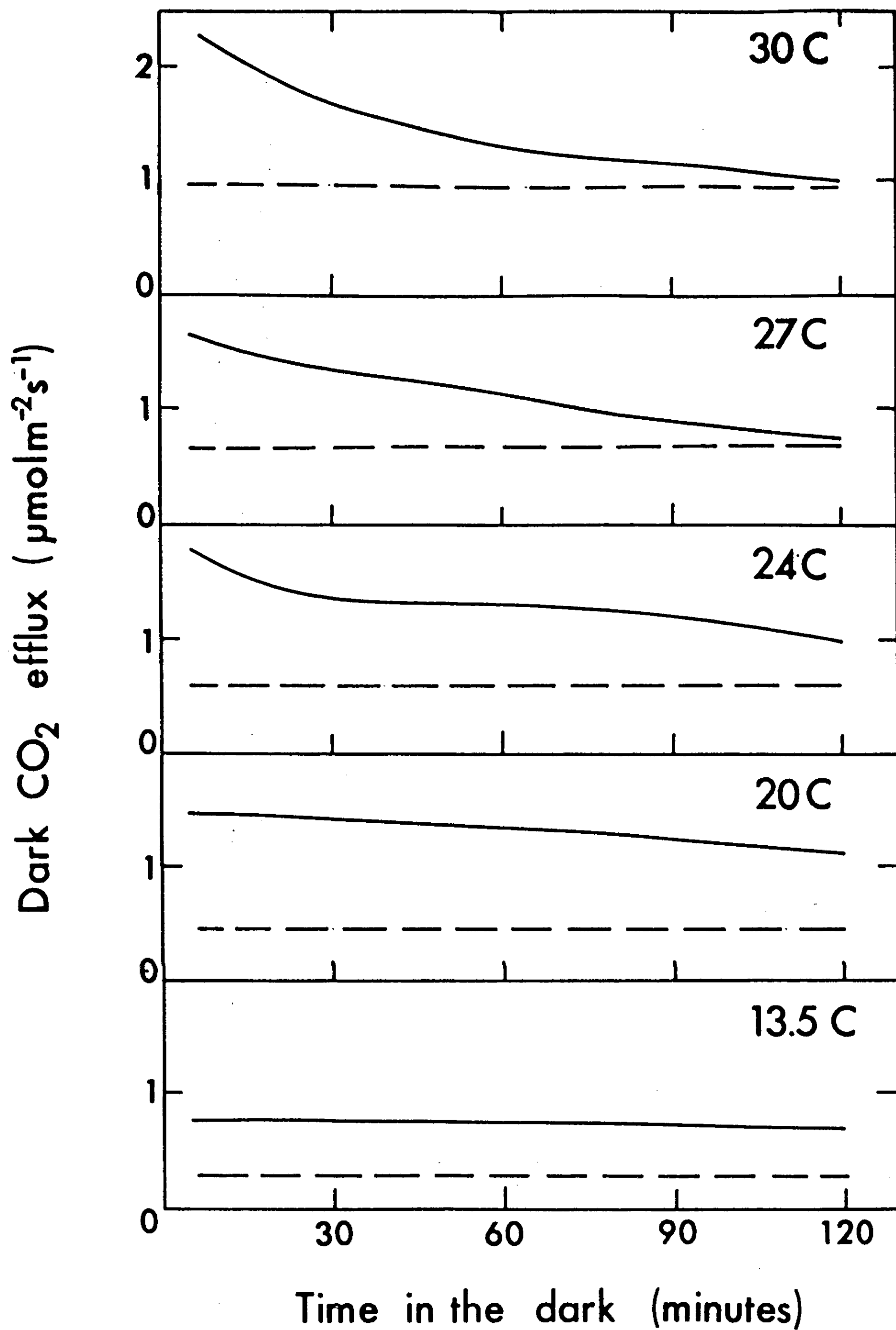

Figure 3.1: Time-course of dark CO_2 efflux of mature wheat leaves after a period of photosynthesis of 6.25 h at ambient CO_2 and O_2 levels (solid lines). The dashed lines correspond to the rate of dark CO_2 efflux at the end of the preceding night period. See Section 3.2.2 for other experimental details.

statistical variation of the data was very small and it is not shown. The standard errors were less than 5% of the absolute values and ranged between 0.01 and 0.08 $\mu mol\ CO_2.m^{-2}.s^{-1}$, the lower values being more common especially at lower temperatures. This increase occurred at all temperatures studied. However, the increase in total dark CO_2 efflux due to the effect of photosynthetic activity was relatively higher at lower temperatures (e.g. 20 °C). At higher temperatures (e.g. 30 °C) the rate of dark CO_2 efflux returned to the level at the end of the night within 2 h. At lower temperatures it took longer (e.g. 5 h at 20 °C). That is, the effect of the photosynthetic activity on dark CO_2 efflux was more accentuated and lasted longer at lower temperatures.

It was commonly found especially at high temperatures (e.g. 30 °C) that the rate of dark CO_2 efflux was higher in the first 30 min after illumination and did not attain a steady slow rate of change until after about 60 min of darkness. When the O_2 concentration of the atmosphere was lowered from 21% to 3% during the last 20 min of the light period and the rate of dark CO_2 efflux measured at 30 °C in either 21 or 3% O_2, a different pattern was obtained (Figure 3.2). The rate of dark CO_2 efflux was lower within 30 min after darkening and attained a slow rate of change after about 10 min of darkness. Similar results were obtained at 20 °C except that the rate of dark CO_2 efflux after a light period in which the O_2 concentration during the last 20 min was 3%, was initially low and increased within 30 min to the level of leaves kept in 21% O_2 throughout the photosynthetic period (Figure 3.2). This suggests that adjustments in the rate of respiration during the light/dark transient take place more rapidly at high temperatures. In all subsequent experiments dark CO_2 efflux rates were measured 30 min after darkening of the leaves.

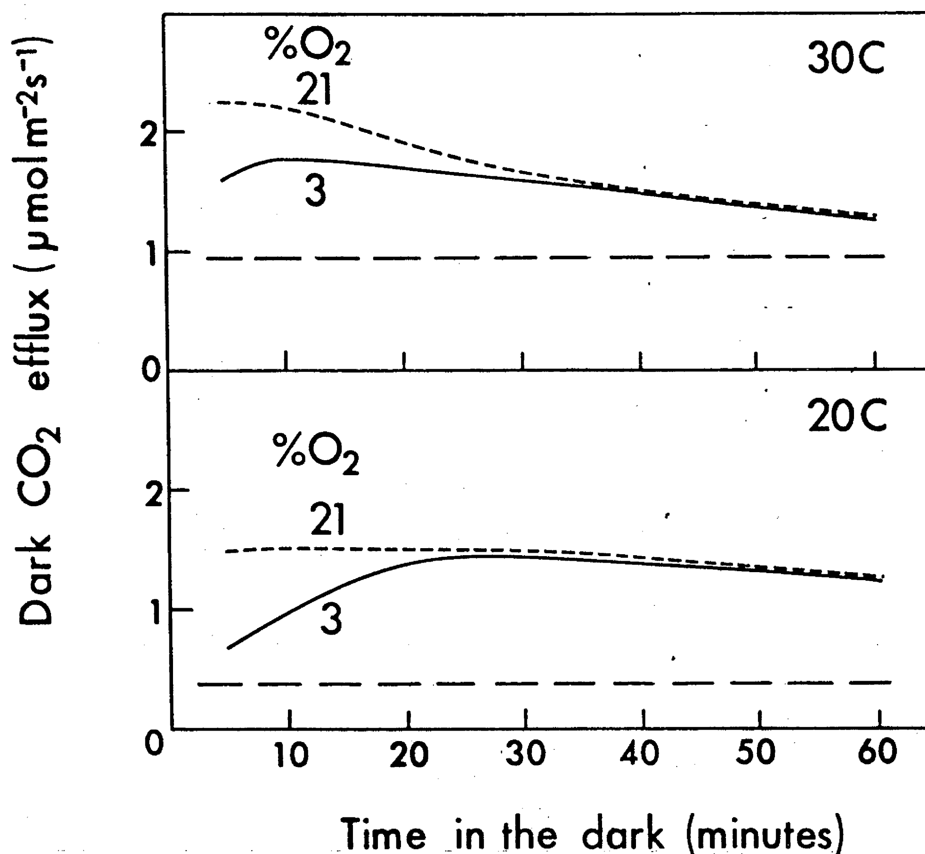

Figure 3.2: Time-course of dark CO_2 efflux of mature wheat leaves after a period of photosynthesis of 6.25 h in which the O_2 concentration in the air in the last 20 min was 3% (solid lines). The time-course of dark CO_2 efflux after a period of 6.25 h of photosynthesis at ambient O_2 concentration (see Figure 3.1) is given for comparison (line of small dashes). The end-of-night level of dark CO_2 efflux is also shown (line of big dashes). See Section 3.2.2 for other experimental details.

Table 3.1

Effect of different photosynthetic pretreatments on the specific leaf weight and total carbohydrate concentration — except fructosans — of mature wheat leaves. Leaf temperature was 21 °C. Mean values are given with standard errors.

	Specific Leaf Weight	Carbohydrate Conc.
	$g.m^{-2}$	g glucose equiv.m^{-2}
At the end of the night	33.8 ± 1.2	0.30 ± 0.13
After 6.25 h of photosynthesis at ambient CO_2 concentrations	40.8 ± 0.4	5.11 ± 0.11
After 5-7 h of photosynthesis at high CO_2 concentration (800 μbar)	44.8 ± 0.9	7.50 ± 0.25

The rate of dark CO_2 efflux 30 min after the termination of the photosynthetic period increased in proportion with the total net CO_2 assimilation which had occurred during this period (Figure 3.3). Dark CO_2 efflux was also positively correlated with specific leaf weight (Figure 3.4) which greatly increased during the light period due to the accumulation of products derived from photosynthesis, mostly carbohydrates (Table 3.1). All measured carbohydrate fractions increased with increased net photosynthesis (see Figure 2.3 of Chapter 2). Dark CO_2 efflux was unspecifically correlated with all measured carbohydrate fractions (Figure 3.5). The relationship between dark CO_2 efflux and fructosans was not investigated here.

3.3.2 Temperature Dependence of Dark CO_2 Efflux

The data from the experiment shown in Figure 3.1 have been used for studying the effect of temperature on the rate of dark CO_2 efflux

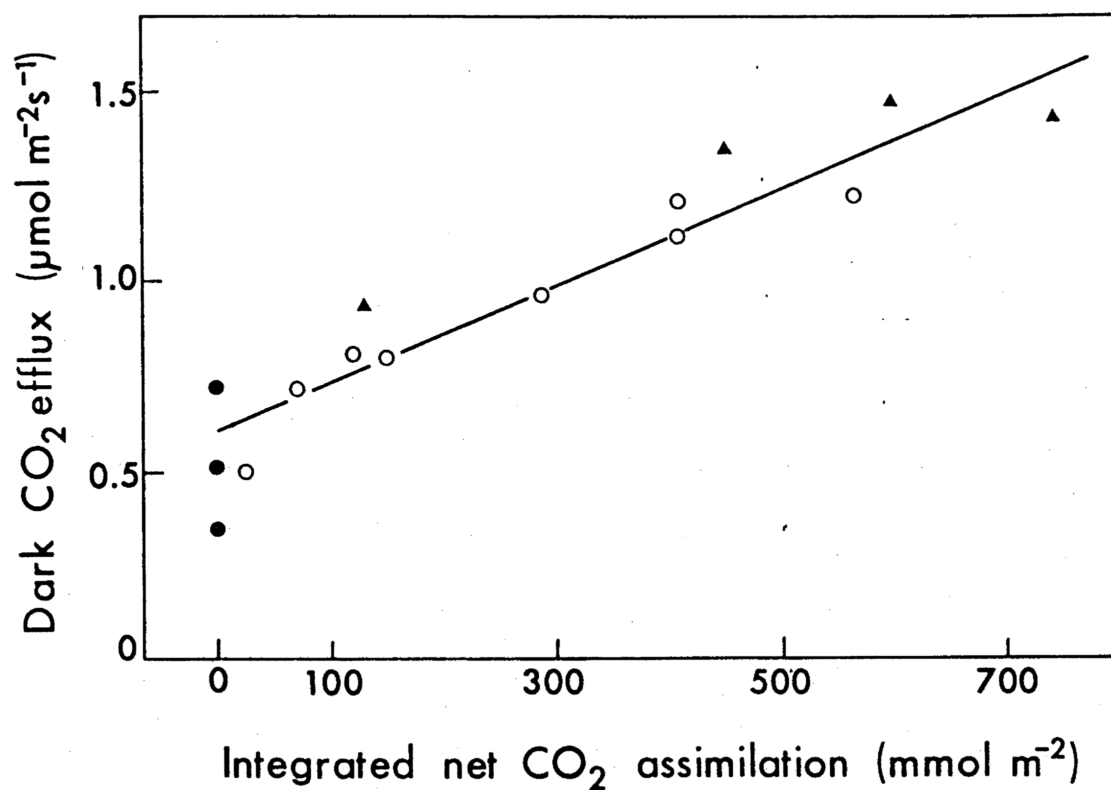

Figure 3.3: Relationship between dark CO_2 efflux and integrated net CO_2 assimilation in mature wheat leaves. Symbols: (●) leaves selected at the end of the night; (○) leaves photosynthesizing at ambient CO_2 levels; (▲) leaves photosynthesizing at 800 μbar CO_2. See Section 3.2.3 for other experimental details.

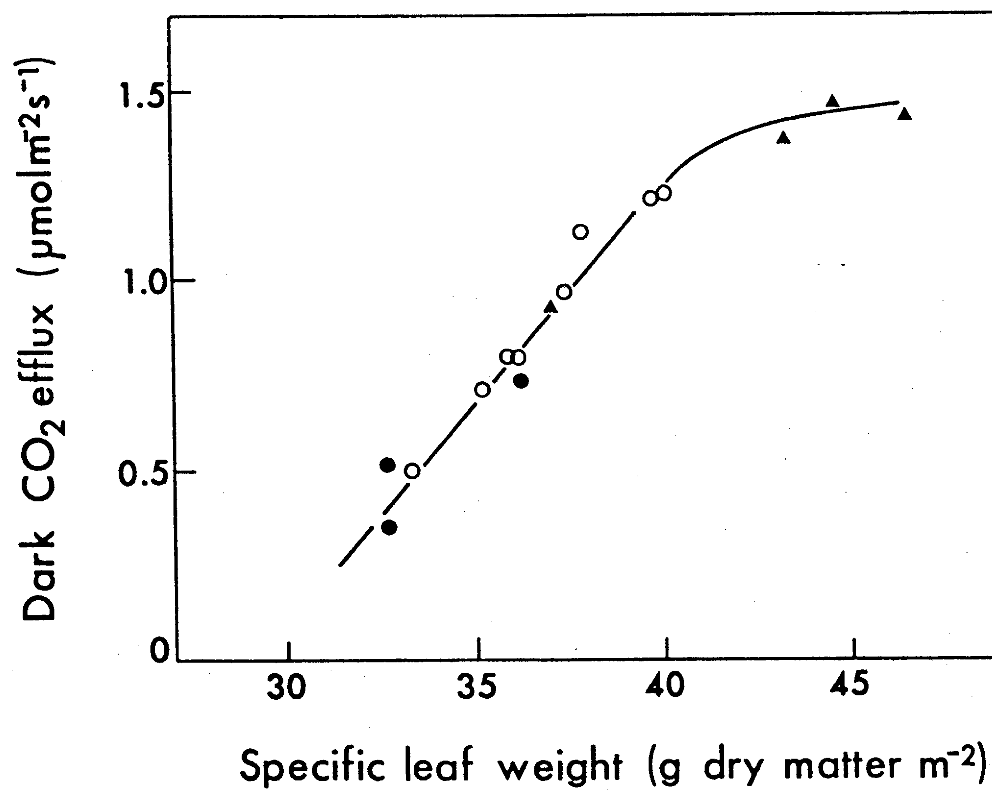

Figure 3.4: Relationship between specific leaf weight and dark CO_2 efflux of mature wheat leaves. Symbols and conditions are the same as in Figure 3.3.

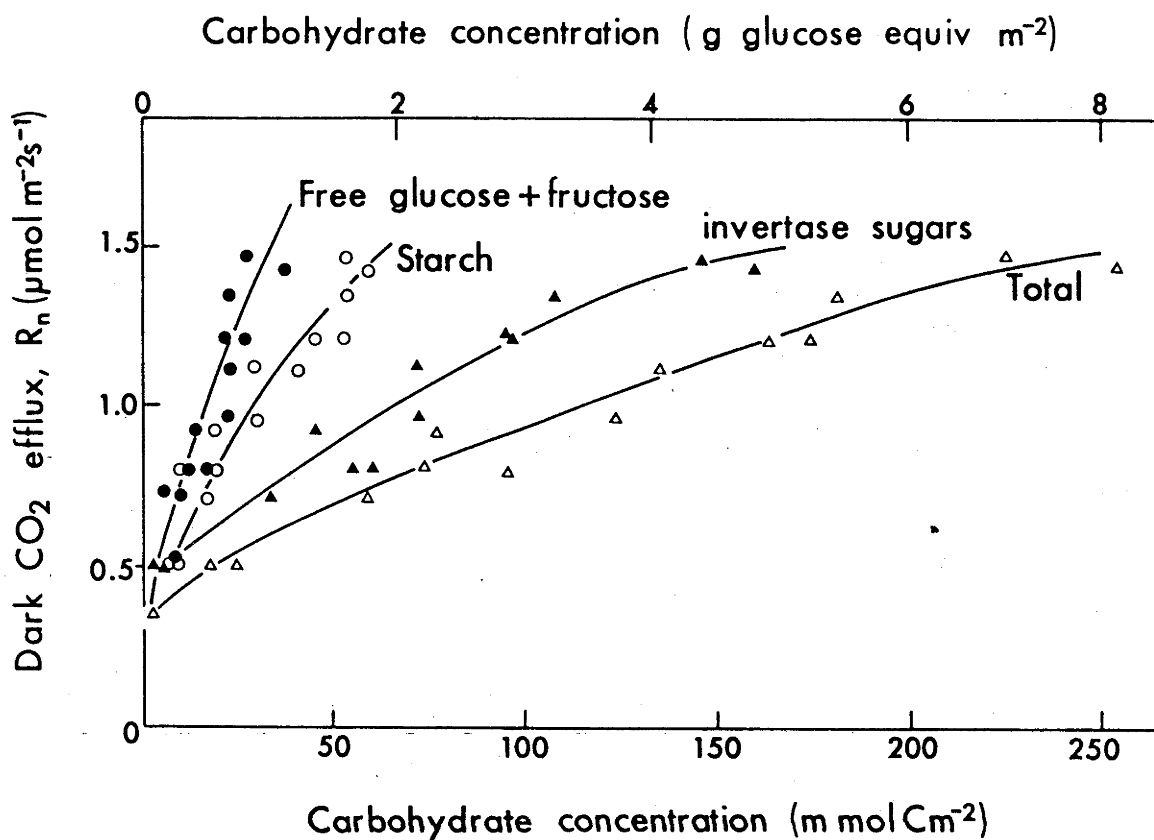

Figure 3.5: Relationship between dark CO_2 efflux and several carbohydrate fractions in mature wheat leaves. See Section 3.2.3 for other experimental details.

at the end of the night and 30 min after the termination of a light period in mature wheat leaves (Figure 3.6), which differed in their carbohydrate content (Table 3.1). The rate of dark CO_2 efflux at the end of the night increased exponentially with temperature with a single apparent activation energy (E_a) of 12.9 $kcal.mol^{-1}$ (which corresponds to a Q_{10} of 2 between 20 and 30 °C). However, dark CO_2 efflux after a light period showed a very irregular pattern in response to temperature, which apparently depended more closely on the 'mean' net CO_2 assimilation rate of the preceding light period (Figure 3.6). This latter parameter did not present a regular dependence with temperature because of slight individual differences in the photosynthetic capacity of the leaves used throughout the experimental period (4 weeks).

These results suggested that dark CO_2 efflux of high carbohydrate leaves is less sensitive to temperature changes than that of low carbohydrate leaves, especially in the range from 20 °C to 30 °C. This suggestion was confirmed in another set of experiments in which the rate of dark CO_2 efflux of a leaf was measured at several temperatures, as described in Section 3.2.4. As previously found, dark CO_2 efflux of end-of-night leaves showed an exponential relationship with temperature (Figure 3.7). However, the rate of dark CO_2 efflux after a period of photosynthesis at ambient CO_2 and O_2 levels was higher at all temperatures but E_a was less in the range from 20 °C to 40 °C and higher in the range from 10 °C to 20 °C (Figure 3.7). E_a in the range from 20 °C to 40 °C was even lower (6.7 $kcal.mol^{-1}$) if the external CO_2 concentration during the light period was 750 μbar.

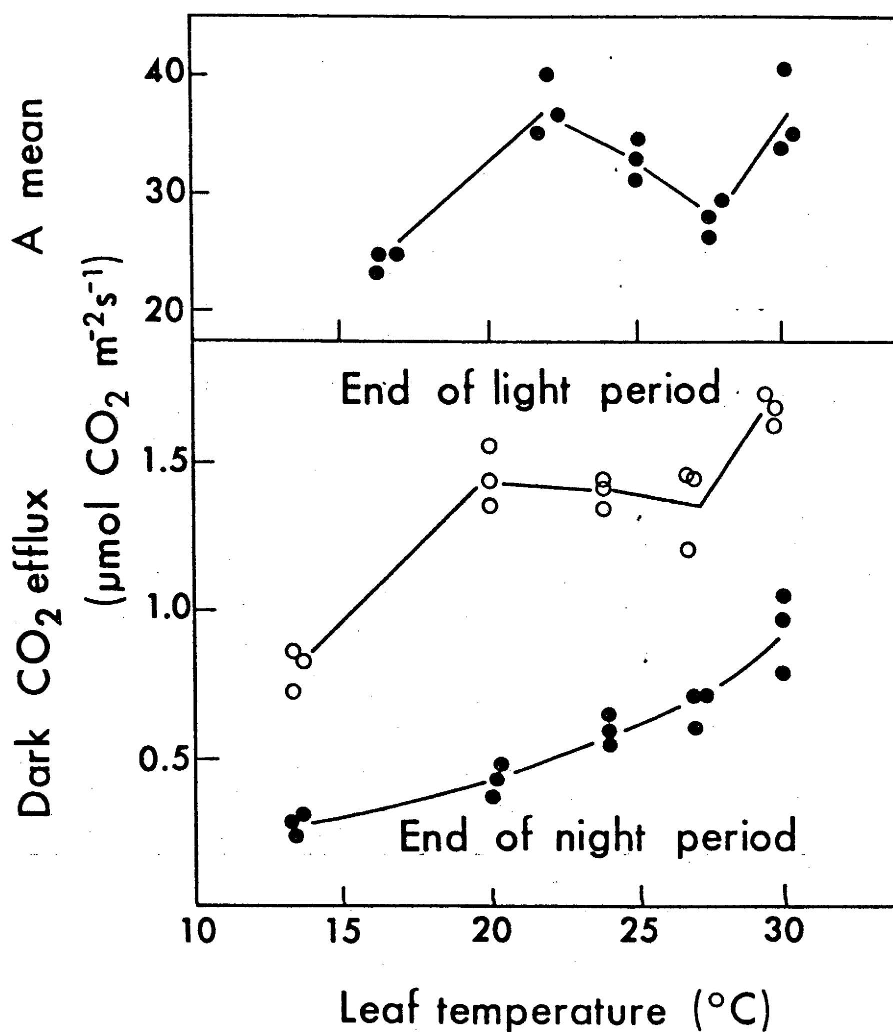

Figure 3.6: Effect of temperature on the rate of dark CO_2 efflux of mature wheat leaves measured at the end of the night and 30 min after the termination of a period of photosynthesis of 6.25 h at ambient CO_2 and O_2 levels. 'Mean' net CO_2 assimilation is also shown. See Section 3.2.2 for other experimental details.

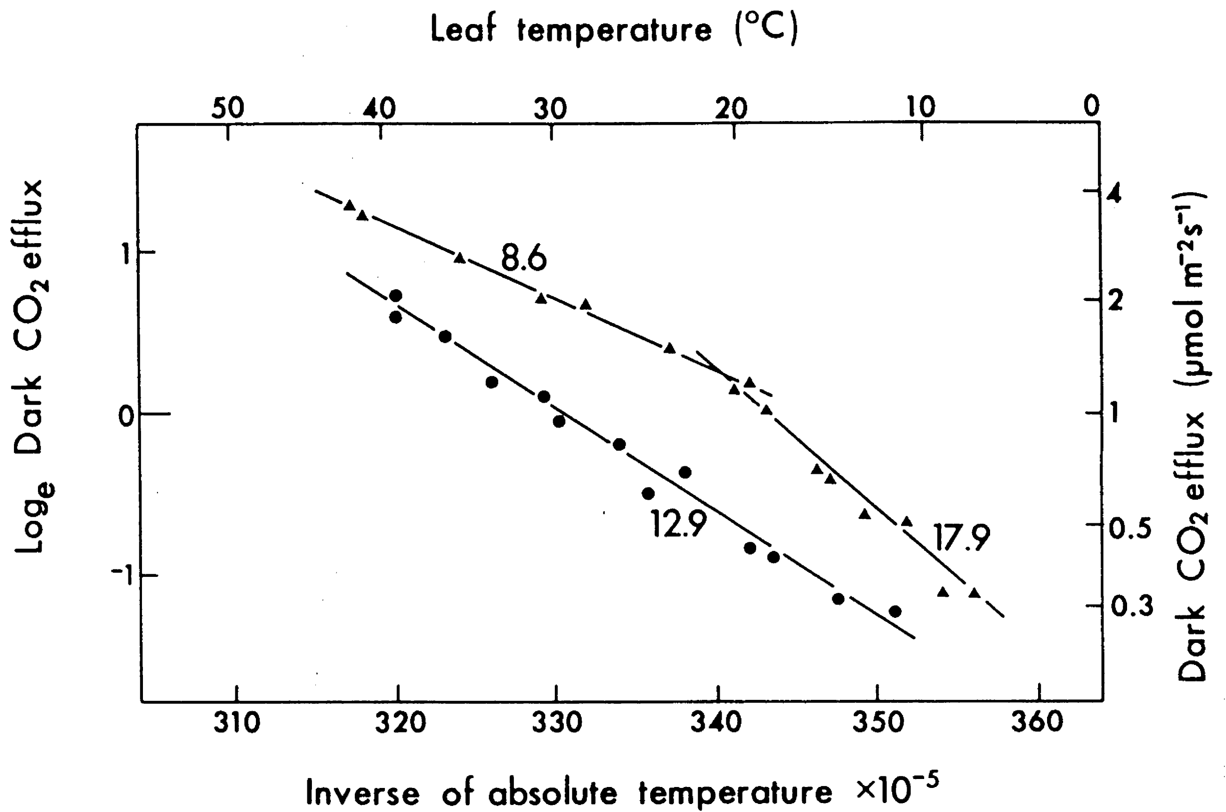

Figure 3.7: Arrhenius plots for dark CO_2 efflux of mature wheat leaves selected at the end of the night period (●) or at the end of a period of photosynthesis of 6.25 h at ambient CO_2 and O_2 pressures (▲). See Section 3.2.4 for experimental details. Apparent activation energies (E_a) are expressed in $kcal.mol^{-1}$. They can be converted to Q_{10} values by using the formula '$\log Q_{10} = 2190.E_a/T_1.T_2$', where $T_1 - T_2 = 10$ K.

3.4 DISCUSSION

Dark CO_2 efflux, R_n, of mature wheat leaves increased considerably after a long period of photosynthesis, as also found in leaves of several species (Stoy, 1965; Ludwig *et al.* 1975; Challa, 1976). At least two groups of substrates contributed to the CO_2 efflux. Approximately 15-20% of the CO_2 evolved in the first 30 min of darkness was abolished if leaves were kept in low oxygen during the latter part of the photosynthetic period. I conclude that this CO_2 arose from photorespiratory substrates. In this sense, the levels of glycine measured in wheat leaves during the light period — 1.5-2 $mmol.m^{-2}$ (M. Berger, personal communication) — are high enough to sustain glycine decarboxylation in the dark for about 30 min at the rates observed in our experiments. This observation contrasts with the idea that the photorespiratory post-illumination burst of CO_2 in leaves is restricted to the first few minutes (2-5) in darkness, as commonly found in many species including wheat (Heichel, 1971a; Doehlert *et al.*, 1979). However, this discrepancy may be explained by the short length of the preceding light period utilized in previous studies (often only a few minutes, i.e. 10-20 min) compared to the present experiments. The variability of the post-illumination burst is also reflected by the fact that it can be eliminated by starving leaves after prolonged darkness (Heichel, 1971a).

The remaining CO_2 efflux, which became the only source of CO_2 after 30 min in the dark, was closely correlated with several carbohydrate fractions. This CO_2 is presumably associated with TCA cycle and pentose phosphate pathway oxidation of carbohydrate derived substrates. A similar correlation between dark CO_2 efflux and carbohydrates has also been found in leaves of *Cucumis sativa* (Challa,

1976), and *Glycine max* (Coggeshall and Hodges, 1980), and also in shoots of *Larrea tridentata* (Cunningham and Syvertsen, 1977), in maize root tips (Saglio and Pradet, 1980) and in whole plants of *Vicia faba*, *Lolium perenne*, *Zea mays* and *Triticum aestivum* (Breeze and Elston, 1978; Penning de Vries *et al.*, 1979). The linear relationship between the rate of photosynthesis and the subsequent rate of dark CO_2 efflux in leaves (see also Ludwig *et al.*, 1975; Challa, 1976) may also be explained in terms of quantitative changes in carbohydrates.

The rate of respiration measured 30 min after darkening the wheat leaves used here showed a positive value in the (extrapolated) absence of carbohydrate accumulation (Figure 3.5). This value may represent maintenance respiration (Penning de Vries *et al.*, 1979). It is unlikely that the enhancement of leaf respiration by carbohydrate accumulation is related to growth respiration because the leaves used here were fully expanded. Nevertheless, this additional respiration might be associated with assimilates in at least two ways. The synthesis of compounds (such as amino acids) in mature leaves for growth in other plant parts, and even the transport of these compounds and sugars may require respiratory energy (Ho and Thornley, 1978). However, respiration increased in leaves and other organs (e.g. wheat stems) when carbohydrate accumulated after treatments (e.g. cooling, lowering sink demand by removing fruits, ears, etc.) which inhibited transport (Birecka, 1968; Birecka *et al.*, 1969; Rook, 1969; Tanaka, 1977; Ho, 1979; Avery *et al.*, 1979). Thus we must also consider the possibility that when carbohydrates accumulate they can be respired without any particular growth, maintenance or other *requirements*.

The temperature dependence of respiration in leaves can also be used to distinguish two types of processes. Respiration of wheat

leaves at the end of the night, which had a very low carbohydrate content in our growth conditions, increased exponentially with temperature. This behaviour is consistent with the temperature dependence of maintenance respiration described by other authors (McCree, 1974; Ryle *et al.*, 1976; Breeze and Elston, 1978). However, when carbohydrates accumulate inside the leaf as a result of the photosynthetic activity, the rate of dark CO_2 efflux increases and the shape of its temperature dependence changes dramatically, showing different apparent activation energies (E_a) above and below 20 °C.

The presence of a break in the Arrhenius plot for dark CO_2 efflux only when the sugar level is high is unlikely to be attributed to membrane phase transitions (Raison, 1980) because mitochondrial respiration is presumably involved in both instances. The mechanism underlying this behaviour may involve the effect of substrate concentration on the temperature dependence of enzymatic reactions. The apparent E_a of an enzymatic reaction decreases at low substrate availability since the K_m of enzymes for their substrates generally increases with temperature (Dixon and Webb, 1979). Therefore, a fixed substrate concentration could be saturating or limiting depending on temperature, and E_a should be consequently affected. In our case, respiration considered as a multi-enzyme system would be saturated by substrates at low temperature after a period of photosynthesis, and its E_a should be very high. However, E_a would decline at higher temperatures as soon as substrates are present at concentrations close to or below the K_m of key enzymes. Respiration in low carbohydrate leaves may have not shown any break because substrates are low to start with ($\leqslant K_m$), and hence K_m increases will not have much effect.

A break in the Arrhenius plot of the cyanide-resistant pathway at

about 17.5 °C in wheat coleoptile mitochondria has been reported (McCaig and Hill, 1977). At higher temperatures the capacity of the alternative pathway declined. Since wheat leaves present an active alternative pathway when their carbohydrate content is high but not when it is low (see Chapter 5) this may also contribute to the temperature dependence of high carbohydrate leaves.

These explanations of the interaction between carbohydrate levels and temperature on respiratory CO_2 efflux assume that there is a direct regulation of respiration by substrate availability. My data suggest that glycolysis and mitochondrial reactions in wheat leaves are not necessarily limited by energy parameters in a very narrow range, at least when substrate levels are low (cf. Beevers, 1974). A similar conclusion was reached by Saglio and Pradet (1980) who have shown that oxygen uptake of maize root tips varied widely in response to sugars while the energy charge remained constant. Atkinson (1977) would say that the fact that energy charge did not change indicates that it is a regulatory parameter. However, in this case, the balance between energy utilization and production is obviously adjusted to meet substrate levels, i.e. somehow energy charge flux responds to substrate availability. It is possible that wheat leaf respiration is regulated in a complex way. Further studies on the nature and regulation of this enhanced respiration will be described in Chapter 5.

In summary, the rate of dark CO_2 efflux from wheat leaves measured 30 min after darkening is correlated with the rate of prior CO_2 assimilation via carbohydrate accumulation. The temperature dependence of dark CO_2 efflux may also be affected by the leaf carbohydrate status.

CHAPTER 4

RELATIONSHIP BETWEEN THE CO_2 AND LIGHT COMPENSATION POINTS AND THE RATE OF CO_2 EFFLUX BY RESPIRATION IN WHEAT LEAVES

4.1 INTRODUCTION

The extent to which respiration occurs in photosynthesizing leaves is uncertain (Kowallik, 1982). Graham (1980) supports the view that the TCA cycle can operate in leaves under steady-state photosynthesis at rates comparable to those in darkness. However, evidence that respiratory CO_2 is released at significant rates from intact illuminated leaves is conflictive. For example, studies in which the specific ^{14}C activity of CO_2 evolved in light and darkness was measured after feeding leaves with $^{14}CO_2$ have been interpreted to show that respiration is suppressed in the light at least 75% (Mangat *et al.*, 1974; Canvin *et al.*, 1976), or as that no inhibition occurs (Ludwig and Canvin, 1971).

The CO_2 compensation point, Γ, is that CO_2 concentration at which the net rate of assimilation of CO_2 is zero. Γ can be used for estimating the rate of CO_2 production by respiration in the light because this parameter reflects a balance between the photosynthetic carboxylation capacity and the total CO_2 production in the light (Azcón-Bieto *et al.*, 1981). This balance can be expressed in mathematical terms (Peisker and Apel, 1980; Tenhunen *et al.*, 1980; Farquhar and von Caemmerer, 1982). Peisker and Apel (1980) studied the relationship between the rate of CO_2 evolution in darkness, R_n,

and the oxygen dependencies of Γ and the carboxylation resistance in wheat leaves and they concluded that respiration is inhibited in the light by about 70%. Peisker *et al.* (1981) observed a relationship between Γ and R_n during the ontogeny of bean leaves. They concluded that CO_2 evolution by leaf respiration was inhibited by about 40-50% at the CO_2 compensation point. Holmgren and Jarvis (1967) reported that Γ of leaves of *Rumex acetosa* decreased after a period of 2 h in darkness; this could reflect a change in the rate of respiration in the light.

The linear relationship between Γ and O_2 concentration frequently extrapolates to values of Γ of zero and this has been used to argue against the operation of sources other than photorespiration in the light (R_d); however, finite values of Γ in the (extrapolated) absence of O_2 have been found in many other cases (see Azcón-Bieto *et al.*, 1981, for a review). The value of this extrapolation probably depends on the ratio between the rate of respiration in the light and the maximal velocity of leaf carboxylation, $R_d/V_{c_{max}}$, rather than on R_d alone, as discussed by Azcón-Bieto *et al.* (1981). These latter authors also concluded that R_d was a significant component of Γ in *Lolium perenne* although no quantification was attempted. Marked seasonal variations of Γ were also interpreted in terms of changes of R_d (Azcón-Bieto *et al.*, 1981).

A new approach for studying the problem has been made in this chapter. As shown in Chapter 3 the rate of CO_2 efflux in darkness, R_n, after a period of photosynthesis, varied in the same leaf. I used this treatment to examine whether the CO_2 and light compensation points responded to varying rates of respiratory CO_2 output.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material and Gas Exchange Apparatus

Triticum aestivum, *Eucalyptus grandis*, *Vicia faba* and *Lolium perenne* plants were grown as described in Section 2.2.1. The open system used for measuring the gas exchanges of leaves has been described in Appendix I.

4.2.2 Measurement of the CO_2 and Light Compensation Points

The CO_2 compensation point, Γ, was either measured by using a closed system and allowing the leaf to equilibrate with its CO_2 atmosphere, or by interpolation of a curve of net CO_2 assimilation vs intercellular CO_2 partial pressure (A vs p_i) to zero assimilation (Figure 4.1). It was noticeable that the initial part of this curve was not straight at both O_2 concentrations, even turning backwards at very low intercellular CO_2 pressures. This phenomenon will be discussed in Section 4.3.2. The gas exchange apparatus was modified by the inclusion of a closed system. A metal bellows pump (Metal Bellows Corp., model MB-21E, Sharon, Massachusetts, U.S.A.) circulated the air through the system. Plug valves (Nupro Co., model B-4P4T, Cleveland, Ohio, U.S.A.) were used to manually switch from open to closed system or vice-versa. Both methods were compared in the same leaf of wheat at two O_2 concentrations (21% and 2.5%) yielding identical results (Table 4.1). In the experiments where Γ was measured in closed system, its value was taken after 60 min in order to obtain perfect steady-state values; then, the rate of dark CO_2 efflux was measured 30 min after the light was switched off, in order to avoid interference with photorespiratory substrates (see Chapter 3).

The light compensation point was measured by interpolation of a

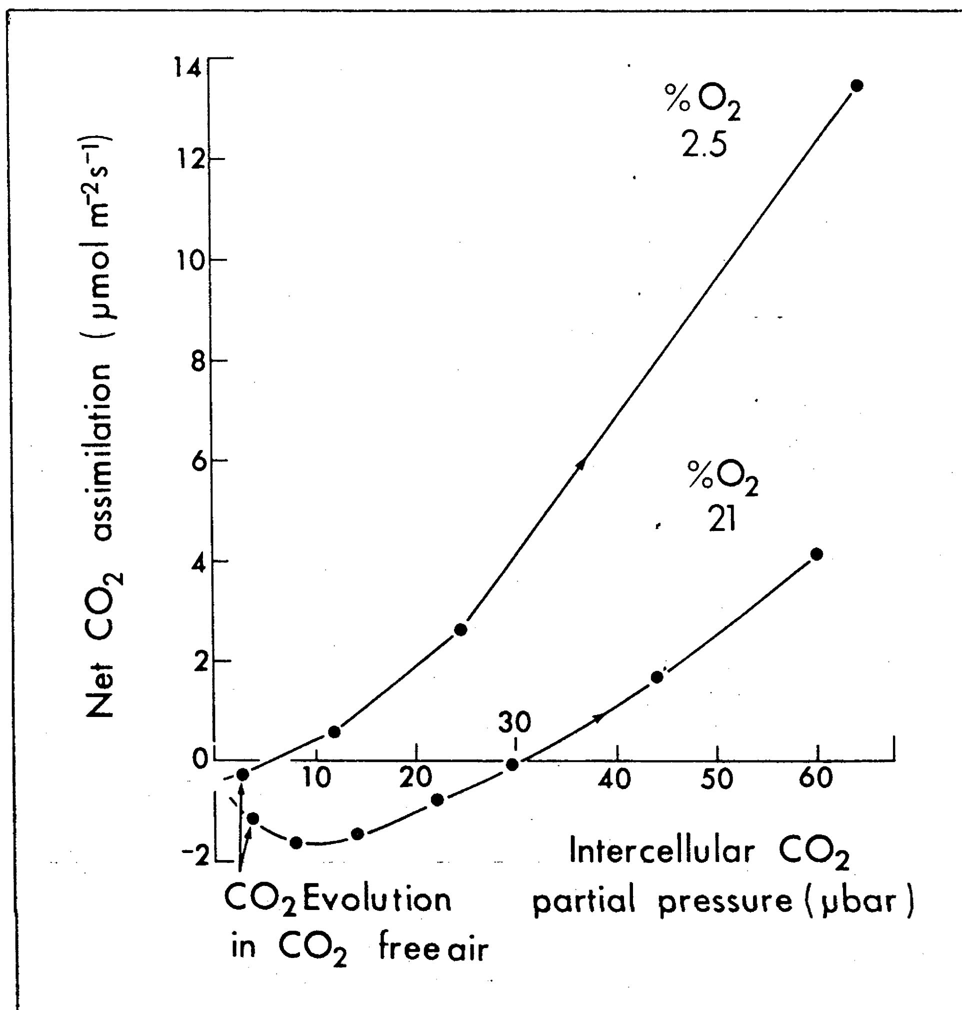

Figure 4.1: Curves of net CO_2 assimilation vs intercellular CO_2 partial pressure of mature wheat leaves at two O_2 concentrations. Leaf temperature was 20 °C and quantum flux was 1000 µE m^{-2} s^{-1}. Leaves were selected at the end of the night period. The arrows indicate the direction in which measurements proceeded. This experiment was performed three times, but only a typical example is shown.

Table 4.1

Comparison of two methods for measuring the CO_2 compensation point, Γ, in mature wheat leaves at two O_2 concentrations.
A. Interpolation of a curve of net CO_2 assimilation vs intercellular CO_2 partial pressure to zero assimilation. B. Closed system. Leaf temperature was 20 °C and irradiance was 1000 μE m^{-2} s^{-1}. Leaves were selected at the end of the night period.

% O_2	CO_2 compensation point	
	A	B
	μbar	
	30	30
21	30	31
	31	31
	6	5.5
2.5	6	6.5
	6	5.5

curve of 'net CO_2 assimilation vs quantum flux' to zero assimilation. Light intensity was changed by interposing copper screens.

4.3 RESULTS

4.3.1 Relationship Between Dark CO_2 Efflux, Carbohydrate Status, and CO_2 and Light Compensation Points

The CO_2 compensation point, Γ, in 21% O_2 increased coincidently with the rate of respiration following a period of photosynthesis (see Chapter 3, Table 4.2, Figure 4.2). Interestingly the values for Γ showed the largest increases after treatments in which photo-respiration would have been least, but the rate of carbohydrate formation would have been maximal (800 μbar CO_2, 2% O_2). These

Table 4.2

Γ and dark CO_2 efflux, R_n, measured at the end of the night and after a period of photosynthesis of 5 h in the same leaf of wheat. Γ was measured in closed system (see Section 4.2.2). Γ and R_n were measured in 21% O_2. Leaf temperature was 21 °C in the light and in the dark. Net CO_2 assimilation rates were about 24 (A) and 30 (B) µmol $CO_2.m^{-2}.s^{-1}$. Quantum flux was 1000 µE $m^{-2}.s^{-1}$. The values shown are means ± standard errors of 3-4 independent experiments.

CO_2 and O_2 levels during the photosynthetic period	At the end of the night		After 5 h light	
	Γ	R_n	Γ	R_n
	µbar	µmol $CO_2.m^{-2}.s^{-1}$	µbar	µmol $CO_2.m^{-2}.s^{-1}$
A. 370 µbar CO_2, 21% O_2	35 ± 1.5	0.65 ± 0.12	38 ± 0.5	0.94 ± 0.04
B. 800 µbar CO_2, 21% O_2 or 800 µbar CO_2, 2% O_2	36.5 ± 2.5	0.62 ± 0.03	42 ± 1.5	1.18 ± 0.16

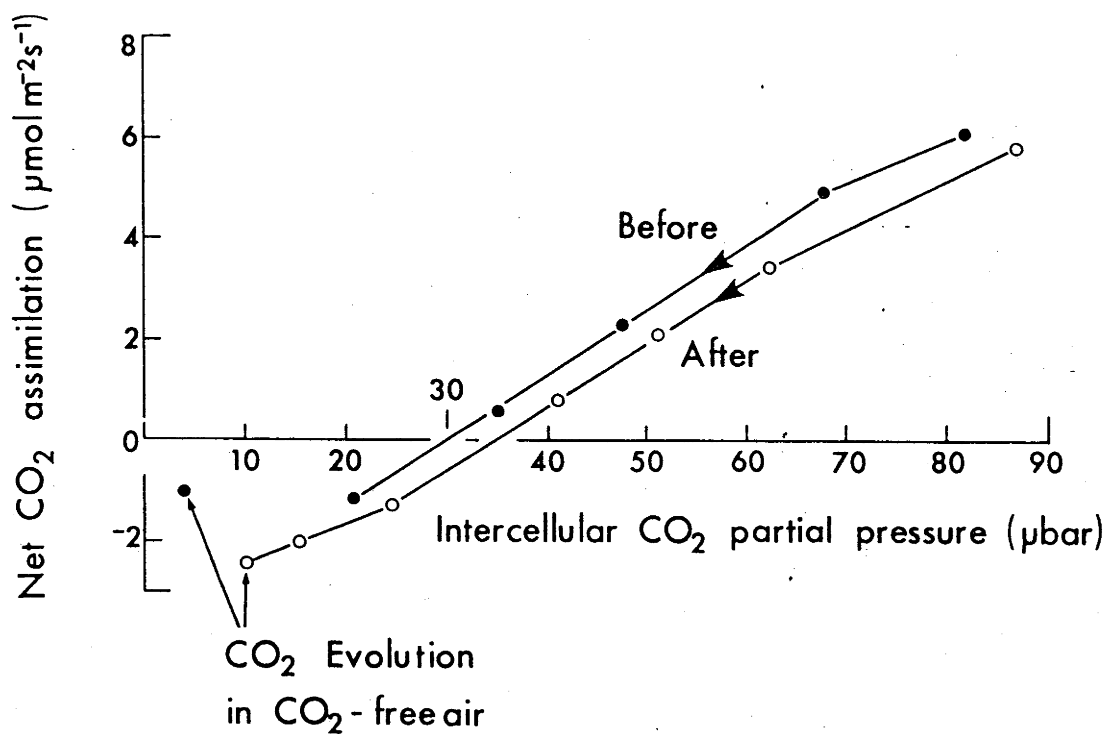

Figure 4.2: Curve of net CO_2 assimilation vs intercellular CO_2 partial pressure measured at the end of the night and after a period of photosynthesis of 3 h at 800 μbar CO_2 in the same leaf of wheat. Leaf temperature was 21 °C. Measurements proceeded from high to low CO_2 partial pressures, as indicated by the arrows.

correlations were confirmed in another set of experiments in which a leaf was initially illuminated for 4 h in air containing 750 μbar CO_2, 21% O_2 (low photorespiration), then in air containing low CO_2 pressures (slightly above Γ) for a second period of 4 h (high photorespiration). Table 4.3 shows again that Γ was higher after the period in which the rates of photorespiration were lower and the rates of carbohydrate formation were higher.

Measurements of Γ from many experiments in which dark CO_2 efflux, R_n, was varied by varying CO_2 and O_2 partial pressures during the period of photosynthesis are shown in Figure 4.3. Extrapolation of this relationship to zero R_n presumably yields the photorespiratory component of Γ in these mature wheat leaves.

The correlation between an increase in Γ in 21% O_2 and R_n following a period of photosynthesis was also observed at temperatures other than 21 °C (e.g. 15 °C and 30 °C) (Table 4.4) and in other species

Table 4.3

Γ and R_n of a mature wheat leaf measured after two consecutive periods of photosynthesis of 4 h at high and low CO_2 partial pressures. O_2 concentration was 21%. The rates of net CO_2 assimilation during the first (A) and second (B) periods were about 23 and 2 μmol $CO_2.m^{-2}.s^{-1}$, respectively. Two independent experiments were performed. The rest of the conditions were the same as in Table 4.2.

CO_2 partial pressure during the photosynthetic period	Γ	R_n
	μbar	μmol $CO_2.m^{-2}.s^{-1}$
A. 750 μbar CO_2	43 ± 1	1.48 ± 0.02
B. 50-75 μbar CO_2	37 ± 2	0.80 ± 0.05

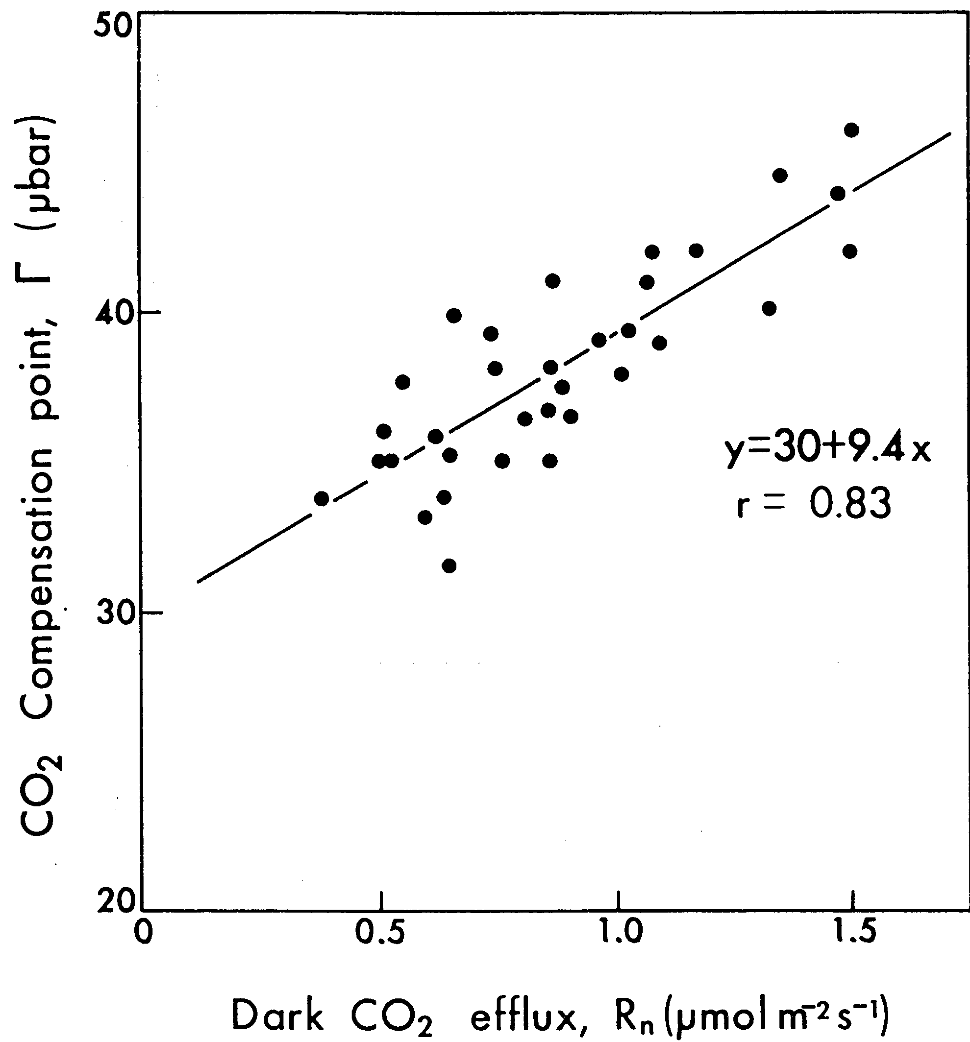

Figure 4.3: Relationship between the CO_2 compensation point and dark CO_2 efflux in mature wheat leaves at 21 °C.

Table 4.4

Γ and R_n of a mature wheat leaf measured at the end of the night and after a period of photosynthesis of 5 h at 800 μbar CO_2, 21% O_2. The rates of net CO_2 assimilation were about 15 and 26 μmol CO_2 $m^{-2}.s^{-1}$ at 15 °C and 30 °C, respectively. The values shown are means ± standard errors of three independent experiments. See Table 4.2 for other experimental conditions.

Leaf Temperature	At the end of the night Γ	R_n	After 5 h in the light Γ	R_n
°C	μbar	μmol $CO_2.m^{-2}.s^{-1}$	μbar	μmol $CO_2.m^{-2}.s^{-1}$
15	33 ± 0.5	0.45 ± 0.03	38 ± 2.0	0.59 ± 0.06
30	50 ± 1.0	1.20 ± 0.13	53 ± 1.0	1.52 ± 0.13

(Table 4.5). However this correlation did not occur when Γ was measured at 2% O_2 (Table 4.6). The increase in Γ in 21% O_2 following a period of photosynthesis was reflected in a decrease in net rate of photosynthesis over a range of CO_2 partial pressures and was not due to a change in the slope of the curve of net CO_2 assimilation vs intercellular CO_2 partial pressure (Figure 4.2, see also Chapter 2). The displacement of this curve was 1.0 ± 0.2 μmol $CO_2.m^{-2}.s^{-1}$, which is an average value obtained in four experiments including that shown in Figure 4.2 (see also Chapter 2). This value compares well with the increase in the rate of dark CO_2 efflux observed after a period of photosynthesis. The rate of CO_2 efflux into CO_2-free air in the light was also higher following a period of photosynthesis (Figure 4.2).

The light compensation point also increased in the same leaf after a period of active photosynthesis (Figure 4.4, see also Chapter 2). Figure 4.5 shows the correlation between the light compensation

Table 4.5

Γ and R_n of mature leaves of several plant species measured at the end of the night and after a period of photosynthesis of 5 h. Temperature was 21 °C. A is the rate of net CO_2 assimilation. The rest of the conditions were the same as in Table 4.2.

Species	CO_2 pressures during the photosynthetic period	A	At the end of the night		After 5 h in the light	
			Γ	R_n	Γ	R_n
	μbar	μmol $CO_2.m^{-2}.s^{-1}$	μbar	μmol $CO_2.m^{-2}.s^{-1}$	μbar	μmol $CO_2.m^{-2}.s^{-1}$
Eucalyptus grandis	340	13	40.5	0.82	45	1.07
	800	17	39	1.20	45	1.50
Vicia faba	800	21	38	0.77	41	1.05
Lolium perenne	800	22	34	0.65	38	0.87

Table 4.6

Γ (measured at 2% O_2) and R_n (measured in ambient air) measured at the end of the night and after a period of photosynthesis of 4 h in the same leaf of wheat. Leaf temperature was 21 °C. CO_2 partial pressures during the light period were either 330 µbar or 800 µbar, but they did not make any difference to the results. The values shown are averages of three independent experiments. For other experimental details see Table 4.2.

	Γ	R_n
	µbar	µmol $CO_2.m^{-2}.s^{-1}$
At the end of the night	6 ± 0.3	0.51 ± 0.01
After 4 h in the light	5 ± 0.2	0.95 ± 0.12

point and dark CO_2 efflux which was varied by the period of prior photosynthesis under different conditions of temperature and CO_2 partial pressures. The displacement of the curve of net CO_2 assimilation vs quantum flux following a period of photosynthesis was not exclusively accounted for by variations in the rate of dark CO_2 efflux (Figure 4.4). The carbohydrate status can also affect the slope of this curve, as discussed in Chapter 2. The correlation between the light compensation point and dark CO_2 efflux shown in Figure 4.5 is even improved if the variation due to the changes in the slope of the curve of net CO_2 assimilation vs quantum flux is taken into account. It was noticeable that the relationship between the rate of photosynthesis and light intensity deviated from linearity near the light compensation point (Figure 4.4, but see also Figure 2.5). This phenomenon, firstly described by Kok (1949) in unicellular algae is known as the 'Kok effect'. Figure 4.6 shows the dependence

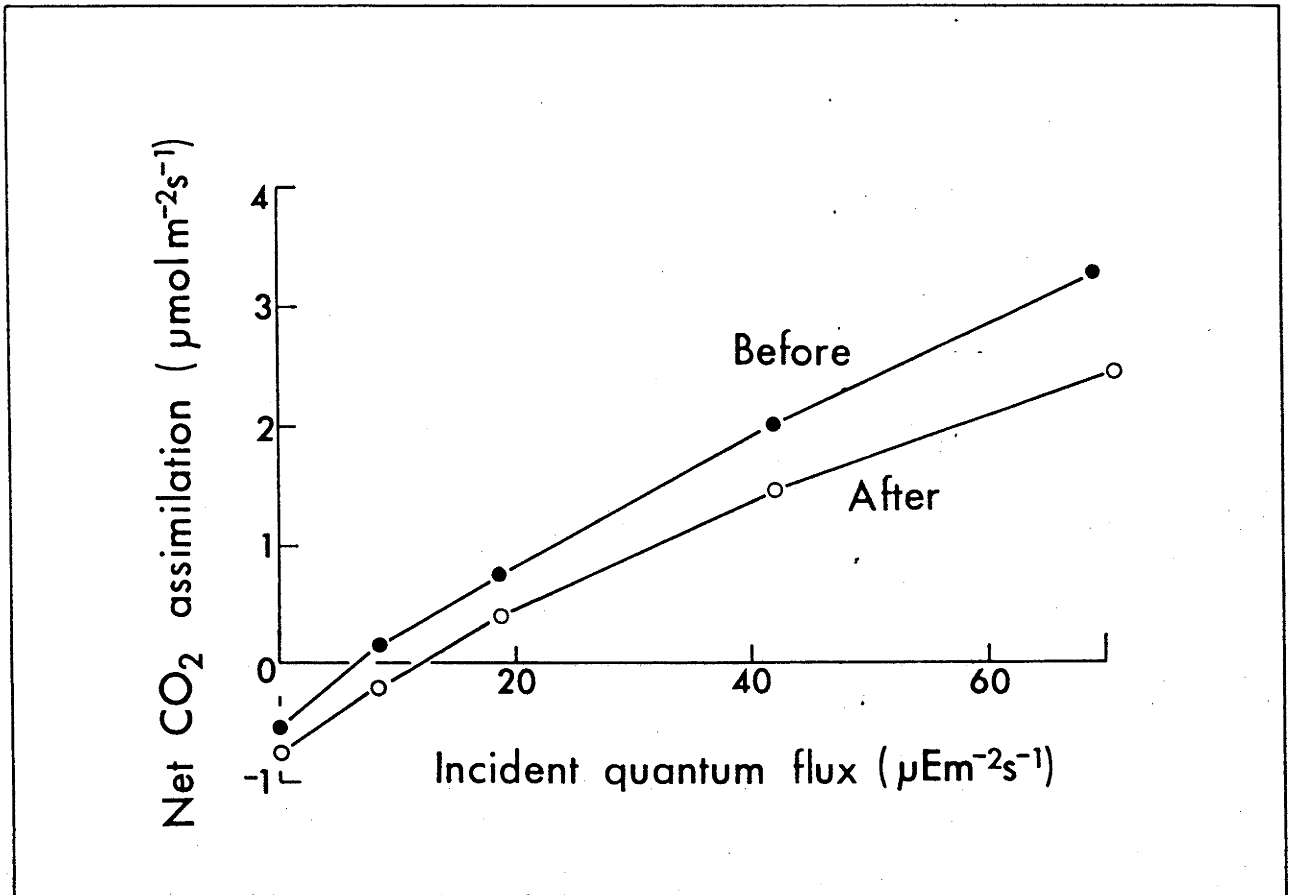

Figure 4.4: Initial part of the curve of net CO_2 assimilation vs quantum flux of a mature wheat leaf, measured before and after a period of photosynthesis of 4 h at ambient CO_2 pressures and saturating quantum flux (1000 μE m^{-2} s^{-1}). Leaf temperature was 20 °C. The leaf was initially selected at the end of the night period.

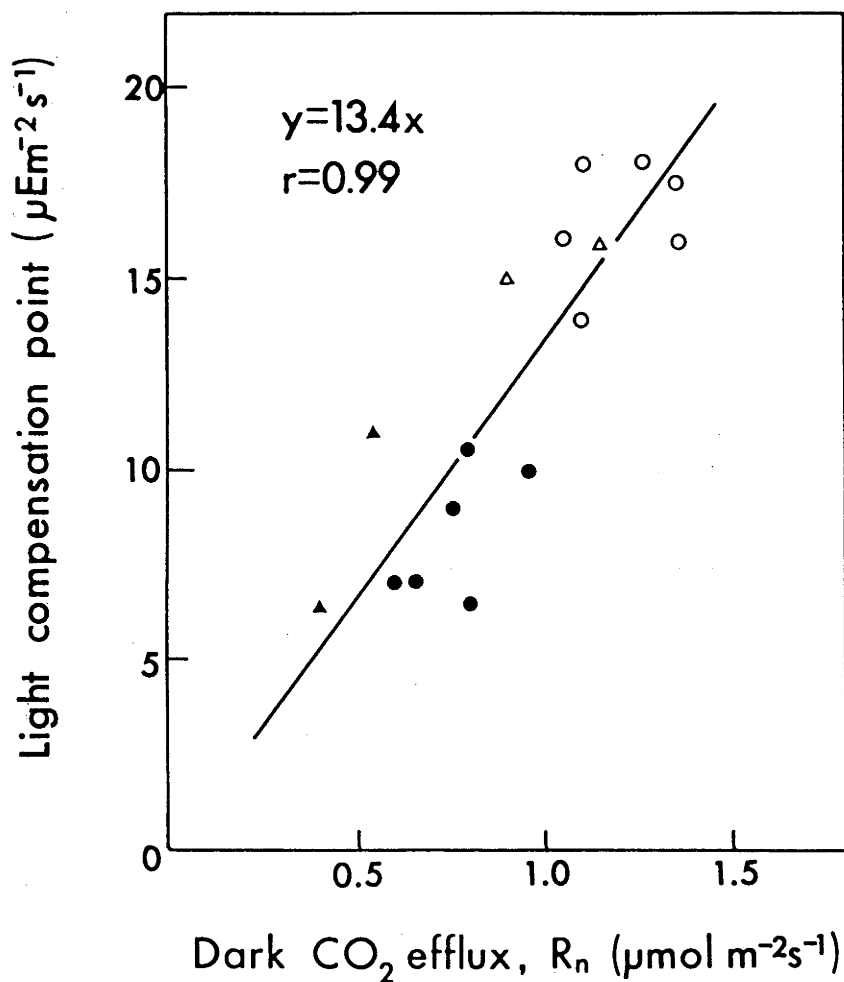

Figure 4.5: Relationship between the light compensation point and dark CO_2 efflux in mature wheat leaves. Symbols: (●) leaf temperature, 20 °C, external CO_2 partial pressure, 330 μbar; (○) 30 °C, 330 μbar CO_2; (▲) 20 °C, 640 μbar CO_2; (△) 30 °C, 640 μbar CO_2.

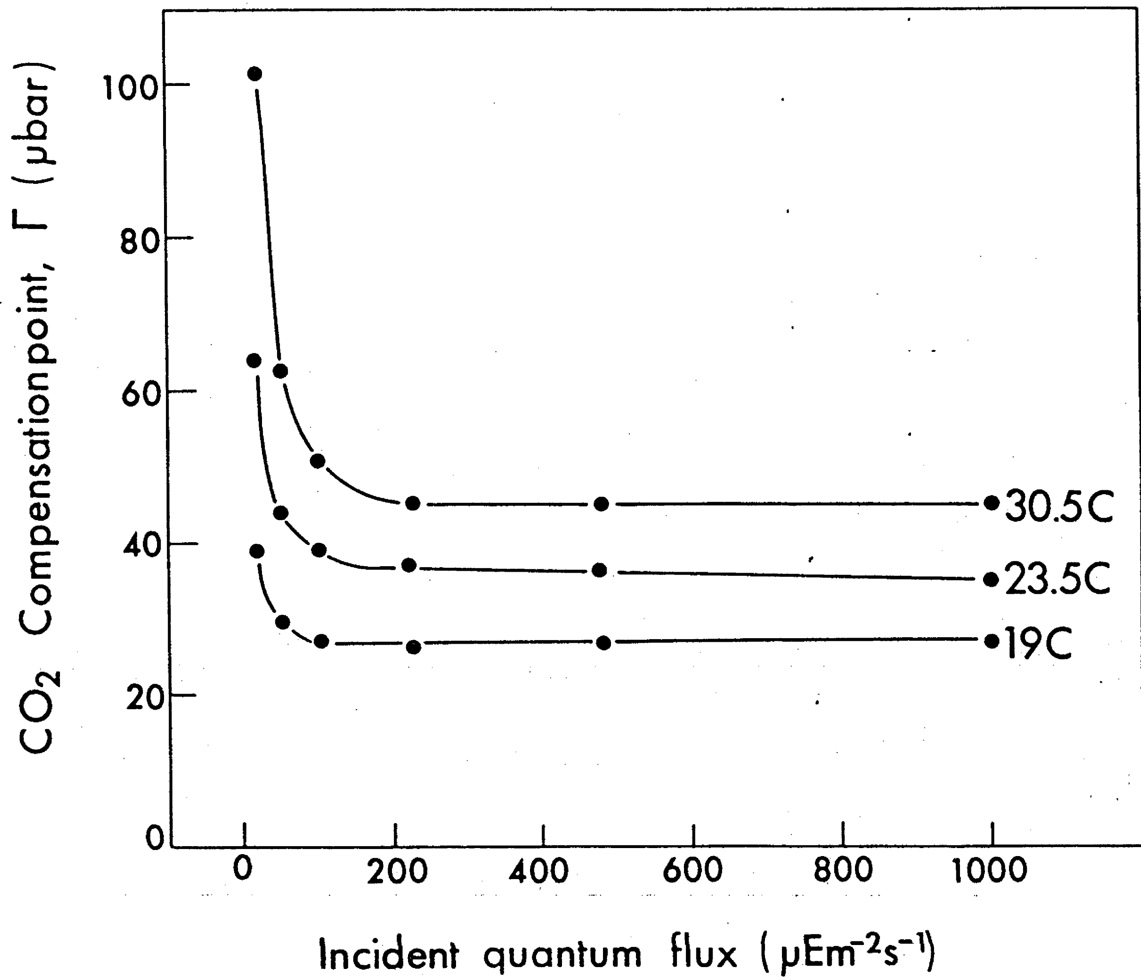

Figure 4.6: Effect of decreasing quantum flux on Γ of mature wheat leaves at three temperatures. Quantum flux was varied by interposing copper screens; leaf temperature was subsequently adjusted.

of the CO_2 compensation point on the quantum flux at three temperatures.

4.3.2 Analysis of the Response of Net CO_2 Assimilation (A) to Low Intercellular Partial Pressures of CO_2 (p_i) in Wheat Leaves

It was a common observation that the slope of the 'A vs p_i' curve deviated from linearity at internal CO_2 pressures near to or below Γ, even turning backwards at very low p_i values (Figures 4.1 and 4.2). This phenomenon has been also observed by other authors in wheat (Meidner, 1970; Doehlert *et al.*, 1979) and in other species (Holmgren and Jarvis, 1967; Meidner, 1970), and it can be explained by considering that the enzyme RuBP carboxylase oxygenase may be not fully activated in the leaf and that RuBP levels may be limiting at very low internal CO_2 pressures (Farquhar and Sharkey, 1982; Badger *et al.*, 1983; Farquhar and von Caemmerer, 1982).

4.4 DISCUSSION

4.4.1 The CO_2 Compensation Point and Respiration

The CO_2 compensation point in 21% O_2 of mature leaves of wheat and other C_3 species varied during the photoperiod, its value being low at the end of the night period, but increasing during the day period. Similar changes of Γ after periods of light or darkness have been reported in leaves of wheat (Peisker and Apel, 1980) and *Rumex acetosa* (Holmgren and Jarvis, 1967). In contrast to these results, Γ did not vary after prolonged exposure to darkness leading to starvation in leaves of *Nicotiana tabacum* (Heichel, 1971a).

In order to investigate the nature of the changes of Γ in wheat leaves, the CO_2 and O_2 partial pressures in the atmosphere were varied

during the photosynthetic period with the object of having different rates of photorespiration and photosynthetic sugar formation. The variations in Γ during the photoperiod are principally related to processes other than photorespiration, and presumably associated with respiration, because Γ and R_n increased maximally after periods in which the gas composition of the air favoured high rates of photosynthetic carbohydrate formation and minimal rates of photorespiration (low O_2 and high CO_2 pressures). Conversely, Γ and R_n were low following a period in which the rate of photorespiration was maximal and the rate of carbohydrate synthesis was very reduced (low CO_2 and ambient O_2 pressures). This conclusion is further supported by the strong correlation found between Γ in 21% O_2 and R_n (Figure 4.3). It appears from this relationship that Γ has a positive value when R_n is zero, which presumably reflects its photorespiratory component.

If a value for R_n of 1 μmol $CO_2.m^{-2}.s^{-1}$ is chosen, which is an average value at 21 °C, the contribution of day respiration, the CO_2 efflux by respiration in the light, R_d, to Γ of mature wheat leaves is about 25%. We have also concluded in an earlier paper (Azcón-Bieto *et al.*, 1981) that R_d is a significant component of Γ in *Lolium perenne*. However, the contribution of R_d to Γ is variable, and it is correlated with the carbohydrate level. This conclusion is consistent with the fact that externally added sugars increase the CO_2 compensation point and the rate of respiration of leaves (Tetley and Thimann, 1974; Smith *et al.*, 1976; see Chapter 5).

The rate of respiration in the light, R_d, can be estimated from the displacement on the curve of net CO_2 assimilation vs intercellular CO_2 partial pressure by varying the rate of respiration in the dark, R_n, through changes in the leaf carbohydrate concentration. It can be

concluded from the results obtained that R_d and R_n are comparable in wheat leaves.

In contrast to my results, Peisker and Apel (1980) analysed the responses of Γ, its oxygen dependence, and respiration in the dark after a dark period and after an extended light period (18 h) at high CO_2 concentrations in wheat leaves, and they concluded that respiration is inhibited by about 70% in the light. For calculating this number they combined the data obtained after both treatments. However, the same analysis would have yielded different results if applied to the data from either of the single treátments considered separately, which suggests that different populations of leaves were probably involved in both cases.

Graham (1980) reviewed the literature and concluded that glycolysis and TCA cycle can operate in illuminated green cells although some modifications probably occur in relation to the dark pattern (see Section 1.3.2.2). This is suggested by the increase in the malate/aspartate ratio and the different labelling patterns after administration of radioactive carbon compounds (e.g. CO_2, TCA cycle intermediates, amino acids, sugars) into citrate and other TCA cycle intermediates and related compounds, such as glutamate, glutamine, etc. (Bidwell, 1963; Graham, 1980). The evidence is consistent with the suggestion that glycolysis and TCA cycle are modified in the light to allow a continuous anaplerotic carbon flow for supplying α-ketoacids which the chloroplast is unable to make (see Section 1.3.2.2). These compounds can be used for a variety of synthetic reactions including amino acid and lipid formation. Important features of this anaplerotic flow are the probable operation of PEP carboxylase in the cytosol and malic enzyme in the mitochondrion to

replenish carbon loss from the TCA cycle (ap Rees, 1980a; Day and Hanson, 1977; see Figure 1.4). It is not known if the TCA cycle operates beyond succinate oxidation because this would depend on the operation of the mitochondrial electron chain in the light, a more uncertain aspect of the problem (see Section 1.3.2.2). The CO_2 arising from the above mentioned reactions (e.g. 1 mol of CO_2 released per mol of glutamine formed) could well be responsible for most of the rate of respiration in illuminated leaves observed in our experiments. Non green cells would also contribute to R_d, but it is not known whether photosynthesis exerts the same influence on their respiratory metabolism as in green cells.

The effect of the photosynthetic activity on the rate of respiration in the light may be mediated by the supply of PEP from recently synthesized triose-phosphate or from sugars. The latter alternative seems more unlikely in view of the fact that exogenous glucose is not metabolized through glycolysis in illuminated leaves including wheat (Bidwell *et al.*, 1955; Graham, 1980). High CO_2 concentration enhances the carbon traffic through the TCA cycle and related compounds, presumably by increasing the supply of substrates for PEP carboxylase (Platt *et al.*, 1977). This may help to explain why some authors have failed to find significant CO_2 efflux by respiration in illuminated leaves into CO_2-free air conditions (Mangat *et al.*, 1974). This also suggests that respiration in daytime, R_d, may be underestimated at the CO_2 compensation concentration.

The observation that Γ measured at 2% O_2 did not increase after a period of photosynthesis was rather surprising. Following the same reasoning used for analysing the diurnal variation of Γ measured at physiological O_2 concentration, we should conclude that respiration in

wheat leaves does not respond to the photosynthetic activity at 2% O_2 as it does at 21% O_2. This reasoning suggests that the production of CO_2 by respiration is probably inhibited when the leaf is at compensation point in 2% O_2. Explanation of this different behaviour is not easy. The partial pressure of CO_2 inside the leaf in equilibrium with air at 2% O_2 was very low (5-6 μbar) and perhaps it is limiting for allowing significant anaplerotic flow through glycolysis and TCA cycle via PEP-carboxylase. This mechanism has been used to explain the lower growth rates of plant tissues in the dark when they are exposed to CO_2-free air (Splittstoesser, 1966; Bown *et al.*, 1974; Bown and Aung, 1974). However, other mechanisms may also be possible because studies on the distribution of photosynthetically fixed $^{14}CO_2$ in wheat leaves have shown that the ^{14}C-incorporation in PEP, malate and aspartate was lower under 2% O_2 than 21% O_2, in contrast to the pattern shown by alanine, which accumulated at 2% O_2 at the expense of PEP (Coudret *et al.*, 1981). This labelling pattern is consistent with a decreased anaplerotic flow through the TCA cycle at low O_2 concentration. Also consistent with this view is the observation that the rate of protein synthesis in *Linum* plants was lower at 2% O_2 than at 21% O_2, in spite of the higher rate of photosynthetic $^{14}CO_2$ fixation observed at low oxygen (Moutot and Jolivet, 1981).

4.4.2 The Light Compensation Point and Respiration

The light compensation point of mature wheat leaves also increased during the day, being correlated to the rate of respiration. This relationship extrapolated to the origin suggesting that respiration is a major component of the light compensation point. These results are

consistent with the increase of the CO_2 compensation point at low light intensities (see also von Caemmerer, 1981; Čatský and Tichá, 1979) which is due to the fact that the respiratory fluxes become relatively more important at these low quantum fluxes, as discussed by von Caemmerer (1981) using a mathematical model of CO_2 assimilation.

The idea that the Kok effect can be interpreted in terms of an inhibition of respiration by light as suggested by Kok (1949) is not supported here. Ishii and Schmid (1981) suggested that the Kok effect seems to be due to the starting of photorespiration rather than to an inhibition of respiration. One of the arguments used is that the Kok effect is suppressed at high CO_2 concentrations. The slight Kok effect at ambient conditions shown in Figure 4.4 was also completely abolished at high CO_2 pressures (see Figure 2.6). Farquhar and Ogawa (unpublished) have shown that the Kok effect does not occur if the intercellular CO_2 partial pressure is kept constant during the determination of the light response curve of photosynthesis. This suggests that the ratio of CO_2/O_2 levels at the fixation sites may be important in determining the presence of the Kok effect.

The shift of the light response curve of photosynthesis following a period of photosynthesis presumably includes a respiratory component, but also can include a component associated with the effect of carbohydrate accumulation on photosynthesis (see Chapter 2). Similar shifts have been also observed in leaves of *Xanthium strumarium* (Sharkey, unpublished) and in rice leaves (Farquhar and Ogawa, unpublished).

In conclusion, the rate of CO_2 production by respiration in the light in 21% O_2 (other than photorespiration per se) is comparable to

that occurring in the dark, and it makes a significant contribution to total CO_2 efflux in illuminated wheat leaves.

CHAPTER 5

THE EFFECT OF CARBOHYDRATE STATUS ON THE RATE, PROPERTIES AND REGULATION OF MITOCHONDRIAL O_2 UPTAKE IN DARKENED WHEAT LEAVES

5.1 INTRODUCTION

Several lines of evidence suggest that there is a direct relationship between the rate of respiration and the carbohydrate content of plant tissues. It has been shown in Chapter 3 that the rate of respiratory CO_2 efflux of mature wheat leaves is correlated with the rate of prior CO_2 assimilation, via carbohydrate accumulation, and similar correlations have been found in leaves of other species (Ludwig *et al.*, 1975; Challa, 1976; Coggeshall and Hodges, 1980; Ferrar, 1980; Moser *et al.*, 1982). Exogenous sugars stimulate CO_2 efflux and O_2 consumption in the dark in the leaves of *Rumex acetosa* (Goldthwaite, 1974) and *Avena sativa* (Tetley and Thimann, 1974), and similar effects have been reported with maize root tips (Saglio and Pradet, 1980).

The biochemical processes responsible for the mentioned respiration in leaves of high carbohydrate status are not known. It is known that the rate of respiration of mature leaves of many plant species is little inhibited or is even frequently stimulated in the presence of cyanide (Allen and Goddard, 1938; MacDonald and De Kok, 1958; Ducet and Rosenberg, 1962; Tetley and Thimann, 1974; Lambers *et al.*, 1979; Kinraide and Marek, 1980). Cyanide is the most frequently

used inhibitor of the cytochrome pathway in studies *in vivo*, and the rate of respiration in its presence gives an estimate of the capacity of the alternative pathway.

The alternative path is a non-phosphorylating cyanide and antimycin A-resistant electron transport pathway, branching from the respiratory chain at ubiquinone (see Section 1.3.1.2). The activity of the alternative pathway can be determined using specific inhibitors such as hydroxamic acids (e.g. salicylhydroxamic acid, SHAM), which were introduced by Schonbaum *et al.* (1971) (see the reviews of Day *et al.*, 1980, and Lambers, 1982).

The alternative pathway of intact leaves has been very little studied, and absolutely nothing is known about the regulation and function of this pathway in leaves *in vivo*. A combination of KCN and SHAM abolished respiration in shoots of *Senecio aquaticus* (Lambers *et al.*, 1979) and in leaves of *Bryophyllum tubiflorum* (Kinraide and Marek, 1980). SHAM alone, however, had no effect on the rate of respiration in both cases, which suggests that the alternative pathway, although present, is not engaged in normal respiration in these leaves.

The experiments described in this chapter investigate the relationship between the carbohydrate status and the rate of mitochondrial electron transport to oxygen in mature wheat leaves. The contribution of the cytochrome and alternative pathways to O_2 consumption under different carbohydrate levels will also be evaluated.

5.2 MATERIALS AND METHODS

5.2.1 Plant Material

Wheat plants were grown as described in Section 2.2.1.

5.2.2 Preparation of Leaf Slices and O_2 Uptake Measurements

Leaves were transversally cut in 1 mm-thick slices with a sharp razor blade under a solution containing 50 mM HEPES, 10 mM MES buffer, pH 6.6, 0.2 mM $CaCl_2$. The slices used in the experiments of Section 5.3.3 were prepared under a 0.2 mM $CaCl_2$ solution. The slices were washed for 10 to 60 min in the same solution, which was renewed several times. The rate of O_2 uptake was measured with a Rank O_2 electrode in 4 ml of an air saturated solution (see legends of figures and tables). A nylon net separated the slices from the stirrer and the electrode. SHAM was added from a stock solution of 1 M in 2-methoxy-ethanol. Typical O_2 electrode traces are shown in Figure 5.4.

5.2.3 Measurement of O_2 Uptake in the Dark in Leaf Segments

Wheat leaf segments (about 70 cm^2 area) were placed in an O_2 electrode cuvette containing 105 ml of reaction medium (50 mM HEPES, 10 mM HEPES buffer, pH 6.6, 0.2 mM $CaCl_2$). The segments (5-8 cm long) were obtained by sectioning leaves perpendicularly to the veins under buffer (see above) with a sharp razor blade. The linear depletion of the O_2 concentration in a closed system was then measured polarographically using a Clark O_2 electrode. The solution was strongly stirred and O_2 became limiting for respiration only at concentrations less than 100 μM. Measurements were made in the dark between 250 and 150 μM O_2. When the effect of SHAM was investigated, the original solution was replaced by a similar solution containing the desired concentration of SHAM, and the rate of O_2 uptake measured again. In the titration experiment (Fig. 5.2) a new batch of leaves

was used for each concentration of SHAM to eliminate the problems caused by accumulation of the inhibitor with time (see Section 5.3.2.1). KCN was added directly to the solution. The rate of O_2 uptake was expressed per leaf area, which was measured with a leaf area meter (Lambda Instruments, model LI-3050A, Lincoln, Nebraska, USA).

5.2.4 Determination of the Respiratory Quotient of a Wheat Leaf Before and After a Period of Light

The rates of dark CO_2 efflux and O_2 uptake were measured independently by using an infrared gas analyser (see Appendix I) and an oxygen electrode (Rank Bros., Cambridge, England), respectively. A leaf was selected at the end of the night and was enclosed in a photosynthetic chamber; the leaf remained attached to the plant and its rate of dark CO_2 efflux was monitored. Simultaneously, the rate of O_2 uptake of a fragment (approx. 2 cm^2) previously cut from the upper portion of this leaf was measured. The leaf fragment was immersed in 4 ml of a solution (10 mM HEPES, 10 mM MES buffer, pH 6.6, 0.2 mM $CaCl_2$) in equilibrium with air; a nylon net separated the leaf fragment from the electrode and the stirrer. The enclosed leaf was then illuminated for 6 h at saturating quantum flux for photosynthesis (1000 $\mu E.m^{-2}.s^{-1}$), and the rate of the latter was recorded periodically. The rate of dark CO_2 efflux was measured again 30 min after the light was switched off to avoid interference from photorespiratory substrates (see Chapter 3). Another fragment of the leaf was transferred immediately into the oxygen electrode cuvette and the rate of O_2 uptake measured. The temperatures during the dark and light periods were 21 °C and 23 °C, respectively.

5.2.5 Isolation of Mitochondria

Wheat leaves (15-20 g) were deribbed and homogenized for 1-2 sec using a Polytron (Kinematica, GmbH, Model K, Kriens-Luzern, Switzerland) with a probe PTA-35/2 (setting #7) in 150 ml of medium containing 0.3 M sorbitol, 50 mM TES buffer, 10 mM KH_2PO_4, 1 mM EDTA, 10 mM isoascorbate, 0.2% (w/v) bovine serum albumin (BSA) and 0.5% PVP-40, adjusted to pH 7.6 with KOH/NaOH. The homogenate was filtered through 4 layers of miracloth and centrifuged at 4000 rpm for 5 min in a Sorvall SS-3 Automatic centrifuge. The supernatant was recentrifuged at 10,000 rpm for 15 min and the pellet resuspended in approximately 40 ml of wash medium (0.3 M sorbitol, 20 mM TES buffer pH 7.2, 0.1% BSA). This suspension was centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in 2-3 ml of wash medium and kept in ice. Temperature during the isolation procedures was 2-4 °C.

O_2 uptake was measured using a Rank O_2 electrode in 2 ml of reaction medium containing 0.3 M sorbitol, 10 mM TES buffer pH 7.1, 5 mM KH_2PO_4, 2 mM $MgSO_4$ and 0.1% BSA, at 22.5 °C. SHAM was added from a stock solution of 1 M in 2-methoxy-ethanol. Other details are provided in the legend of Table 5.2.

5.2.6 Carbohydrate, Protein and Chlorophyll Determination

Free glucose plus fructose, invertase sugars and starch fractions were measured as described in Section 2.2.6.

Protein was estimated according to Lowry *et al.* (1951) with BSA as standard, and chlorophyll according to Arnon (1949). The contribution of contaminating thylakoids to mitochondrial protein estimations was corrected for by assuming a thylakoid protein to chlorophyll ratio of 7 to 1 (Day, 1980).

5.3 RESULTS AND DISCUSSION

5.3.1 The Effect of Carbohydrate Status on the Rate of O_2 Uptake by Leaves

The rate of O_2 uptake by wheat leaves in the dark was much lower in leaves harvested at the end of the night than that in leaves harvested after a period of photosynthesis (Table 5.1; note that respiration was measured about 30 min after the light was turned off, in these experiments). The rate of dark CO_2 efflux also increased, but to a greater extent than the rate of O_2 uptake, resulting in an increase in the respiratory quotient (Table 5.1). Concomitant with this decrease in respiration during the night was a decrease in the carbohydrate content of the leaves (Table 5.1).

5.3.2 The Effect of Carbohydrate Status on Alternative Pathway Activity in Wheat Leaves and Leaf Slices

5.3.2.1 On the validity of using SHAM

The contribution of the cytochrome and alternative pathways to leaf respiration was estimated using the inhibitors KCN and SHAM. SHAM has been the inhibitor most widely used for studying the activity of the alternative path in intact organs (see Lambers, 1982), but there have been some doubts raised about the validity of these techniques *in vivo* (Laties, 1982).

SHAM concentrations higher than 2 mM also inhibit the cytochrome pathway in isolated mitochondria (Schonbaum *et al.*, 1971; Lambers *et al.*, 1982). However, the concentrations of SHAM needed in studies *in vivo* have been frequently ten times or higher, as in the present study; this presumably reflects the poor penetration of this inhibitor into intact tissues, and/or its sequestration within the tissues.

Table 5.1

The effect of a period of photosynthesis of 6 h on the rate of dark respiration, the respiratory quotient (R.Q.), and carbohydrate levels of wheat leaves. Net CO_2 assimilation during the light period was 33 ± 1.5 $\mu mol.m^{-2}.s^{-1}$. External CO_2 concentration was 375 μbar and irradiance was 1000 $\mu E.m^{-2}.s^{-1}$. The values of respiration are means ± SE of 3 experiments. The experimental procedures are detailed in Section 5.2.4. Carbohydrate levels were determined in leaves selected from the growth cabinet and the values shown represent averages of 5 to 10 leaves.

Parameter Measured	At the end of the night	After 6 h in the light
CO_2 efflux ($\mu mol.m^{-2}.s^{-1}$)	0.51 ± 0.06	1.32 ± 0.18
O_2 uptake ($\mu mol.m^{-2}.s^{-1}$)	0.55 ± 0.06	0.73 ± 0.08
R.Q.	0.93 ± 0.03	1.80 ± 0.21
Free glucose and fructose (mmol $C.m^{-2}$)	4.8	16
Invertase sugars	2	58
Starch	3.2	18
Total carbohydrate	10	92

The titration of respiration with SHAM in the presence and in the absence of a constant cyanide concentration (Bahr and Bonner, 1973; Theologis and Laties, 1978) can be used as a method for determining the influence of SHAM on the cytochrome path. A plot of both sets of values should yield a straight line if SHAM does not affect the cytochrome pathway* (Day *et al.*, 1980). The slope of this line (ρ) represents the extent to which the alternative pathway is engaged in the absence of inhibitors. Figure 5.3 shows that this is the case for wheat leaves, and similar results have been reported for maize and wheat roots (Lambers *et al.*, 1983). These latter authors also

* See Appendix IV for a detailed discussion of this analysis.

examined the SHAM sensitivity of mitochondria isolated from wheat and maize roots, and concluded that SHAM concentrations above 2 mM are not reached within the tissues studied when exposed to high external SHAM concentrations for short periods of time. The results that I have obtained in wheat leaves (see later) support this conclusion.

Unfortunately, SHAM, as well as the rest of the hydroxamic acids, also inhibit other oxidase systems as well as the alternative oxidase, including lipoxygenase (Parrish and Leopold, 1978). This enzyme which seems to be localized in other cell compartments (Douillard, 1981; Neuburger *et al.*, 1982) often contaminates washed mitochondria and may be of importance in damaged tissues. Goldstein *et al.* (1980) have shown that the cyanide-insensitive O_2 uptake measured with mitochondria obtained from wheat seedlings by differential centrifugation was due to the oxygenation of linoleate by lipoxygenase; this contaminating lipoxygenase could be eliminated by purifying the mitochondria using a linear Percoll density gradient. On this basis, Goldstein *et al.* (1982) proposed a model which can account for most of the kinetic data obtained with isolated mitochondria. However, this model depends on lipoxygenase being associated with the mitochondria. This may happen with isolated mitochondria since contamination with other cell compartments may occur during organelle preparation. *In vivo*, the situation is most unlikely to arise because lipoxygenase is confined either to plastids, vacuoles or lysozomes (Douillard, 1981). Laties (1982) also concluded that lipoxygenase should not be a problem in intact tissues in most conditions.

Fortunately, the compound tetraethylthiuram disulfide (disulfiram) inhibits the alternative pathway (Grover and Laties, 1981), but does not affect lipoxygenase (Miller and Obendorf, 1981).

It is important to note in this context that SHAM and disulfiram inhibited to the same extent the antimycin A-resistant rate of O_2 uptake of isolated wheat leaf mitochondria (Table 5.2), suggesting that the contribution of lipoxygenase to O_2 uptake by these mitochondria was negligible. Similar results have been reported for pea leaf mitochondria (Lambers *et al.*, 1983) and for aged potato slice mitochondria (Shingles *et al.*, 1982). Unfortunately, the use of disulfiram for studies *in vivo* is not possible due to either a limited penetration into the tissue or its dissipation in the cytosol (Grover and Laties, 1981). The contribution of plastid or vacuolar lipoxygenase to O_2 uptake *per se* is likely to be very small in intact tissue due to lack of appropriate substrate. The respiratory quotient measured in wheat leaves (Table 5.1) suggests that carbohydrate was the main source of O_2 uptake, as does the correlation between carbohydrate status and O_2 uptake.

I therefore conclude that the techniques used in this chapter are suitable for correctly estimating the contributions of the alternative and cytochrome pathways to wheat leaf respiration. The fact that SHAM concentrations as high as 25 mM (in the absence of KCN) did not inhibit respiration of wheat leaves in which the alternative pathway was not engaged (see Fig. 5.2A) supports this conclusion, as does the observation that O_2 uptake was completely inhibited by the presentation of SHAM and KCN together (i.e. residual respiration is negligible in these leaves). In the following sections and chapters, therefore, it can be assumed that the O_2 uptake resistant to KCN represents alternative path activity, while the O_2 uptake resistant to SHAM represents cytochrome path activity.

Table 5.2

Comparison of antimycin A-resistant O_2 uptake by mitochondria isolated from wheat leaves harvested at different times of the photoperiod. O_2 uptake was measured in the presence of 10 mM malate, 10 mM glutamate, 10 mM succinate, 10 mM glycine, 0.1 mM thiamine pyrophosphate and 1.5 μmole ADP. Antimycin A and SHAM were added at 5 μM and 1.8 mM, respectively. Disulfiram was used at 100 μM (see line marked by the asterisk). Mitochondria showed respiratory control ratios with glycine as substrate of about 2.

Leaves harvested	O_2 uptake		
	State 3	+ Antimycin A	+ Antimycin A + SHAM (or disulfiram*)
	nmol.min^{-1}.mg^{-1} protein		
At the end of the night	76	23	6.5
	73	23	7 *
After 5 h light	82	26	11
	86	29	12 *

5.3.2.2 Results obtained with wheat leaf segments, slices, and isolated mitochondria

Figure 5.1 shows that the alternative oxidase capacity of wheat leaves, that O_2 uptake not inhibited by KCN, was very substantial, being about 80% of total measured respiration in leaves harvested after a period in the light (Fig. 5.1B). In leaves harvested at the end of the night, the capacity of the alternative pathway was greater than the flux of reducing equivalents to the respiratory chain, as shown by the fact that KCN had either no effect on O_2 uptake or slightly stimulated it (Fig. 5.1A). This stimulation presumably was via a Pasteur-type effect, since a decrease in the production of ATP

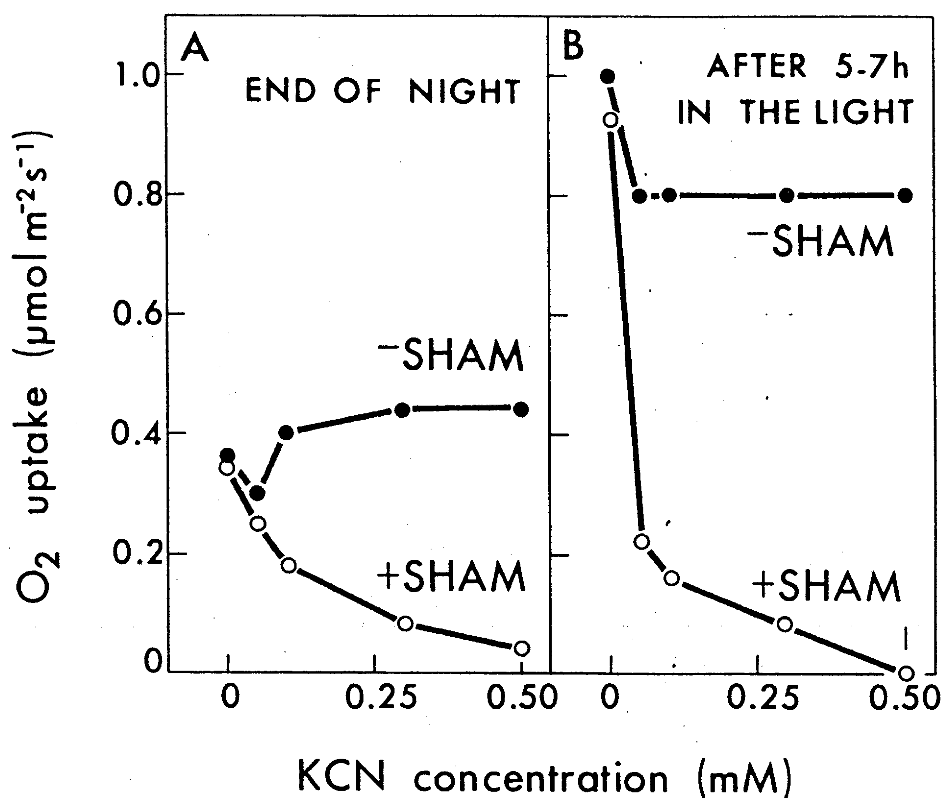

Figure 5.1: The effect of KCN concentration on the rate of oxygen uptake of wheat leaves in the presence (o) and in the absence (•) of 20 mM SHAM. Leaves were selected at the end of the night (A) or after 5-7 h in the light (B). Each curve represents a typical experiment with one batch of leaves. Temperature was 22.5 °C.

might enhance glycolytic flux (Day *et al.*, 1980). When appropriate levels of KCN and SHAM were used together, oxygen uptake was completely inhibited.

Titrating respiration with SHAM (Fig. 5.2) showed that the alternative pathway contributed substantially to the observed respiratory rate in leaves harvested after a period of photosynthesis; that is, SHAM inhibited in the absence of KCN (Fig. 5.2B). A plot of the rates of O_2 uptake in the presence of SHAM alone, against those obtained in the presence of both KCN and SHAM, yields a straight line (Fig. 5.3), whose slope (ρ) is 0.53. That is, after a period in the light, the alternative pathway was used at approximately half its capacity. In leaves harvested at the end of the night, on the other hand, SHAM alone had no effect ($\rho = 0$), indicating that the alternative path was not engaged (Fig. 5.2A). The lack of effect of SHAM in the end-of-night leaves was not due to poor penetration of the inhibitor, since subsequent addition of KCN inhibited O_2 uptake (Fig. 5.1A). A comparison of Figures 5.2A and 5.2B shows that much of the increase in respiration observed after a period of photosynthesis was due to the engagement of the alternative pathway.

Residual respiration, defined as the rate of O_2 uptake resistant to a combination of SHAM and KCN (Theologis and Laties, 1978) was absent or very small in leaf segments, and always absent in leaf slices (see later). The residual component observed sometimes is probably the result of either penetration problems of inhibitors into the leaves or of some possible chemical interactions between KCN and SHAM (Lambers *et al.*, 1983) rather than as the result of the operation of an oxidase system other than the cytochrome and alternative oxidases. As discussed above, the straight line obtained in the ρ-

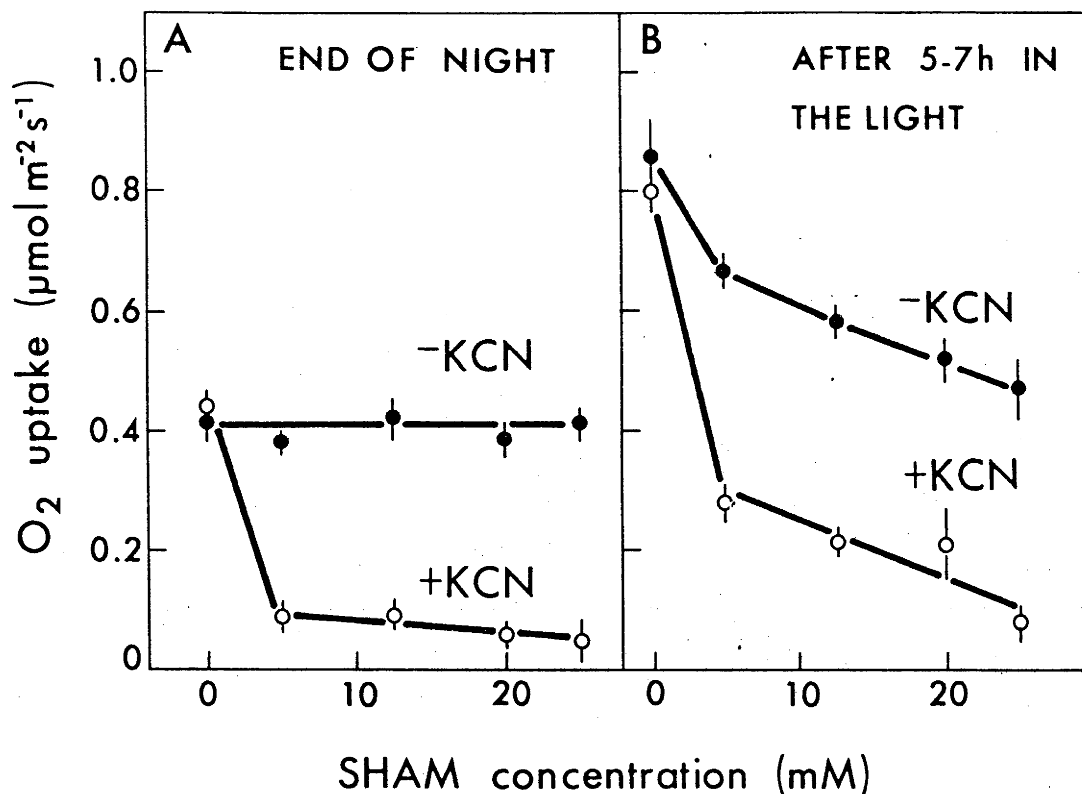

Figure 5.2: The effect of SHAM concentration on the rate of O_2 uptake of wheat leaves in the presence (○) and in the absence (●) of 0.3 mM KCN. Leaves were taken from the growth cabinet at the end of the night (A) or after 5-7 h in the light (B). Temperature was 22.5 °C. The rate of O_2 uptake was firstly recorded in the absence of any inhibitor. Then SHAM and KCN were sequentially added. Only one concentration of SHAM was used for every batch of leaves. Mean values ± S.E. of 2-3 observations are shown. For other details see Section 5.2.3.

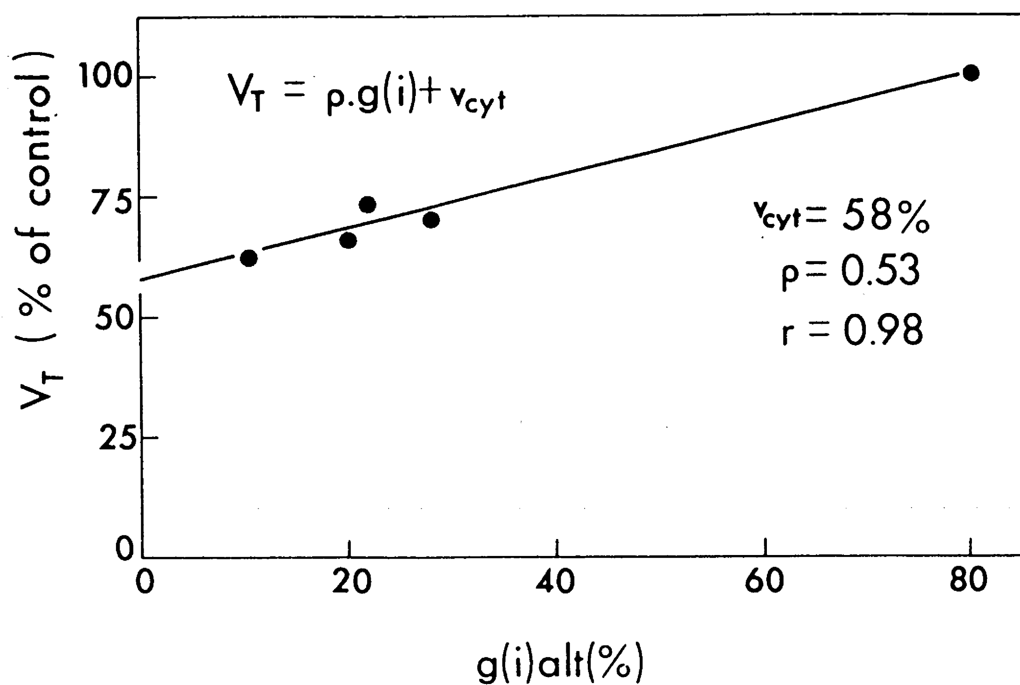

Figure 5.3: Plot of the rate of O_2 uptake of wheat leaves harvested after 5-7 h in the light at different SHAM concentrations (V_T) versus a similar set of values obtained in the presence of 0.3 mM KCN [$g(i)_{alt}$]. v_{cyt} is the rate of the cytochrome pathway. For other details see legend of Figure 5.2.

plot indicates that SHAM did not affect the cytochrome pathway. This, together with the observation that SHAM alone did not inhibit O_2 uptake in the end-of-night leaves, and the lack of significant residual respiration indicate that SHAM inhibition gives a reliable estimate of alternative path activity in wheat leaf tissue.

When slices were prepared carefully as described in Section 5.2.2, their rates of O_2 uptake and their sensitivity to KCN and SHAM were very similar to those measured with intact leaves (Table 5.3). This indicates that wounding effects on slice respiration were negligible. The lower concentration of SHAM which was required to fully inhibit cyanide-resistant respiration in slices (5 mM), compared to leaves (20 mM; Table 5.3, Fig. 5.1), probably reflects increased permeability to the inhibitor in the slices. Typical O_2 electrode traces obtained with slices are shown in Figure 5.4. Slice respiration had similar properties to leaf respiration, the overall rate and SHAM-sensitivity increasing after a period of photosynthesis.

Adding sucrose to slices cut from leaves harvested at the end of night stimulated O_2 uptake almost to the rate observed with slices cut from leaves harvested after several hours in the light (Fig. 5.4 and Table 5.4). Subsequent addition of SHAM reduced the rate of O_2 uptake to a value similar to that observed before adding sugars. That is, the respiration of slices taken at the end of night resembled that of slices taken after light treatment, when sucrose was provided externally. A similar effect of sugars was seen with slices from *Panicum miliaceum* (O_2 uptake increased from 0.53 to 0.70 $\mu mol.m^{-2}.s^{-1}$ with 45 mM glucose) and starved pea leaves (see Chapter 6). Table 5.5 shows the effect of various sugars on the respiration of wheat leaf slices; only sucrose, glucose, and fructose gave significant

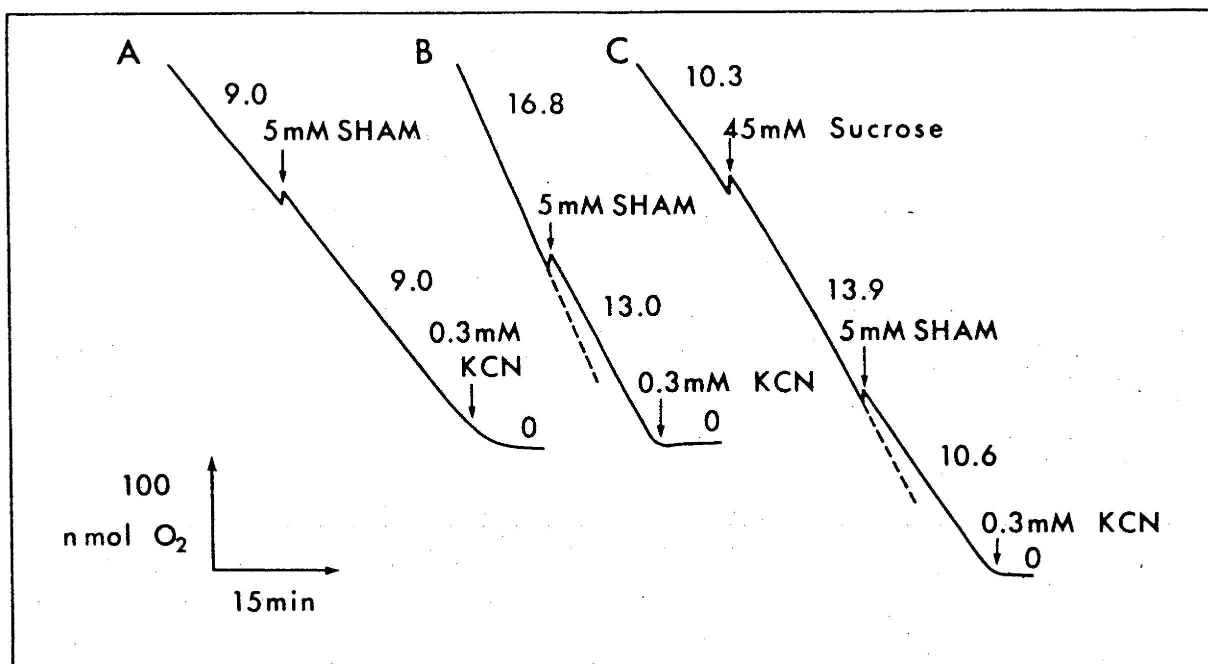

Figure 5.4: Typical traces of oxygen consumption by wheat leaf slices. Leaves were selected at the end of the night (A and C) and after 5-7 h in the light (B). Temperature was 22 °C. Rates shown on traces are expressed as nmol $O_2.min^{-1}$. Leaf areas were 4.05, 3.44 and 2.72 cm^2 for A, B and C, respectively. For further details see Section 5.2.2 and Table 5.3.

Table 5.3

Respiration of wheat leaf slices. The slices were prepared as described in Section 5.2.2. The reaction medium contained 50 mM HEPES, 10 mM MES buffer pH 6.6, 0.2 mM $CaCl_2$. Temperature was 22 °C. The rates of O_2 uptake shown are means ± S.E. of 2-4 determinations.

Additions to vessel	Oxygen Consumption	
	Leaves from end of night	Leaves after 5-7 hours in the light
	$\mu mol.m^{-2}\ s^{-1}$	
Expt 1:		
None	0.39 ± 0.06	0.81 ± 0.07
5 mM SHAM	0.39 ± 0.06	0.62 ± 0.06
5 mM SHAM + 0.3 mM KCN	0.0	0.0
Expt 2:		
None	0.49 ± 0.06	0.97 ± 0.02
0.3 mM KCN	0.49 ± 0.06	0.84 ± 0.05
0.03 mM KCN + 5 mM SHAM	0.0	0.0

Table 5.4

Effect of SHAM on the sucrose-dependent stimulation of O_2 uptake in wheat leaf slices harvested at the end of the night. The values are means ± S.E. of three independent experiments. For further details see Table 5.3.

Sequential Additions to Vessel	Oxygen Consumption
	$\mu mol.m^{-2}.s^{-1}$
None	0.51 ± 0.06
45 mM sucrose	0.74 ± 0.07
5 mM SHAM	0.61 ± 0.04
0.3 mM KCN	0.0

Table 5.5

Effect of different sugars on the rate of O_2 consumption of wheat leaf slices. Slices were cut from leaves harvested at the end of the night. Mean values ± S.E. (n = at least 3) are shown. For further details see Table 5.3.

Sugar Added	Oxygen Consumption	
	Control rate	+ 45 mM sugar
	$\mu mol.m^{-2}.s^{-1}$	
Sucrose	0.45 ± 0.05	0.68 ± 0.06
Glucose	0.38 ± 0.07	0.53 ± 0.07
Fructose	0.49 ± 0.02	0.58 ± 0.02
Ribose	0.51 ± 0.01	0.52 ± 0.01
Mannitol	0.52 ± 0.03	0.50 ± 0.03

stimulations. The lack of effect of mannitol shows that the stimulation did not involve osmotic effects on the slices; only those sugars which can be readily metabolized through glycolysis stimulated O_2 uptake.

The effect of varying sucrose concentration on the rate of O_2 uptake in flag wheat leaf slices is shown in Figure 5.5. Preliminary studies indicated that the maximal response of respiration to exogenously added sucrose occurred at about pH 5.5-6.0. Respiration was stimulated maximally by 60 mM sucrose at 22 °C. High concentrations of mannitol, on the other hand, slightly inhibited O_2 uptake (Fig. 5.5A); this may be due to an osmotic effect on the slices and may also occur with sucrose, in which case the stimulation by sucrose would be underestimated. Sucrose also stimulated considerably the rate of O_2 uptake by leaf slices at 13 °C and 30 °C (Fig. 5.5B).

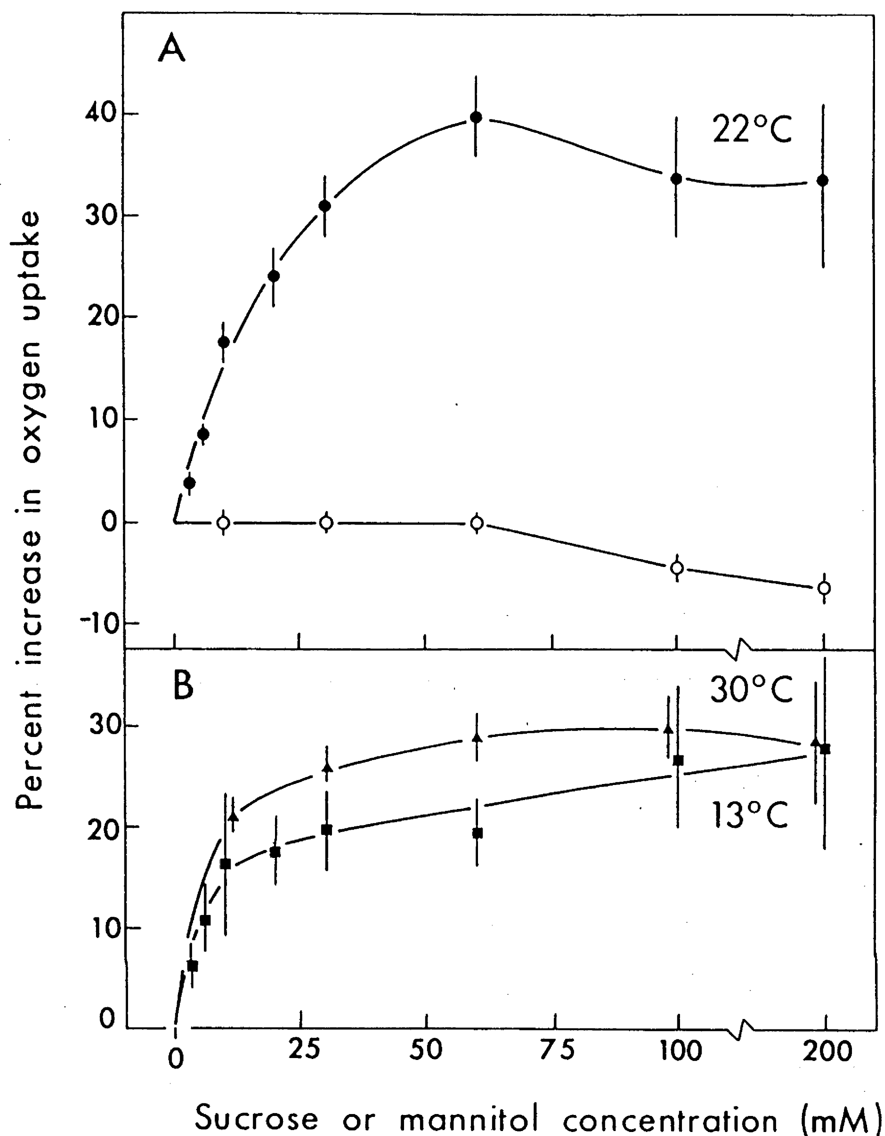

Figure 5.5: The effect of sucrose and mannitol concentration on the rate of O_2 uptake (expressed as per cent of the basal rate) of flag wheat leaf slices selected at the end of the night. A. The effect of sucrose (●) and mannitol (o) at 22 °C. B. The effect of sucrose at 13 °C (■) and at 30 °C (▲). The basal rates (expressed as $\mu mol\ O_2.m^{-2}.s^{-1}$) were 0.36 ± 0.01 at 13 °C: 0.80 ± 0.05 at 22 °C; 1.19 ± 0.16 at 30 °C. Each point is an average of 3-10 determinations. The bars show the standard errors. For other details see Table 5.6.

Mitochondria isolated from wheat leaves showed no significant differences in the capacity of the alternative pathway, regardless of whether the organelles were prepared from leaves harvested at the end of the night or after several hours in the light (Table 5.2; see also Table 6.6). As mentioned earlier in this section, contribution of lipoxygenase to the antimycin A-insensitive O_2 uptake of these mitochondria appeared to be negligible on the basis of disulfiram inhibition of this portion of respiration (cf. Miller and Obendorf, 1981). That is, disulfiram and SHAM inhibited to the same extent. The results in Tables 5.2 and 6.6 show that the changes observed in alternative path activity in wheat leaves, following a period of photosynthesis, were not due to changes in the mitochondria themselves.

5.3.3 Regulation of Flag Leaf Slice Respiration

The interactions between the effects of sucrose, glycine, the uncoupler FCCP, and SHAM on slice respiration were examined in detail using slices cut from flag wheat leaves. The rates of O_2 uptake per leaf area of these slices were slightly higher than those of slices used in the experiments reported above but their respiratory properties in relation to added sugars and inhibitors of electron transport were the same (Table 5.6, experiments 1, 2 and 3). That is, the rate of O_2 uptake of end-of-night leaf slices was stimulated by added KCN and sucrose, and was not inhibited by SHAM; respiration of slices from leaves harvested after a period of light increased, and showed sensitivity to both KCN and SHAM.

Sucrose stimulated respiration of end-of-night leaf slices through an increase in the activities of both the cytochrome and alternative pathways (Table 5.6, Expt 3). However, sucrose did not

Table 5.6

Respiratory properties of flag wheat leaf slices. The slices were prepared as described in Section 5.2.2. The reaction medium contained 10 mM HEPES, 10 mM MES buffer pH 6, 0.2 mM $CaCl_2$. Temperature was 22 °C. KCN and SHAM were added at 0.3 mM and 5 mM, respectively. Sucrose and glycine were added at 60 mM and 100 mM, respectively. Values shown are means ± S.E. of 3-8 measurements.

Sequential Additions		Oxygen Uptake by Leaf Slices: At the end of the night	Oxygen Uptake by Leaf Slices: After 5-7 h in the light
		$\mu mol.m^{-2}.s^{-1}$	
Expt 1:	None	0.69 ± 0.03	0.97 ± 0.02
	KCN	0.76 ± 0.10	0.84 ± 0.05
	SHAM	0.0	0.0
Expt 2:	None	0.72 ± 0.02	1.12 ± 0.06
	SHAM	0.72 ± 0.02	0.80 ± 0.02
	KCN	0.0	0.0
Expt 3:	None	0.65 ± 0.05	1.04 ± 0.08
	Sucrose	0.93 ± 0.10	1.04 ± 0.08
	SHAM	0.77 ± 0.07	-
	KCN	0.0	-
Expt 4:	None	0.71 ± 0.05	0.90 ± 0.02
	Glycine	0.87 ± 0.01	0.93 ± 0.02
	SHAM	0.78 ± 0.02	-
	KCN	0.0	-

stimulate the rate of respiration of slices from leaves harvested after a period of light of several hours (Table 5.6, Expt 3), which already had the alternative pathway engaged to some extent (Table 5.6, Expts 1 and 2). The average level of endogenous sugars (fructose, glucose and sucrose) was much higher in these latter leaves than in

leaves harvested at the end of the night period — these values were 52 and 6.2 mmol carbon.m^{-2}, respectively.

External glycine also stimulated the rate of O_2 uptake in end-of-night slices but not in slices from leaves harvested after a light period (Table 5.6, Expt 4). The stimulatory effect of glycine on slice respiration occurred very rapidly upon addition, and it is presumably due to the action of glycine decarboxylase, as suggested by studies made in pea leaf slices (Grodzinsky and Woodrow, 1981).

The absence of stimulation of respiration in high carbohydrate leaf slices by added sucrose or glycine cannot be explained by considering that the electron transport chain was saturated, since the alternative pathway at least was not fully engaged in these slices (Table 5.6, Expts 1 and 2). The uncoupler FCCP was used to test the possibility that respiration was under adenylate control. FCCP produced only a slight stimulation of respiration in slices from leaves harvested at the end of the night or after a light period (Table 5.7); when sucrose or glycine was added to the slices, however, FCCP stimulated respiration substantially in all cases (Tables 5.7 and 5.8). The small effect of FCCP in the absence of added sugars suggests that the level of endogenous substrates was never in excess for slice respiration. It also appears that respiration of slices from leaves harvested after a light period was more tightly controlled by adenylates than that of end-of-night slices, since respiration could only be stimulated by exogenous substrates when this control was removed by using an uncoupler. In end-of-night slices, on the other hand, exogenous sucrose stimulated in the absence of FCCP.

Although FCCP alone had very little effect on the rate of O_2 uptake, it prevented subsequent inhibition by SHAM (Table 5.8, Expt 1).

Table 5.7

Effect of FCCP plus sucrose or glycine on the rate of O_2 uptake of flag wheat leaf slices. FCCP was added at 1 µM. Values shown are means ± S.E. of 3-6 measurements. For other details see Table 5.6.

Sequential Additions		Oxygen Uptake by Leaf Slices	
		At the end of the night	After 5-7 h in the light
		$\mu mol.m^{-2}.s^{-1}$	
Expt 1:	None	0.68 ± 0.03	0.97 ± 0.03
	FCCP	0.75 ± 0.06	1.04 ± 0.04
	Sucrose	1.25 ± 0.04	1.25 ± 0.04
Expt 2:	None	-	0.96 ± 0.08
	FCCP	-	1.05 ± 0.05
	Glycine	-	1.17 ± 0.07

However, SHAM clearly inhibited respiration in the presence of FCCP when sucrose was added (Table 5.8, Expt 2) and in the absence of both FCCP and added sucrose (Table 5.6). The rate of respiration resistant to SHAM, which is an estimate of the activity of the cytochrome pathway (the SHAM-resistant O_2 uptake was completely inhibited by KCN) was about 30% higher in slices exposed to FCCP than that of slices not treated with FCCP (Table 5.8; see also Table 5.6).

The results in Tables 5.6 to 5.8 are summarized in Table 5.9. Here I use V_t to signify the total measured rate of O_2 uptake in the absence of inhibitors, while v_{cyt} and v_{alt} (determined using KCN and SHAM as explained in the legend of Table 5.9) represent the activities of the cytochrome and alternative paths respectively. In leaves harvested at the end of the night, when sugars and respiratory rates are low, V_t is due solely to O_2 uptake by the cytochrome pathway

Table 5.8

Effect of sucrose and uncoupler on the respiration of slices from flag wheat leaves harvested after 5-7 h in the light. For experimental details see Tables 5.6 and 5.7. Values shown are means ± S.E. of 3-6 independent measurements.

Sequential Additions		Oxygen Uptake
		$\mu mol.m^{-2}.s^{-1}$
Expt 1:	None	1.02 ± 0.09
	FCCP	1.06 ± 0.09
	SHAM	1.00 ± 0.07
	Sucrose	1.03 ± 0.07
	KCN	0.0
Expt 2:	None	1.06 ± 0.06
	Sucrose	1.06 ± 0.06
	FCCP	1.28 ± 0.06
	SHAM	1.04 ± 0.02
	KCN	0.0
Expt 3:	None	1.13 ± 0.06
	SHAM	0.80 ± 0.02
	FCCP	0.90 ± 0.04
	Sucrose	0.97 ± 0.10
	KCN	0.0

(Table 5.9A). In leaves harvested after several hours in the light, V_t increases, and this increase is due to higher cytochrome pathway activity and to engagement of the alternative path (Table 5.9B). This situation also holds for end of night leaves supplied with sucrose exogenously (not shown in Table 5.9, but see Table 5.6, Expt 3). When FCCP is added to leaves harvested after a period of light, V_t remains unchanged but v_{cyt} increases at the expense of v_{alt} (Table 5.9C). That is, electrons are re-routed from the alternative to the

Table 5.9

Estimation of the activities of respiratory pathways in flag wheat leaf slices. The values shown were calculated from experiments similar to those described in Tables 5.6, 5.7 and 5.8, and are means ± S.E. from 6 to 29 independent measurements. V_t is the measured rate of O_2 uptake in the absence of inhibitors. v_{cyt} is the activity of the cytochrome path, estimated by measuring O_2 uptake in the presence of SHAM. v_{alt} is the activity of the alternative pathway, and is calculated by subtracting v_{cyt} from V_t. ρ is the fraction of the maximum capacity of the alternative path that is expressed; $\rho = v_{alt}/V_{alt}$, where V_{alt} is the capacity of the alternative path, estimated by measuring O_2 uptake in the presence of KCN, sucrose and FCCP. $V_{alt} = 0.84\ \mu mol\ O_2.m^{-2}.s^{-1}$.

Experimental Conditions	V_t	v_{cyt}	v_{alt}	ρ
	$\mu mol\ O_2.m^{-2}.s^{-1}$			ratio
A. Leaves from end of night	0.69 ± 0.02	0.69 ± 0.02	0.0	0
B. Leaves after 6 h light	1.03 ± 0.03	0.78 ± 0.02	0.25 ± 0.02	0.3
C. Leaves after 6 h light + FCCP	1.06 ± 0.09	1.0 ± 0.07	0.0	0.0
D. Leaves after 6 h light + FCCP + sucrose	1.25 ± 0.04	1.02 ± 0.04	0.23 ± 0.04	0.27

cytochrome path when the latter is uncoupled (the alternative path *per se* is not coupled to phosphorylation of ADP; Day *et al.*, 1980). This strongly suggests that in these leaves the cytochrome path is restricted by oxidative phosphorylation, and this suggestion is supported by the observation that glycine (which feeds electrons directly to the respiratory chain via glycine decarboxylase, and therefore bypasses glycolysis) only stimulates respiration in these leaves when FCCP is also added (Table 5.7, Expt 2). When both sucrose and FCCP are added to leaves harvested after light treatment, V_t is

increased, and this increase is due to engagement of the alternative path (Table 5.9D).

The data shown in Table 5.9 imply that respiration in wheat leaves is regulated in the following manner. When carbohydrate (probably free sugars are the more important fraction in this respect, as we will see in the next chapters) levels are low, respiration is restricted by substrate supply from glycolysis to the mitochondria, and all O_2 uptake occurs via cytochrome oxidase. When leaf carbohydrate levels are increased (e.g. by a period of photosynthesis), substrate supply to the mitochondria is increased, but the activity of the cytochrome chain is restricted by cytosolic adenylate levels; under these conditions the glycolytic supply of reducing equivalents to the mitochondria is greater than that which can be handled by the cytochrome chain, and electrons therefore spill-over into the alternative path. However, even when leaf carbohydrate levels are high (e.g. in leaves harvested after a period of photosynthesis, in the presence of exogenous sugars), the mitochondrial electron transport pathways are not fully expressed, and they are not further stimulated by the addition of exogenous sugars in the absence of uncoupler. Only when uncoupler is also added is respiration stimulated. This suggests that glycolysis as well as the cytochrome chain is restricted by adenylate levels; if so, FCCP presumably stimulates via a Pasteur-type of effect (Day *et al.*, 1980). Alternatively, alternative path activity may be limited by respiratory control of electron transport through the first site of energy transduction; that is, under these conditions the flux of reducing equivalents from substrates to ubiquinone (the branch point of the alternative path and cytochrome path) may be less than the capacity of the pathways from ubiquinone to O_2 (Laties, 1982).

5.4 GENERAL DISCUSSION

In summary, the results obtained in this chapter suggest that wheat leaf respiration is subject to control by both carbohydrates (e.g. sugars) and adenylates, the relative influence of the two substrates varying with the photoperiod and the rate of prior photosynthesis.

Although the results suggest that adenylate levels regulate glycolysis as well as the cytochrome chain, the control of the latter appears to be tighter. That is, regulation of glycolytic flux is more flexible than that of the cytochrome pathway. Similar conclusions have been drawn concerning the regulation of root respiration (Lambers *et al.*, 1983). An important consequence of this is that the rate of glycolysis can exceed the activity of the cytochrome chain, thus allowing expression of the non-phosphorylating alternative path. How this is achieved in the face of cytosolic energy charge values high enough to restrict the cytochrome path is not certain. Very high ATP/ADP ratios are needed to significantly inhibit coupled mitochondrial electron transport *in vitro* (Dry and Wiskich, 1982) and one might have expected glycolysis also to be restricted under these conditions. A possible mechanism may involve a bypass of the pyruvate kinase step in glycolysis. PEP could be converted to malate via PEP carboxylase and malate dehydrogenase in the cytosol, and the malate could then be decarboxylated via NAD-linked malic enzyme in the mitochondrial matrix to yield pyruvate (Day and Hanson, 1977; ap Rees, 1980a; see Section 1.3.1.1 and Figure 1.4). Plant phosphofructokinase is less tightly controlled by adenylates than its animal counterpart (Turner and Turner, 1980), and hence need not be tied so tightly to ATP turnover (Pradet and Raymond, 1982). Whatever the mechanism, it

is apparent that in wheat leaves which have an active alternative pathway, the rate of glycolysis is not strictly matched to meet the energy demands of the cell.

The regulation of the distribution of electrons between the cytochrome and alternative pathways in wheat leaves is in accord with the model of branched electron transport proposed by Bahr and Bonner (1973) from studies in isolated mitochondria. This model suggests that the alternative pathway only operates when the cytochrome pathway is either saturated (e.g. state 3 or uncoupled respiration in the presence of adequate substrates) or restricted (e.g. by oxidative phosphorylation). This hypothesis was also supported by work with slices from storage tissues (Theologis and Laties, 1978) where it has been shown that the alternative pathway is unoperative unless the cytochrome pathway is saturated with electrons. In this case saturation of the cytochrome pathway and engagement of the alternative pathway was observed only upon adding an uncoupler, suggesting that in these tissue slices glycolysis is restricted by adenylate energy charge (uncouplers stimulate via a Pasteur effect). The model of De Troostembergh and Nyns (1978), which proposes that the assignment of the electrons takes place at the level of ubiquinone in proportion to the capacity of the cytochrome and the alternative pathways (i.e. that the two pathways compete for reduced ubiquinone) is not supported by the results obtained in wheat leaves.

The higher respiratory quotient in the high carbohydrate leaves (Table 5.1) implies that the carbohydrates in these leaves are used in processes other than mitochondrial respiration. Oxidative pentose-phosphate pathway activity in the chloroplasts and cytosol, and pentan biosynthesis (ap Rees, 1980a), may contribute substantially to CO_2

release here; obviously the activity of these processes is also regulated at least in part by leaf carbohydrate levels. Some electrons could also be used for reducing nitrate and nitrite in the dark (Aslam *et al.*, 1979) instead of reducing oxygen. The rate of these reactions has been found to depend on the carbohydrate status in barley seedlings under dark aerobic conditions (Aslam *et al.*, 1979).

In summary, my results suggest that wheat leaf respiration in the dark is regulated by both cell carbohydrate levels and by adenylate control of the mitochondrial respiratory chain. When leaf carbohydrate levels are substantial, the alternative path becomes engaged because the cytochrome chain is restricted. When leaf sugar levels are low, respiration is limited by substrate supply to the mitochondria, and the alternative pathway is not expressed.

CHAPTER 6

RESPIRATION OF PEA AND SPINACH LEAVES AND ISOLATED MITOCHONDRIA

6.1 INTRODUCTION

The results obtained in the previous chapters have shown that marked diurnal variations in the rates of total (uninhibited) and alternative pathway respiration occur in mature wheat leaves, and these variations are directly correlated with prior photosynthesis via carbohydrate accumulation.

The purpose of the work described in this chapter is to determine the participation of the alternative path in respiration of leaves of several C_3 species, and to study the possible occurrence of diurnal variations in leaves of peas and spinach. These species were chosen because they are suitable material for extraction of mitochondria and the respiratory properties of isolated mitochondria will be examined at different times of the diurnal cycle.

The methods used for determining the contribution of the various pathways to leaf and mitochondrial respiration involved the utilization of inhibitors of the cytochrome chain (KCN and antimycin A) and the alternative path (SHAM and disulfiram) as discussed previously (Chapter 5). The experiments shown in this chapter were made in collaboration with Drs. D.A. Day and H. Lambers.

6.2 MATERIALS AND METHODS

6.2.1 Plant Material

Plants of *Spinacea oleracea* L. (cv. Hybrid 102, New World seeds, Australia) and *Pisum sativum* L. (cvs. Alaska and Massey) were grown for 4-5 weeks in half-strength Hewitt's solution (see Section 2.2.1 for the composition of the full-strength solution). Seedlings of *Pisum sativum* (cv. Massey) were also grown for two weeks in vermiculite. *Triticum aestivum* L. (cv. Gabo) plants were grown in pots of soil as described in Section 2.2.1. In all these cases, quantum flux (400-700 mm) was about 600-700 $\mu E.m^{-2}.s^{-1}$. The day/night temperature regime was 25/20 °C with a daylength of 13 h. Relative humidity was between 60 and 80%.

Plants of *Cucumis sativus* L. (cv. Green Gem), *Gossipium hirsutum* L. (cv. Deltapine), *Helianthus annuus* L. (cv. Suncross 52), *Lycopersicum esculentum* Mill. (cv. Grosse Lisse), *Phaseolus aureus* L. (cv. Celera), *Phaseolus vulgaris* L. (cv. Epicure) and *Spinacea oleracea* L. (Hybrid 102, New World seeds) were also grown in culture solution (see above). However, the temperature during growth was 25 °C (day and night). The light intensity was 350-500 $\mu E.m^{-2}.s^{-1}$ supplied by Sylvania Gro-Lux and Sylvania Cool White (ratio 3:8) fluorescent tubes during 16 h per day. Mature leaves were used in the experiments.

6.2.2 Measurements of O_2 Uptake by Intact Leaves and Isolated Mitochondria

The rate of O_2 uptake by intact leaves was described in Section 5.2.3. Slices from leaves were obtained as shown in Section 5.2.2. Mitochondria were isolated as described in Section 5.2.5. Carbohydrate concentration was determined following the procedures described in Section 2.2.6.

6.3 RESULTS AND DISCUSSION

6.3.1 Effect of a Period of Photosynthesis on the Respiration of Intact Leaves and Mitochondria

After several hours in the light leaf respiration displayed considerable cyanide-resistance in all species examined (except perhaps in *Phaseolus aureus*) (Table 6.1), as also observed in wheat (see Chapter 5; Allen and Goddard, 1938), and in other species (MacDonald and De Kok, 1958; Ducet and Rosenberg, 1962; Tetley and Thimann, 1974; Lambers *et al.*, 1979; Kinraide and Marek, 1980). Leaf respiration was sensitive to SHAM, with the exceptions of *Phaseolus vulgaris* and *Helianthus annuus* (Table 6.1). Low SHAM sensitivity has also been observed in shoots of *Senecio aquaticus* (Lambers *et al.*, 1979) and in leaves of *Bryophyllum tubiflorum* (Kinraide and Marek, 1980). Some residual respiration (resistant to a combination of SHAM and KCN) was observed in some species (Table 6.1). This is probably due to the fact that SHAM concentration used (10 mM) may have not been saturating in these species, as found in titration experiments performed later in wheat leaves (see Fig. 5.2).

The rate of O_2 uptake in spinach leaves was lower at the end of the night than after a period of several hours in the light (Table 6.2). Concomitant with this increase in respiration was an increase in all the carbohydrate fractions of the spinach leaves (Table 6.3). This trend was similar to that found in wheat leaves (see Chapter 5). In pea leaves, on the other hand, the variation in total carbohydrates after a period of light was less marked, and the levels of free glucose and fructose actually decreased (Table 6.3). In these leaves there was no increase in respiratory rates after a light period (Table 6.2).

Table 6.1

Respiration of intact mature leaves of several species harvested after several hours in the light. KCN and SHAM were used at 0.3 mM and 10 mM respectively. The values shown are means ± S.E. of at least 3 determinations. Temperature was 25 °C.

Species	O_2 Uptake	SHAM-Inhibition	CN-Resistance	Residual Respiration
	$\mu mol.m^{-2}.s^{-1}$	%	%	%
Gossipium hirsutum	0.6 ± 0.04	14 ± 5	65 ± 3	-
Helianthus annuus	1.1 ± 0.05	8 ± 4	40 ± 4	20 ± 1
Lycopersicum esculentum	1.1 ± 0.03	17 ± 4	52 ± 5	21 ± 4
Phaseolus aureus	1.1 ± 0.09	13 ± 6	17 ± 6	-
Phaseolus vulgaris	0.8 ± 0.05	0 ± 5	74 ± 6	15 ± 3
Spinacea olereacea	1.2 ± 0.09	24 ± 6	40 ± 4	-

Table 6.2

Respiration of intact pea and spinach leaves at 23 °C. The values are means ± S.E. of 3-4 determinations. The values in brackets are the per cent contribution of SHAM-sensitive respiration to total O_2 uptake.

Leaves Harvested	O_2 Uptake		
	None	+ 0.4 mM KCN	+ 20 mM SHAM
		$\mu mol.m^{-2}.s^{-1}$	
Spinach			
at the end of the night	0.51 ± 0.05	0.34 ± 0.05	0.37 ± 0.01(27)
after 5-7 h in the light	0.80 ± 0.07	0.36 ± 0.03	0.57 ± 0.09(29)
Peas			
at the end of the night	0.71 ± 0.05	0.66 ± 0.09	0.55 ± 0.07(23)
after 5-7 h in the light	0.67 ± 0.05	0.66 ± 0.10	0.46 ± 0.08(31)

Table 6.3

Carbohydrate levels in pea and spinach leaves at different times of the photoperiod. The values shown are averages of 6 to 20 leaves selected at random from the cabinet.

Leaves Harvested	Free Glucose + Fructose	Invertase Sugars	Starch	Total
		$mmol\ C.m^{-2}$		
Spinach				
at the end of the night	0.9	1.7	3.0	5.6
after 6-10 h in the light	3.3	15.0	25.5	43.8
Peas				
at the end of the night	11.0	17.5	18.0	46.5
after 6-10 h in the light	9.3	55.0	29.8	94.1

The addition of exogenous sugars did not affect the rate of O_2 uptake of slices obtained from pea leaves harvested at the end of the night or after a light period (not shown). However, when peas were kept in the dark for 48 h, the free sugar levels did decrease to very low values, and respiratory rates of leaf slices declined to 0.49 ± 0.03 µmol $O_2.m^{-2}.s^{-1}$ ($n = 4$). Addition of sugars (glucose, sucrose) to these starved slices increased the rate of O_2 uptake to almost the values observed in non-starved leaves (0.64 ± 0.03 µmol. $m^{-2}.s^{-1}$). It thus appears that the level of free fructose and glucose is a controlling factor of the rate of respiration in the leaves of the species examined.

The capacity of the alternative pathway was estimated using KCN and did not vary after a light period in either spinach or pea leaves (Table 6.2). The extent of engagement of the alternative pathway (determined by SHAM inhibition) in spinach leaves increased after a period of photosynthesis although the per cent contribution to total respiration was the same (Table 6.2). The rate of O_2 uptake resistant to SHAM, which is an estimate of the activity of the cytochrome pathway, increased after a period of photosynthesis. In peas, where no significant change in respiration occurred after the light treatment, alternative pathway contribution was approximately the same before and after the light period (Table 6.2). Thus, similarly to wheat leaves, alternative path involvement in these leaves seems to be correlated with the level of free sugars in the leaf.

Mitochondria were isolated from the same batches of spinach and pea leaves used in the respiration studies with intact tissues. No significant differences in the mitochondrial properties (e.g. responses to several substrates, contribution of the cytochrome and

alternative pathways — see next section) were noticed, regardless of whether the organelles were prepared from leaves harvested at the end of the night or after several hours in the light (Tables 6.4 and 6.5). Similar results were obtained with wheat leaf mitochondria (Table 6.6; see also Table 5.2). These results show that the changes observed in alternative path activity following a period of photosynthesis were not due to changes in the mitochondria themselves. The mitochondrial data intrinsically presented some interesting characteristics which I decided to investigate further.

6.3.2 Oxidation of Different Substrates by Isolated Mitochondria: Effects on the Alternative Pathway

Mitochondria isolated from mature leaves of spinach, pea and wheat oxidized glycine in the presence of ADP at faster rates than TCA cycle substrates (Tables 6.4, 6.5 and 6.6). These mitochondria showed good respiratory control and ADP/O values (see legends of tables; and Fig. 6.2). Some apparently low RCR values (for instance, 1.7 in some pea leaf mitochondria) are probably due to the large engagement of the alternative pathway (see below).

Simultaneous oxidation of two or more substrates gave O_2 uptake rates faster than those observed with any single substrate, as observed also in mitochondria from other tissues (Day and Wiskich, 1977, 1981; Wiskich and Day, 1982), especially when glycine was added in the presence of malate, α-ketoglutarate and succinate (Tables 6.7, 6.8 and 6.9). The fastest rates of O_2 uptake by pea leaf mitochondria were observed in the presence of malate, succinate, NADH, and glycine, added in this order (Table 6.7).

The antimycin A-resistant rate of O_2 uptake, which is an estimate of the capacity of the alternative path when residual respiration is

Table 6.4

Comparison of alternative pathway activity of mitochondria isolated from spinach leaves at different times of the photoperiod. O_2 uptake was measured in the presence of various substrates (10 mM) as indicated and 2 µmol ADP. When the malate was used, 10 mM glutamate and 0.1 mM thiamine pyrophosphate were also added to the vessel. Antimycin and SHAM were used at 10 µM and 1.5 mM, respectively. RCR (with glycine) was 2.9. The pH of the reaction medium was 7.1 and temperature was 23 °C. The values in brackets represent the rates of O_2 uptake sensitive to SHAM. ρ is the fraction of the maximum capacity of the alternative pathway that is expressed, and is calculated as the ratio between the SHAM-sensitive and antimycin A-resistant rates of O_2 uptake. The residual respiration (resistant to both antimycin A and SHAM) when present was subtracted from the antimycin A-resistant rate.

Substrates	O_2 Uptake				
	State 3	+ SHAM	+ Antimycin A	+ SHAM + Antimycin A	ρ
	$nmol.min^{-1}.mg^{-1}$ protein				ratio
AT THE END OF THE NIGHT					
malate	66	45(21)*	-	0	-
glycine	104	85(19)	20	0	1
malate + succinate	83	-	0	-	-
malate + succinate + glycine	136	110(26)	26	0	1
AFTER 5 h IN THE LIGHT					
malate	83	58(25)	-	0	-
glycine	133	108(25)	25	1	1
malate + succinate	112	-	-	-	-
malate + succinate + glycine	176	139(37)	34	0	1

* State 3 rate minus rate in the presence of SHAM.

Table 6.5

Comparison of alternative pathway activity of mitochondria isolated from pea (cv. Alaska) leaves at different times of the photoperiod. RCR (with glycine) was 1.7. For other details see Table 6.4.

Substrates	O_2 Uptake				ρ
	State 3	+ SHAM	+ Antimycin A	+ SHAM + Antimycin A	
	nmol.min^{-1}.mg^{-1} protein				ratio
AT THE END OF THE NIGHT					
malate	113	93(20)	64	9	0.36
succinate	89	71(18)	52	3	0.37
glycine	155	116(39)	55	6	0.80
malate + succinate	172	-	-	-	-
malate + succinate + glycine	232	176(56)	108	3	0.53
AFTER 5 h IN THE LIGHT					
malate	118	92(26)	79	6	0.36
succinate	116	87(29)	66	0	0.44
glycine	170	120(50)	71	7	0.78
malate + succinate	196	-	-	-	-
malate + succinate + glycine	257	181(76)	133	0	0.57

Table 6.6

Comparison of alternative pathway activity of mitochondria isolated from wheat leaves at different times of the photoperiod. RCR (with glycine) was 2. Antimycin A and SHAM were used at 5 μM and 1.8 mM, respectively. Other details are the same as in Table 6.4.

Substrates	Oxygen Uptake		
	State 3	+ Antimycin A	+ Antimycin A + SHAM
	nmol.min^{-1}.mg^{-1} protein		
AT THE END OF THE NIGHT			
malate	47	-	-
glycine	70	31	13
malate + succinate	66	33	14
malate + succinate + glycine	114	52.5	15
AFTER 5 h IN THE LIGHT			
malate	30	-	-
glycine	69	23.5	8
malate + succinate	56	28	9.5
malate + succinate + glycine	97	38	7

taken into account, was found to vary depending on the substrate(s) being oxidized. This rate was similar when malate, succinate and glycine were oxidized separately by leaf mitochondria from two pea cultivars (Tables 6.5, 6.7 and 6.8), but was slightly lower with NADH (Table 6.7), as observed with other tissues (see Day *et al.*, 1980).

In mitochondria from mature pea leaves concurrent oxidation of malate and succinate either did not affect or only slightly increased the antimycin A-resistant O_2 uptake rate (Table 6.7, Fig. 6.1A and B), but further addition of glycine considerable stimulated this rate (Tables 6.5, 6.7 and Fig. 6.1A and B). Similar results were obtained in wheat leaf mitochondria (Table 6.6) and, to a lesser extent, in spinach leaf mitochondria (Table 6.4). However, mitochondria from leaves of pea seedlings (two weeks of age) in contrast to those of older plants, showed less resistance to antimycin A, and the antimycin A-resistant rate was not significantly altered depending on the substrate(s) being oxidized (Table 6.8, Fig. 6.1C). Reasons for these differences are not obvious, but they might be related to the developmental status of the plant.

The extent of engagement of the alternative path (ρ) in pea leaf mitochondria was estimated by measuring the rate of O_2 uptake suppressed by SHAM or disulfiram (since the effects of these compounds were found to be the same, I conclude that lipoxygenase is not at issue). When single substrates were oxidized, SHAM (or disulfiram) had a greater effect on S_3 rates with glycine than those with either malate or succinate. That is, ρ was substantially higher with glycine as substrate (Tables 6.5, 6.7, 6.8); in fact, with this substrate, the alternative path was almost fully expressed. When a cocktail of malate, succinate and glycine was used, the absolute rate of O_2 uptake

Table 6.7

Alternative path activity of pea (cv. Massey) leaf mitochondria. Mature leaves from four week-old plants were selected. RCR (with glycine) was 1.7. Antimycin A and disulfiram were used at 6 μM and 150 μM, respectively. The pH of the medium was 7.1 and temperature was 23 °C. The values in brackets represent the rates of O_2 uptake sensitive to disulfiram. The order of substrate additions was as indicated. When malate was used, 10 mM glutamate and 0.1 mM thiamine pyrophosphate were also added to vessel. NADH was used at 1 mM.

Substrates	O_2 Uptake				
	State 3	+ Disulfiram*	+ Antimycin A	+ Disulfiram + Antimycin A	ρ
	nmol.min^{-1}.mg^{-1} protein				ratio
glycine	171	131(40)	47	7	1.
malate	95	78(17)	55	11	0.4
NADH	169	144(25)	37	12	1
malate + succinate	122	102(20)	55	11	0.45
malate + succinate + glycine	205	141(64)	77	11	1
malate + succinate + NADH	189	-	-	-	-
malate + succinate + NADH + glycine	222	-	70	17	-

* The same results were obtained using SHAM.

Table 6.8

Alternative path activity of pea (cv. Massey) leaf mitochondria. Leaves from two week-old seedlings were selected. RCR (with glycine) was 2.3. Temperature was 25 °C. Antimycin A and disulfiram were used at 6 μM and 100 μM, respectively. Other details were the same as in Table 6.7.

Substrates	O_2 Uptake				
	State 3	+ Disulfiram	+ Antimycin A	+ Disulfiram + Antimycin A	ρ
	nmol.min^{-1}.mg^{-1} protein				ratio
glycine	230	180(50)	52	0	1
malate	165	145(20)	72	0	0.3
succinate	137	116(21)	46	0	0.46
malate + glycine	337	253(84)	74	0	1
malate + glycine + succinate	367	301(66)	61	0	1

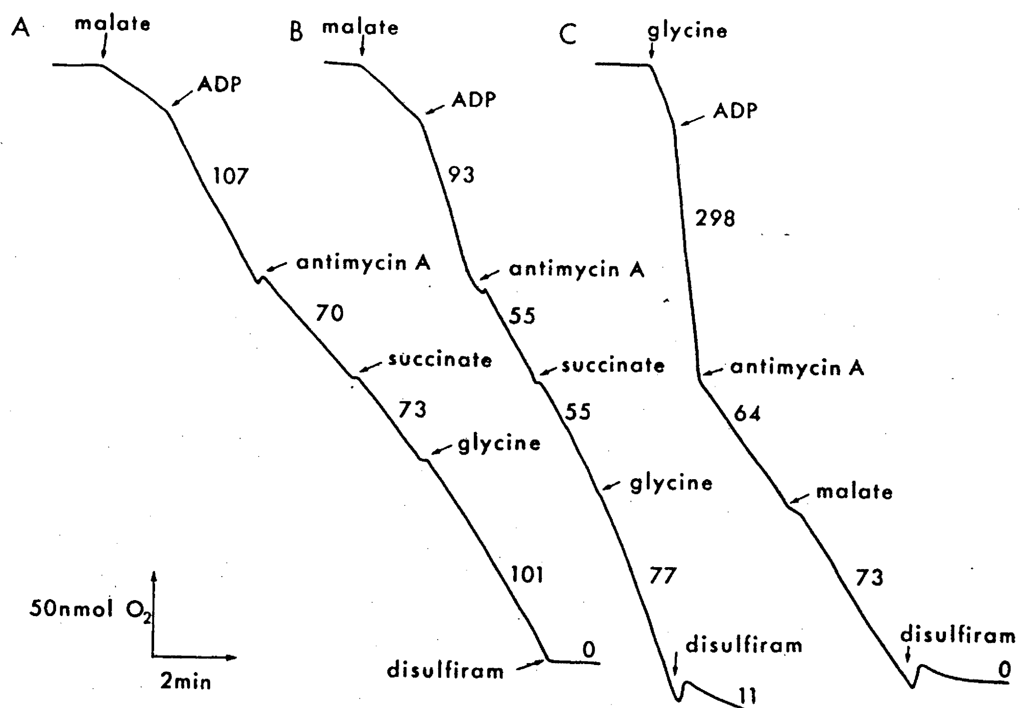

Fig. 6.1: Effect of several substrates on the antimycin A-resistant rate of O_2 uptake by pea leaf mitochondria. A: cv. Alaska; B and C: cv. Massey; A and B: four week-old plants; C: two week-old seedlings. Assay conditions as in Tables 6.7 (A and B) and 6.8 (C). Numbers on traces refer to nmol $O_2.min^{-1}.mg^{-1}$ protein.

inhibited by SHAM was greater than with any single substrate (i.e. more electrons were flowing to the alternative oxidase with multiple substrates); however, the maximal capacity of the alternative pathway was greater when these three substrates were used together, and thus ρ was less than that with glycine alone (actually, ρ with glycine + succinate + malate approximated the numerical average of the ρ values calculated with the substrates individually). SHAM-sensitive O_2 uptake was also stimulated when glycine was used with either succinate or malate alone (not shown). However, SHAM sensitivity with malate plus succinate was not much different from that with these substrates individually.

The cytochrome pathway, estimated as the rate of O_2 uptake resistant to SHAM or disulfiram, also increased substantially when glycine was used either alone or in combination with other substrates (Tables 6.4, 6.5, 6.7 and 6.8).

These results suggest that, in mature leaf mitochondria, some electron transport capacity (both cytochrome and alternative pathways) is accessible to glycine but not to TCA cycle substrates. That is the activity of both the cytochrome and alternative pathways is higher when glycine is being oxidized.*

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* The conclusion related to the alternative path's engagement is correct only if SHAM and disulfiram have no effect on the glycine decarboxylation reaction itself. Some kinetic studies (Grover and Laties, 1981; Dizengremel *et al.*, 1982) show that these compounds inhibit the alternative pathway by different mechanisms. Since SHAM and disulfiram inhibited glycine oxidation to the same extent (Table 6.7), it is very likely that their effects are only on the alternative pathway.

6.3.3 Interactions Between Oxidation of NAD-Linked Substrates in Pea Leaf Mitochondria

Malate oxidation in plant mitochondria is catalysed by two enzymes — malate dehydrogenase (MDH) and NAD-linked malic enzyme (ME). Under the conditions employed here (pH 7.1), both enzymes will be active (Wiskich, 1980). A problem associated with the operation of MDH in isolated mitochondria is the production of oxalacetate (OAA). *In vivo*, of course, OAA is condensed with acetyl CoA to form citrate, but in isolated mitochondria OAA can accumulate and inhibit O_2 uptake by reversing the MDH reaction (Wiskich, 1980). To prevent this, glutamate was added together with malate to remove OAA via transamination. Glutamate itself contributes little to O_2 uptake, while the oxidation of α-ketoglutarate (α-KG, a product of the transamination of OAA and glutamate) does not proceed unless thiamine pyrophosphate (TPP, a cofactor leached from the mitochondria during isolation) is also added (Hanson and Day, 1980). The product of ME is pyruvate, and TPP is also necessary for its oxidation (Wiskich, 1980). Thus, in the presence of glutamate plus malate, but no TPP, O_2 uptake is largely due to malate oxidation alone (note, however, that when TPP is also present, the oxidation of pyruvate, citrate and α-KG may also contribute to O_2 uptake).

Oxidation of α-KG by mitochondria produces succinate, whose oxidation may also contribute to O_2 uptake. Thus when α-KG was used, small quantities of malonate were added to inhibit succinic dehydrogenase. When ADP was present, the malate-dependent rate of O_2 uptake was considerably inhibited by subsequent addition of α-ketoglutarate and citrate (Table 6.9, Expt 1), but was greatly stimulated by glycine (Table 6.9, Expt 2), as also observed by Day and Wiskich

(1981). The oxidation of α-ketoglutarate was slow and was further inhibited by citrate (Table 6.9, Expt 4), but stimulated by malate (Table 6.9, Expt 5). Glycine always stimulated O_2 uptake on top of malate, α-ketoglutarate and citrate, added in any order (Table 6.9, Expts 1, 4 and 5).

Why α-KG and citrate inhibited O_2 uptake with malate is not readily apparent, but may be due, at least in part, to competition for transport across the inner membrane (Wiskich, 1977). However, despite such competition, one would still expect to see a stimulation of O_2 uptake if α-KG and citrate had access to NAD and/or NADH dehydrogenase molecules not used during malate oxidation, since such stimulation is seen when succinate (which can also compete with malate for transport) is added to mitochondria oxidizing malate. Succinate oxidation, of course, is FAD-linked, and hence malate and succinate only compete for electron transport capacity at or beyond the ubiquinone step (Day and Wiskich, 1977). Since O_2 uptake is greater with malate plus succinate than either substrate alone, the electron transport chains beyond (i.e. on the O_2 side) of ubiquinone cannot be saturated by oxidation of only a single substrate. Since O_2 uptake with malate plus α-KG plus citrate (all NAD-linked) is either the same, or less, than that with malate alone, I conclude that these substrates compete for a common pool of NAD and/or NADH dehydrogenase(s). Similar conclusions have been drawn from studies with mitochondria from other plant tissues (Day and Wiskich, 1977; Wiskich and Day, 1982).

However, addition of glycine with other NAD-linked substrates always stimulated O_2 uptake. This suggests that glycine has access to either an NAD pool or to NADH dehydrogenases not accessible to the TCA cycle substrates. Day and Wiskich (1981) also suggested this. On the

Table 6.9

Concurrent oxidation of several NAD-linked substrates by pea (cv. Alaska) leaf mitochondria in the presence of ADP (initially added at 2 μmole). All substrates were added to 10 mM. 0.1 mM thiamine pyrophysphate and 1 mM malonate were included when α-ketoglutarate was used. Glutamate (10 mM) was added with malate. The pH of the medium was 7.1. Temperature was 23 °C.

	Sequential Additions to Vessel	State 3 Oxygen Uptake
		$nmol.min^{-1}.mg^{-1}$ protein
Expt 1:	malate + ADP	85
	α-ketoglutarate	70
	citrate	54
	glycine	135
Expt 2:	malate + ADP	87
	glycine	154
	α-ketoglutarate	147
	citrate	134
Expt 3:	glycine + ADP	96
	α-ketoglutarate	106
	malate	125
Expt 4:	α-ketoglutarate + ADP	33
	citrate	16
	malate	68
	glycine	145
Expt 5:	α-ketoglutarate + ADP	26
	malate	87
	citrate	62
	glycine	128

other hand, S_3 rates with glycine plus TCA cycle substrates was less than the sum of the individual rates (Table 6.9; see also Tables 6.4 to 6.8), showing that glycine does compete eventually for electron transport. That is, although glycine has access to additional electron transport capacity in these preparations, it can also use those shared by the TCA cycle substrates.

The state 4 rate of O_2 uptake with malate (Fig. 6.2A) and succinate (not shown) was also stimulated substantially by addition of glycine. However, the stimulated rate was very sensitive to disulfiram (Fig. 6.2B), and the disulfiram-resistant rate was only slightly higher than the state 4 rate with malate alone (Fig. 6.2B).

The glycine-dependent state 4 rate of O_2 uptake was also stimulated by malate (or succinate), but again the increase was fully sensitive to disulfiram (Fig. 6.2C). As expected, malate had practically no effect on the S_4 rate when disulfiram was added during the previous state 3 with glycine (Fig. 6.2D). The effects of disulfiram suggest that the stimulation of S_4 oxygen uptake by addition of a second substrate was due to the engagement of additional alternative path electron transport; since this pathway is non-phosphorylating, S_4 rates are higher with multiple substrates. These results also suggest that control of electron flow through the first site of energy transduction (i.e., that part of the respiratory chain between internal NADH dehydrogenase and ubiquinone) by oxidative phosphorylation is not great enough to prevent operation of the alternative pathway. The recently demonstrated bypass of the first site of energy transduction (H^+ translocation) may be involved under these conditions (Palmer and Møller, 1982).

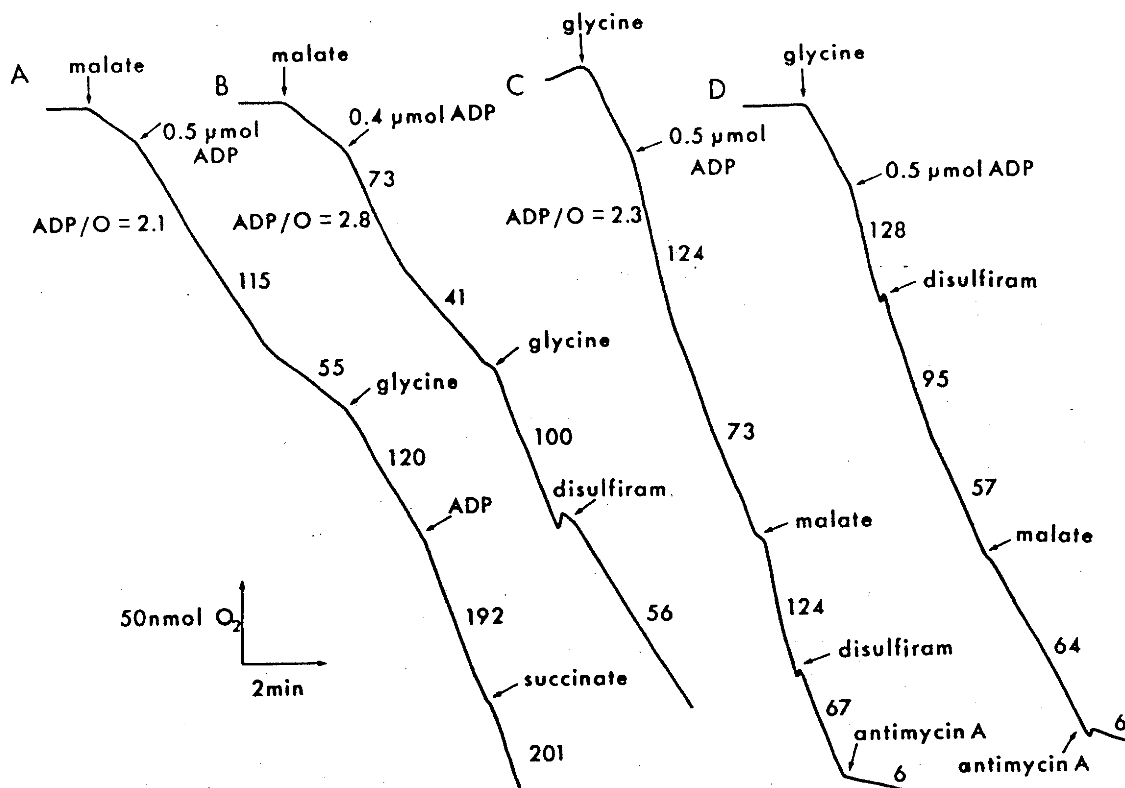

Fig. 6.2: Concurrent oxidation of malate and glycine by pea leaf mitochondria in the absence of ADP. A: cv. Alaska; B, C and D: cv. Massey; C and D are experiments from the same preparation. Assay conditions as in Table 6.7. Numbers on traces refer to nmol $O_2.min^{-1}.mg^{-1}$ protein.

6.3.4 Implications for Photorespiration

The results presented in this chapter indicate that leaf mitochondria can oxidize glycine and TCA cycle substrates simultaneously via the respiratory chain, either in the presence or in the absence of ADP. Possible mechanisms for avoiding (in part) competition between these substrates may include independent electron transport (cytochrome and alternative) pathways, and perhaps different NAD pools and/or NADH dehydrogenases. However, at this time it is not possible to say whether the indicated compartmentation of glycine and TCA cycle intermediates occurs in the same mitochondrion, or whether two separate populations (one primarily involved in photorespiration *in vivo*) were present in the mitochondrial preparations used. What is clear, however, is that there is in leaf cells some mitochondrial electron transport (including the alternative oxidase) exclusively associated with glycine oxidation.

The mechanism(s) by which NADH produced during glycine decarboxylation is reoxidized *in vivo* are still not known. As discussed in the Introduction, NADH can be reoxidized either via the respiratory chain or via a substrate shuttle *in vitro*, but there is little information to indicate which mechanism operates *in vivo*.

Recent experiments with pea leaves (H. Lambers, D.A. Day, J. Azcón-Bieto, unpublished) have shown that the decarboxylation of accumulated photorespiratory intermediates during the immediate post-illumination period (Chapter 3) is linked to O_2 uptake; O_2 uptake immediately following a 20 min period of photosynthesis was substantially greater than that before illumination, and the alternative path was fully engaged. That is, decarboxylation of glycine *in vivo* in the dark is linked to O_2 uptake via the respiratory

chain. Whether this is indicative of a similar mechanism operating in the light is not known. It is possible that the cell may switch from a shuttle-supported system to an electron transport-supported system as soon as the light is switched off. However, it is most unlikely that a specific electron transport capacity for the oxidation of glycine in leaf mitochondria evolved only to be used for a few minutes a day. It seems more probable that at least some of this electron transport is coupled to glycine decarboxylation during the light period, as well as in the dark. In the extreme case that oxidative phosphorylation is totally restricted by high cytosolic ATP/ADP ratios in the light, glycine could still be oxidized by the non-phosphorylating alternative pathway with little or no competition from TCA cycle substrates. This idea is supported by the observation that isolated leaf mitochondria can simultaneously oxidize TCA cycle substrates and glycine in state four, but only when the alternative pathway is not inhibited (Fig. 6.2).

However, it is unlikely that the reoxidation of the NADH produced in the glycine decarboxylation occurs exclusively via the alternative oxidase, because the capacity of this pathway in leaves is not big enough to account for the photorespiratory fluxes observed *in vivo* (Day and Wiskich, 1981). Further, this capacity appears to be very variable depending on the species and probably on the developmental status of the plant (see Sections 6.3.1 and 6.3.2). Therefore, the alternative pathway needs to be supplemented by either the cytochrome chain or by a shuttle system (see below) for handling high photorespiratory fluxes.

Addition of oxaloacetate (OAA) to mitochondria oxidizing glycine greatly inhibits O_2 uptake while not affecting (or sometimes

stimulating) glycine decarboxylation (Woo and Osmond, 1976; Moore *et al.*, 1977; Day and Wiskich, 1981). The equilibrium constant of the reaction catalysed by the enzyme malate dehydrogenase (MDH) favours the formation of malate from OAA under these conditions. It has been proposed that the reducing equivalents produced in the glycine decarboxylation can be transferred to the peroxisomes using shuttle systems involving malate (Day and Wiskich, 1981; Journet *et al.*, 1981). The reducing equivalents produced in other mitochondrial reactions (e.g. TCA cycle) can also be transferred to the cytosol using these shuttles (Woo *et al.*, 1980). The potential capacity of these shuttles in the pea leaf mitochondria used in the present study, appears to be very high and adequate to support simultaneous oxidation of glycine and TCA cycle substrates, since addition of OAA to mitochondria oxidizing either glycine alone or in combination with malate and succinate greatly inhibited O_2 uptake, the small resistant rate being the same in both cases (Table 6.10). That is, the capacity of MDH and the intramitochondrial NAD pool is adequate to cope with the NADH produced by both glycine decarboxylation and malate oxidation.

It therefore appears that malate dehydrogenase would not limit the *in vivo* operation of the shuttles described. However, the degree of coupling between mitochondria and peroxisomes, and the capacity of the carriers involved in these shuttles, could greatly influence the activity of these shuttles *in vivo*. The extent to which these limitations occur is not known, but a reconstituted OAA/malate shuttle in pea leaf mitochondria (Day and Wiskich, 1981) appeared to be limited by the rate of malate efflux from the mitochondria, and "whole cell" rates of glycine decarboxylation with the shuttle were less than those calculated for the respiratory chain.

Table 6.10

Effect of oxaloacetate on the respiration of pea (cv. Massey) leaf mitochondria. Assay conditions as in Table 6.7. Oxaloacetate was provided at 2 mM.

Substrates	State 3 Oxygen Uptake	
	Control	+ Oxaloacetate
	nmol $min^{-1}.mg^{-1}$ protein	
glycine	118	32
glycine + malate + succinate	231	32

I propose on the basis of the evidence presented here that the alternative pathway contributes to the reoxidation of NADH generated in the decarboxylation of glycine, but may act as an overflow of other quantitatively more important mechanisms (e.g. cytochrome pathway and/or shuttle mechanisms).

Finally, I wish to make the point that the reactions of the TCA cycle are not expected to be significantly restricted in the light, either in the presence or in the absence of ADP, as a result of concomitant photorespiratory glycine decarboxylation, because these reactions do not apparently compete at the mitochondrial level. The reducing equivalents generated in the TCA cycle (or in the anaplerotic reactions of this cycle, as discussed in Chapter 4) can be oxidized by alternative and cytochrome chains which are probably different to those used by glycine, or can be transferred to the cytosol using a shuttle (see above). The findings that there is turnover of organic acids via the TCA cycle in the light (Graham, 1980) and that significant CO_2 is evolved from illuminated leaves from sources other

than photorespiration (see Chapter 4) are consistent with these conclusions.

CHAPTER 7

RESPIRATORY CHARACTERISTICS DURING DEVELOPMENT OF BEAN LEAVES

7.1 INTRODUCTION

The preceding chapters described several aspects of respiration in mature leaves of wheat and other species. This chapter mainly deals with a study of respiratory responses of bean leaves, a common material for these sort of studies, from the initial stages of development to maturity. Wheat leaves have not been used here because they do not develop uniformly along their length.

It has been known since 1921 that the rate of respiration in the dark of a growing leaf decreases from high values to a relatively constant low value when maturity is reached (Kidd *et al.*, 1921). However, the contribution of the cytochrome and alternative pathways to respiration in developing leaves has not been evaluated.

Several reports indicate that respiration of young leaves is severely inhibited by cyanide, while that of mature leaves is little affected or even stimulated by cyanide (Marsh and Goddard, 1939; McDonald and De Kok, 1958; Ducet and Rosenberg, 1962). These observations suggest that the balance between the cytochrome and alternative pathways may change during development. Interestingly, Marsh and Goddard (1939) proposed the existence of an oxidase system other than cytochrome oxidase which was insensitive to cyanide; they suggested that both oxidases operate in young leaves, but the cyanide-

sensitive oxidase is lost during leaf development. The latter part of this hypothesis is obviously wrong because the phosphorylating cytochrome pathway must be present in mature leaves (see Chapters 5 and 6). Of course Marsh and Goddard could not foresee that the electrons can be diverted from the cytochrome pathway to the alternative path when the former was inhibited.

The CO_2 compensation point, Γ, includes a respiratory component (see Chapter 4) and, therefore, it can be used for monitoring changes in respiration in the light during leaf development. Several studies have shown that Γ follows a pattern similar to that of respiration in the dark (see Tichá and Čatský, 1981, for a review). A linear relationship between Γ and dark CO_2 efflux has been observed sometimes during leaf ontogeny (Čatský *et al.*, 1976; Peisker *et al.*, 1981).

The experiments described in this chapter investigate the developmental patterns of respiratory pathways and the relationship between Γ and dark CO_2 efflux in bean leaves.

7.2 MATERIALS AND METHODS

7.2.1 Plant Material

Phaseolus vulgaris (cv. Hawkesbury Wonder) plants were grown in pots of soil as described in Section 2.2.1. Leaflets of the fourth trifolium (in chronological order of appearance) were used. *Phaseolus vulgaris* (cv. Epicure) plants were grown hydroponically as described in Section 6.2.1. Leaflets of the second trifolium were used. Leaves were utilized after a period of several hours in the light. New leaves were used for every individual measurement.

Leaf age was calculated in days, and "day zero" was arbitrarily established as the first day in which the leaflets of the selected

trifolium were fully unfolded; this occurred approximately 3 or 4 days after the appearance of the corresponding foliar bud. Leaf area at day zero was less than 5% of maximal area which occurred at about days 6-7.

7.2.2 Measurement of O_2 Uptake in the Dark in Leaves and Leaf Slices

The rate of O_2 uptake of intact bean leaves (cv. Epicure) was measured at 25 °C as described in Section 5.2.3. O_2 uptake by bean leaf slices (cv. Hawkesbury Wonder) was measured at 22 °C as described in Section 5.2.2. Leaves were cut under a 0.2 mM $CaCl_2$ solution. The reaction medium contained 10 mM HEPES, 10 mM MES buffer pH 6, 0.2 mM $CaCl_2$.

7.2.3 Gas Exchange Techniques

The open system used for measuring CO_2 and water exchanges of intact attached leaves has been described in Appendix I. Γ was measured at 21% O_2 by interpolation of a curve of net CO_2 assimilation versus intercellular CO_2 partial pressure to zero assimilation. Γ was also measured at 2% O_2 using a closed system (see Section 4.2.2). Assuming a linear relationship between Γ and O_2 concentration (Azcón-Bieto *et al.*, 1981), a coefficient γ expressing the O_2 dependence of Γ was calculated as $\Delta\Gamma/\Delta[O_2]$. Dark CO_2 efflux was measured 30 min after the light was turned off in ambient air (330 μbar CO_2, 21% O_2).

7.2.4 Carbohydrate and Chlorophyll Determination

Free glucose plus fructose, invertase sugars, and starch fractions were measured as described in Section 2.2.6. Chlorophyll was measured using the method of Arnon (1949).

7.3 RESULTS

7.3.1 Relationship between the CO_2 Compensation Point and Dark CO_2 Efflux during Development

The photosynthetic capacity of young bean leaves was low, but progressively increased with leaf expansion (Fig. 7.1). In contrast, dark CO_2 efflux (R_n) and the CO_2 compensation point at 21% O_2, Γ_{21}, decreased rapidly over this period (Fig. 7.2). Γ measured at 2% O_2, Γ_2, was low in young bean leaves and decreased more slowly than Γ_{21} with leaf age. Similar low values of Γ_2 were also found in young leaves of *Populus* (not shown). The slope of the relationship (γ) between Γ and O_2 concentration also declined very rapidly as leaf growth progressed (Fig. 7.3). Similar developmental patterns to those described here have been found by other authors in leaves of beans and other species (Dickmann *et al.*, 1975; Čatský *et al.*, 1976; Smith *et al.*, 1976; Peisker *et al.*, 1981; Tichá and Čatský, 1981). The relationship between Γ_{21} and R_n was very strong in young leaves, but it was not strictly linear over the range of leaf ages studied, in contrast to the results of Peisker *et al.* (1981). This relationship mainly deviated from linearity because Γ_{21} reached a constant value earlier than R_n (Fig. 7.2), which has also been observed in other species (Dickmann *et al.*, 1975; Smith *et al.*, 1976).

The relationship between Γ_2 and R_n also appeared to be linear over a certain range of R_n values, but Γ_2 increased less dramatically as R_n increased (Fig. 7.3). A similar observation was made by Peisker *et al.* (1981).

7.3.2 Properties of Leaf Respiration during Development

The rate of O_2 uptake by bean leaves and leaf slices in the dark decreased during their growth period (see V_t column in Tables 7.3 and

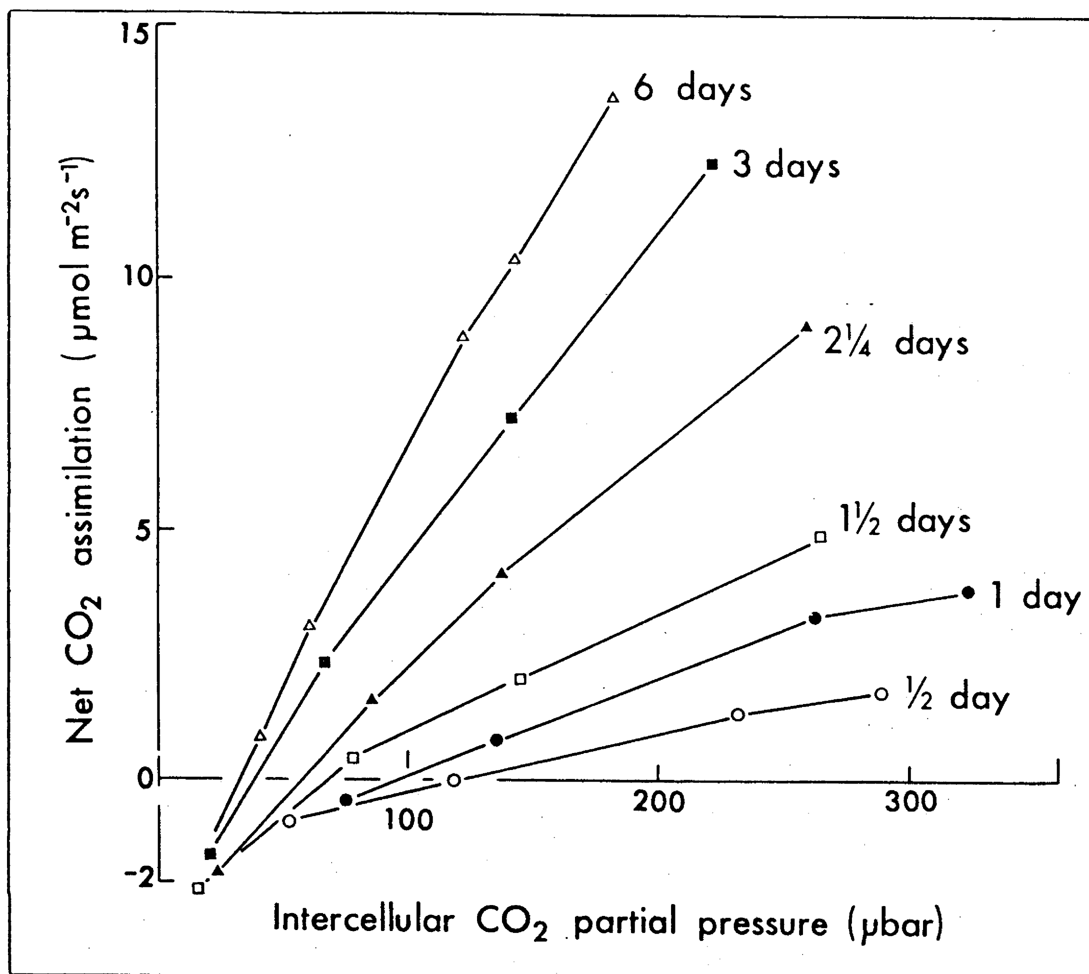

Figure 7.1: Relationship between net CO_2 assimilation and intercellular CO_2 partial pressure in growing leaves of *Phaseolus vulgaris* (cv. Hawkesbury Wonder). Leaf temperature was 25 °C and quantum flux was 1000 $\mu E.m^{-2}.s^{-1}$. O_2 concentration was 21%. See Sections 7.2.1 and 7.2.3 for further experimental details.

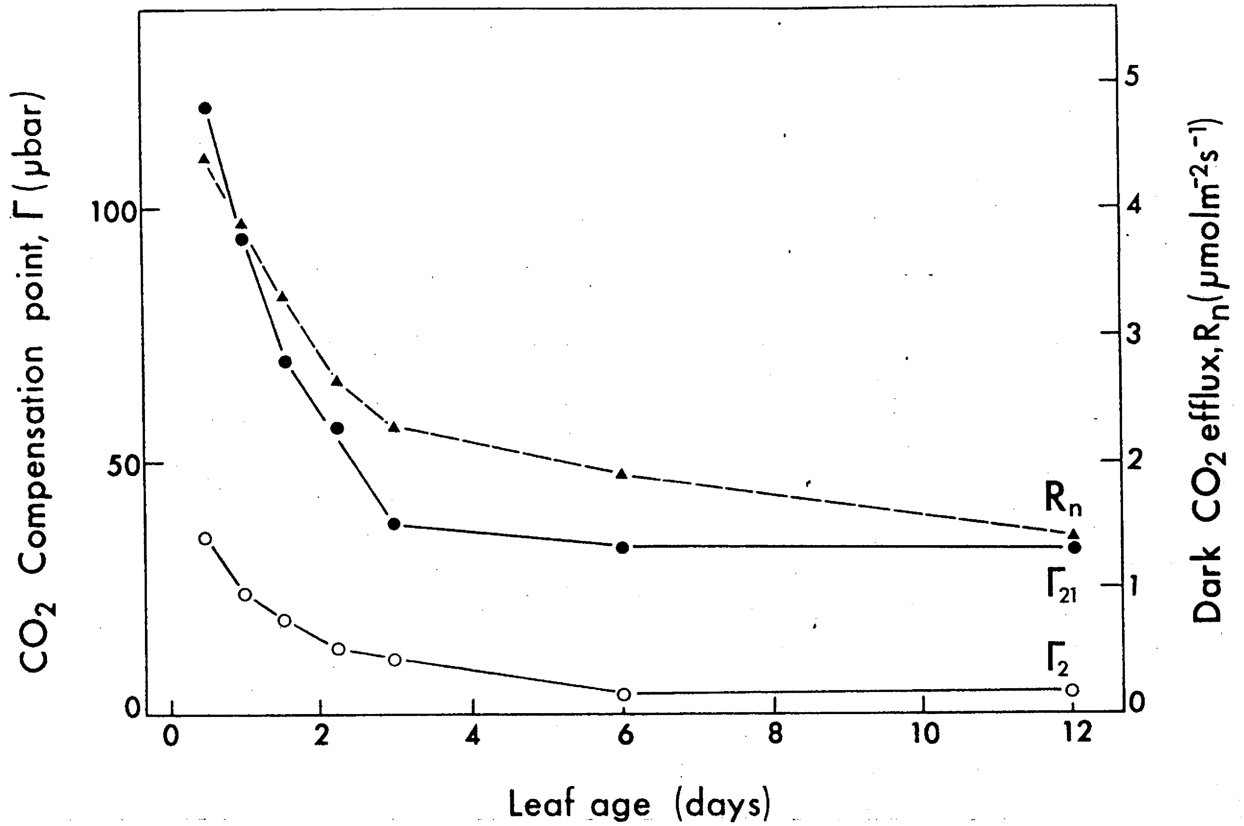

Figure 7.2: Changes in the CO_2 compensation point and dark CO_2 efflux with age in bean leaves. Γ was measured at 21 and 2% O_2. For other details see legend of Figure 7.1.

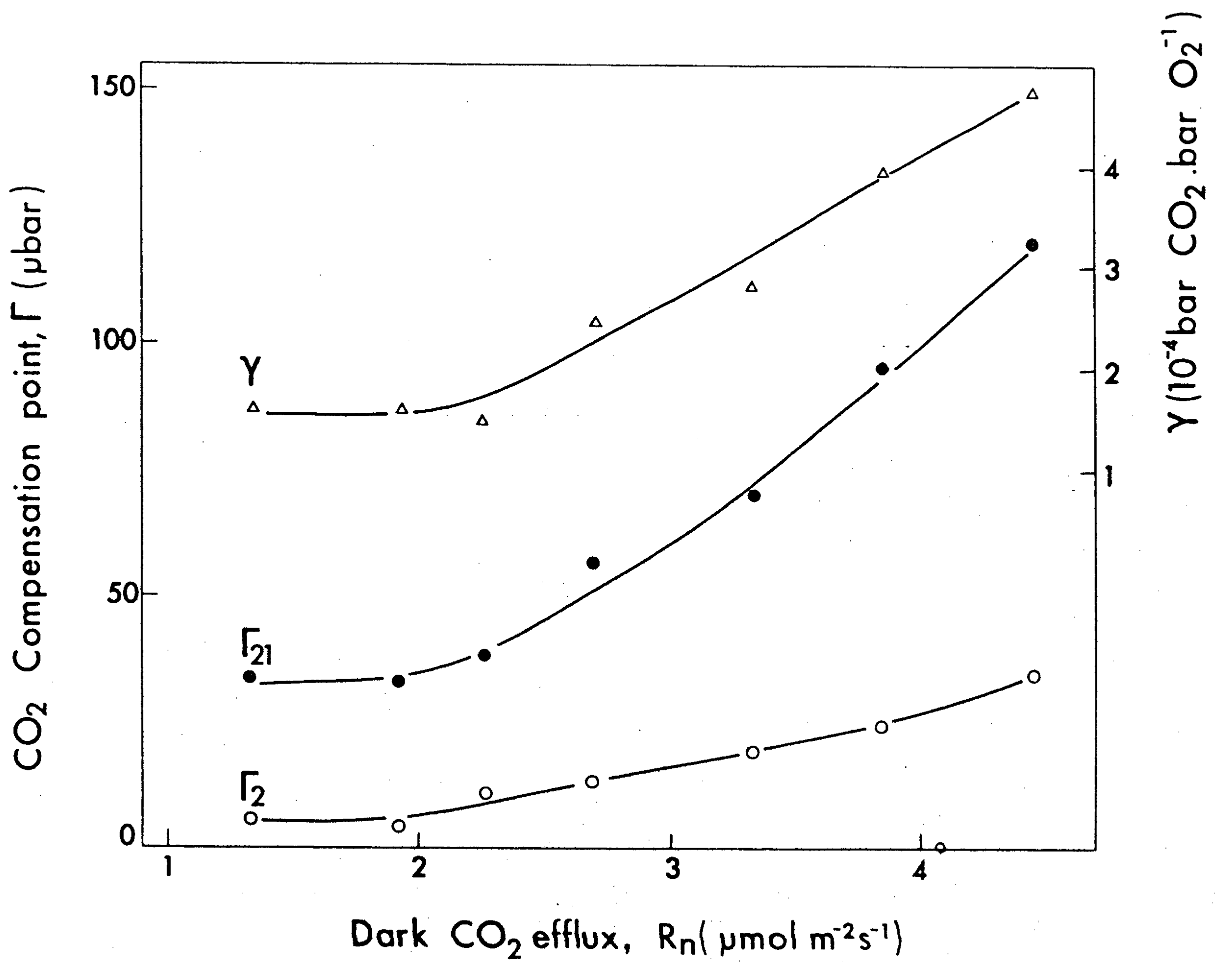

Figure 7.3: Relationships between the CO_2 compensation point at 21 and 2% O_2, the O_2 dependence of Γ (γ) and the rate of dark CO_2 efflux in growing bean leaves. For other details see legend of Figure 7.1.

7.5), showing a similar pattern to that of dark CO_2 efflux (Fig. 7.2). Expressing the rate of respiration on a chlorophyll basis does not alter the observed ontogenetic trend of respiration, since chlorophyll concentration per unit leaf area did not vary significantly throughout leaf development (Table 7.1).

The contribution of the cytochrome and alternative pathways to leaf respiration was estimated using the inhibitors KCN and SHAM. The reliability of this method in intact leaves and leaf slices has been discussed in Section 5.3.2.1. The percentage cyanide-resistance of respiration of leaves and leaf slices progressively increased with leaf maturation (see Experiment 1 of Tables 7.2 and 7.4). Sensitivity of respiration to SHAM alone was slightly different in intact leaves and in leaf slices. Whereas the rate of O_2 uptake of leaf slices showed some sensitivity to SHAM at all developmental stages studied, that of intact leaves was only inhibited by SHAM at 1-2 days of age (see Experiment 2 of Tables 7.2 and 7.4). These slight differences might have been due either to the fact that different bean cultivars were used or to the different growth conditions.

The combination of SHAM and KCN did not inhibit O_2 uptake completely, the residual component being about 10% of total respiration (Tables 7.2 and 7.4). Increasing SHAM and KCN concentrations did not abolish this residual respiration.

Respiration of bean leaf slices was stimulated by the uncoupling agent FCCP, the stimulation being more pronounced in young leaves (Table 7.4, Experiment 3). Uncoupled leaf slice respiration was also sensitive to SHAM alone (Table 7.4, Experiment 3). Exogenous sucrose (60 mM) did not stimulate the rate of O_2 uptake of leaf slices at any age either in the presence or in the absence of FCCP (not shown).

Table 7.1

Chlorophyll concentration and chlorophyll a/b ratio in bean leaves during development. The values shown are averages of at least 3 leaves.

Leaf Age	Cultivar			
	Hawkesbury Wonder		Epicure	
	Chl a+b	Chl a/b	Chl a+b	Chl a/b
days	$mmol.m^{-2}$	ratio	$mmol.m^{-2}$	ratio
½	0.22	2.51	0.42	2.48
1 - 1½	0.34	2.64	0.42	2.44
2 - 3	0.30	2.47	0.44	2.62
3 - 4	0.30	2.66	0.37	2.80
6	0.32	2.70	0.44	2.84
14	-	-	0.29	2.94

The results obtained in Tables 7.2 and 7.4 are summarized in Tables 7.3, 7.5 and 7.6 taking into account residual respiration. Here I use V_t to signify the total measured rate of O_2 uptake in the absence of inhibitors, while v_{cyt} and v_{alt}, determined as explained in the legend of Table 7.3, represent the activities of the cytochrome and alternative pathways, respectively. V_t in the absence of FCCP declined with leaf expansion mainly due to the decrease in the activity of the cytochrome path (Tables 7.3 and 7.5). The activity of the alternative path and the rate of residual respiration, V_{res}, also tended to decline during this period. However, the capacity of the alternative path, V_{alt}, remained more or less constant throughout leaf development. The ratio v_{cyt}/V_{alt}, therefore, declined, and this explains why the percentage cyanide-resistance of respiration increases with leaf expansion (see Introduction).

Table 7.2

Respiration of intact bean leaves during development.* KCN and SHAM were added at 0.5 mM and 10 mM, respectively. For other details see Section 7.2.2. Values shown are means ± S.E. of 3-4 measurements.

Additions	Days of Age					
	0 - ½	1	2	3 - 4	6	14
			$\mu mol\ O_2.m^{-2}.s^{-1}$			
Expt 1:						
None	2.69 ± 0.20	2.31 ± 0.09	1.63 ± 0.09	1.48 ± 0.21	0.66 ± 0.06	0.79 ± 0.15
KCN	0.92 ± 0.12	0.76 ± 0.14	0.54 ± 0.07	0.65 ± 0.08	0.45 ± 0.03	0.57 ± 0.14
Expt 2:						
None	2.71 ± 0.06	2.25 ± 0.12	1.77 ± 0.09	1.33 ± 0.07	0.83 ± 0.08	0.82 ± 0.04
SHAM	2.69 ± 0.05	1.82 ± 0.18	1.44 ± 0.07	1.27 ± 0.13	0.87 ± 0.08	0.82 ± 0.01
SHAM + KCN	0.31 ± 0.17	0.21 ± 0.12	0.07 ± 0.04	0.11 ± 0.06	0.17 ± 0.07	0.10 ± 0.03

* This experiment was performed in collaboration with Dr. H. Lambers.

Table 7.3

Estimation of the activities of respiratory pathways in bean leaves. The values shown were calculated from the experiments described in Table 7.2, and are means ± S.E. of 3-6 measurements. V_t is the measured rate of O_2 uptake in the absence of inhibitors. v_{cyt} is the activity of the cytochrome path, estimated by measuring O_2 uptake in the presence of SHAM. v_{alt} is the activity of the alternative pathway, and is calculated by subtracting v_{cyt} from V_t (which was corrected for V_{res}; see below). ρ is the fraction of the maximum capacity of the alternative path that is expressed; $\rho = v_{alt}/V_{alt}$, where V_{alt} is the capacity of the alternative path, estimated by measuring O_2 uptake in the presence of KCN. V_{res} is the rate of O_2 uptake resistant to both SHAM and KCN, and it was taken into account for calculating v_{cyt}, v_{alt} and V_{alt}.

Leaf Age	V_t	v_{cyt}	v_{alt}	V_{alt}	V_{res}	ρ
days			μmol $O_2.m^{-2}.s^{-1}$			ratio
0 - ½	2.70 ± 0.09	2.38 ± 0.28	0.0	0.61 ± 0.12	0.31 ± 0.17	0
1	2.28 ± 0.07	1.61 ± 0.28	0.43 ± 0.09	0.55 ± 0.14	0.21 ± 0.12	0.78
2	1.70 ± 0.06	1.38 ± 0.11	0.33 ± 0.13	0.47 ± 0.07	0.07 ± 0.04	0.70
3 - 4	1.41 ± 0.11	1.16 ± 0.18	0.06 ± 0.06	0.54 ± 0.08	0.11 ± 0.06	0.11
6	0.77 ± 0.06	0.70 ± 0.08	0.0	0.28 ± 0.03	0.17 ± 0.07	0
14	0.81 ± 0.05	0.72 ± 0.0	0.0	0.47 ± 0.14	0.10 ± 0.03	0

Table 7.4

Respiration of bean leaf slices during development. KCN and SHAM were added at 0.3 mM and 5 mM, respectively. FCCP was added at 1 μM. For other details see Section 7.2.2. Values shown are means ± S.E. of 4-5 measurements.

Sequential Additions		Days of Age 0 – ½	2	4 – 5	7
		$\mu mol\ O_2.m^{-2}.s^{-1}$			
Expt 1:	None	2.80 ± 0.10	2.19 ± 0.15	1.57 ± 0.07	1.16 ± 0.08
	KCN	1.28 ± 0.09	1.25 ± 0.13	1.42 ± 0.07	1.15 ± 0.08
	SHAM	0.33 ± 0.05	0.28 ± 0.06	0.15 ± 0.04	0.10 ± 0.02
Expt 2:	None	2.67 ± 0.06	2.32 ± 0.09	1.75 ± 0.08	1.07 ± 0.04
	SHAM	2.43 ± 0.06	2.04 ± 0.03	1.59 ± 0.05	0.96 ± 0.02
	KCN	0.29 ± 0.05	0.25 ± 0.02	0.22 ± 0.02	0.12 ± 0.01
Expt 3:	None	2.61 ± 0.09	2.26 ± 0.12	1.52 ± 0.04	1.15 ± 0.04
	FCCP	3.07 ± 0.05	2.69 ± 0.13	1.80 ± 0.08	1.29 ± 0.06
	SHAM	2.76 ± 0.09	2.39 ± 0.10	1.55 ± 0.06	1.19 ± 0.06
	KCN	0.19 ± 0.04	0.21 ± 0.02	0.10 ± 0.06	0.15 ± 0.01

The described developmental patterns of respiratory pathways were not altered by the presence of FCCP (Table 7.6). It is remarkable that the activity of the cytochrome path was higher when FCCP was present, and that the alternative pathway remained similarly engaged in these conditions. Since the alternative path was also at least partially engaged in the presence of FCCP, I assume that the estimated flux through the cytochrome path in Table 7.6 represents the total capacity of this pathway.

Carbohydrate levels were measured in the same leaves used for obtaining the slices (Table 7.7). Free fructose plus glucose concentration decreased but starch levels increased with leaf

Table 7.5

Estimation of the activities of respiratory pathways in bean leaf slices. The values shown were calculated from experiments described in Table 7.4. Symbols and definitions are the same as in Table 7.3.

Leaf Age	V_t	v_{cyt}	v_{alt}	V_{alt}	V_{res}	ρ	v_{cyt}/V_{alt}
days	$\mu mol\ O_2\ m^{-2}.s^{-1}$					ratio	ratio
0 - ½	2.70 ± 0.05	2.14 ± 0.04	0.23 ± 0.03	0.95 ± 0.06	0.28 ± 0.03	0.24	2.25
2	2.23 ± 0.07	1.79 ± 0.03	0.28 ± 0.11	0.96 ± 0.08	0.25 ± 0.02	0.29	1.86
4 - 5	1.61 ± 0.04	1.37 ± 0.03	0.16 ± 0.06	1.27 ± 0.05	0.15 ± 0.03	0.13	1.08
7	1.13 ± 0.03	0.84 ± 0.02	0.11 ± 0.02	1.05 ± 0.07	0.12 ± 0.01	0.10	0.80

Table 7.6

Estimation of the activities of respiratory pathways in bean leaf slices in the presence of 1 μM FCCP. The values shown were calculated from the experiments described in Table 7.4. Symbols and definitions are the same as in Table 7.3. The values of V_{alt} and V_{res} used for calculations were the same as in Table 7.3.

Leaf Age	V_t	v_{cyt}	v_{alt}	ρ	v_{cyt}/V_{alt}
days		$\mu mol\ O_2.m^{-2}.s^{-1}$		ratio	ratio
0 - ½	3.07 ± 0.05	2.57 ± 0.07	0.31 ± 0.05	0.33	2.71
2	2.69 ± 0.13	2.18 ± 0.09	0.30 ± 0.05	0.31	2.27
4 - 5	1.80 ± 0.08	1.45 ± 0.02	0.26 ± 0.06	0.20	1.14
7	1.29 ± 0.06	1.04 ± 0.05	0.11 ± 0.02	0.10	1.00

Table 7.7

Carbohydrate levels in bean leaves (cv. Hawkesbury Wonder) during development. The values shown are averages of at least 5 leaves.

Leaf Age	Free Fructose plus Glucose	Invertase Sugars	Starch	Total
days		$mmol\ carbon.m^{-2}$		
0 - ½	19.2	8.1	9.9	37.2
2	15.0	2.0	20.5	37.5
4 - 5	11.2	16.1	28.5	55.8
7	4.5	10.5	78.0	93.0

expansion. Sucrose showed a more irregular pattern, tending to increase in mature leaves. Therefore, the rate of respiration of bean leaf slices was correlated with the free fructose plus glucose fraction. The activity of the cytochrome pathway (Table 7.5), the extent to which FCCP stimulated respiration (Table 7.4, Experiment 3),

and the degree of engagement of the alternative path (Tables 7.5 and 7.6), were all correlated with the levels of free sugars, which feed glycolysis directly.

7.4 DISCUSSION

Bean leaf respiration seems to be regulated in a complex manner during development. The evidence suggests that both coarse and fine controls of respiration (see ap Rees, 1980b) operate in developing bean leaves.

The capacity of the phosphorylating cytochrome pathway per unit of leaf area declined with leaf expansion (Table 7.6), and this was a major factor influencing respiration in bean leaves. Geronimo and Beevers (1964) found that the amount of several electron transport enzymes, including cytochromes, decreased with age in pea leaves. In contrast, the capacity of the non phosphorylating alternative pathway remained constant during development, suggesting that the rates of synthesis and/or turnover of the components of these two pathways are subject to different control mechanisms as the leaf ages.

Adding sucrose does not stimulate O_2 uptake in the presence of FCCP. This latter observation contrasts with the fact that sucrose did stimulate respiration (mainly the alternative path) of wheat leaves in the presence of FCCP. These different responses may be due to several reasons. First, active sucrose uptake may have been inhibited by the uncoupling action of FCCP (Delrot and Bonnemain, 1981); the passive transport of sucrose may have not been sufficient to stimulate respiration in bean leaf slices (however, this seems unlikely because it appeared to be sufficient in wheat leaf slices). The second possibility, which seems more feasible, suggests that the

level of endogenous substrates was high enough to saturate glycolysis in the presence of FCCP, at all leaf ages. Since O_2 uptake rates in the presence of FCCP and added sugars decrease with leaf age but are always lower than the maximal capacity of mitochondrial electron transport at any age (determined as the sum of the *capacities* of the cytochrome and alternative paths), it is likely that the capacity of glycolysis also declines with leaf age. For example, levels of glycolytic enzymes may decrease (Smillie, 1962). The levels of free sugars, which feed glycolysis directly, varied in accordance with the coarse capacity of glycolysis. Despite the relatively large capacity of the alternative path, its engagement in normal respiration was relatively small or sometimes absent. Perhaps the apparently parallel decline in the capacities of glycolysis and cytochrome path during aging prevented full expression of the alternative path (at least under the conditions examined here). It is unclear why the alternative pathway is maintained throughout leaf development, but it is possible that the leaf cell might need to use the alternative path fully under some conditions (perhaps during the light period?).

There is also evidence that fine control of respiration operates in bean leaves at all developmental stages, in a similar manner to that observed in wheat leaf slices (see Chapter 5). The alternative path was partially engaged on top of a restricted cytochrome pathway in bean leaf slices at all developmental stages. Addition of FCCP increased the flux of electrons through both the cytochrome and alternative pathways, the greater effect being observed in young leaves (Tables 7.4, 7.5 and 7.6). Similar results were found in mature wheat leaves harvested after a period of photosynthesis, except that addition of FCCP stimulated the activity of the cytochrome path

at the expense of the alternative path, which then was not engaged (see Table 5.9B,C). This suggests that the amount of endogenous respiratory substrates was high enough in bean leaves, but not in wheat leaves, to keep the alternative path engaged on top of a saturated cytochrome pathway.

Since exogenous sucrose had no effect on the rate of O_2 uptake of bean leaf slices in the absence of FCCP, but FCCP stimulated in the absence of exogenous sucrose, it appears that glycolysis was also controlled by adenylates in bean leaves. However, glycolysis appears to be less tightly regulated by adenylates than oxidative phosphorylation in young bean leaves and mature wheat leaves (glycolytic flux must have been in excess of that through the cytochrome path for the alternative pathway to be engaged). In mature bean leaves, on the other hand, the rate of glycolysis appears to meet more closely the energy demands of the cell (FCCP stimulates only slightly and the alternative path is less active: Tables 7.5 and 7.6).

It is not known if either of the mitochondrial electron transport pathways operates in illuminated growing leaves (see Section 1.3.2.2). Some information in relation to the fluxes of CO_2 can be obtained by studying the CO_2 compensation point, Γ. The strong correlation found between Γ (measured at 21% O_2) and the rate of dark CO_2 efflux, R_n, during leaf aging suggests that considerable CO_2 production by respiration occurs in illuminated bean leaves. However, I do not expect that the ontogenetic changes of Γ are exclusively due to changes in respiration (but see Charles-Edwards, 1978), since the value of Γ in leaves of C_3 plants also depends on the kinetic properties of the enzyme rubisco (Farquhar *et al.*, 1980; Peisker *et al.*, 1981). The capacity for carboxylation (and presumably for

oxygenation) also varies during ontogeny, as indicated by the CO_2 responses of net CO_2 assimilation (Fig. 7.1), and this is probably correlated with increases in the rubisco concentration per unit leaf area during leaf expansion (O'Toole *et al.*, 1977; Secor *et al.*, 1982). The lack of linearity in the relationship between Γ and R_n over the whole range of ages studied supports this conclusion. Peisker *et al.* (1981) also concluded that the changes of Γ during leaf development are probably related to simultaneous and opposite variations of the rates of respiration and carboxylation, despite the fact that they observed a linear relationship between Γ and R_n during leaf ontogeny.

The model of Γ (Farquhar *et al.*, 1980; Azcón-Bieto *et al.*, 1981) also predicts that the values of Γ measured at 2% O_2, Γ_2, at a given age should be considerably higher (approximately double) than those observed experimentally if the same rate of respiratory CO_2 efflux is assumed to occur in the light at 21 and 2% O_2. This suggests that CO_2 production by respiration is less when the leaf is in compensation point at 2% O_2 than that occurring at 21% O_2. A similar conclusion was reached with mature wheat leaves by studying the effect of a period of photosynthesis on the value of Γ_2 (see Section 4.4). The mechanism of this inhibition is unknown, but it must be related to the presence of light because the rates of CO_2 efflux in the dark of bean and wheat leaves were found not be initially affected by decreasing O_2 concentrations to 2% (see also Dickmann *et al.*, 1975).

In summary, respiration of bean leaves during growth appears to be regulated mainly by coarse control of the capacities of glycolysis and the cytochrome pathway. However, fine control of respiration also occurs at all developmental stages within the limits imposed by the

coarse control and also involves the alternative path. It appears that both glycolysis and oxidative phosphorylation are restricted by adenylates. The availability of substrate for respiration was never a limiting factor in the conditions examined. The contribution of respiratory processes to decarboxylation in photosynthesizing bean leaves appears to be of considerable magnitude, as judged by the responses of Γ_{21} to changes in the subsequent rate of dark CO_2 efflux. However, nothing is known about the operation of mitochondrial electron transport in the light. The answer to this question may help to explain the apparent paradox that the capacity of a relatively little used pathway in the dark (the alternative path) is maintained constant during leaf expansion while the capacity of active pathways (glycolysis and cytochrome path) decreases under these conditions.

CHAPTER 8
SYNTHESIS AND SPECULATION

8.1 CARBOHYDRATE STRESS IN WHEAT LEAVES

The results obtained in this thesis suggest that accumulation of carbohydrates in wheat leaves during rapid photosynthesis is directly responsible for a reduction in the rate of photosynthesis and in the stomatal conductance, and an increase in the rate of respiration. The decline in the rate of photosynthesis was not apparently due to photo-inhibition, or stomatal or timing effects. The increase in respiration in the light, which can be attributed to the higher substrate availability from recently fixed carbon, was not sufficiently large to account for all the observed inhibition of photosynthesis. Rather, the responses are consistent with the occurrence of end product inhibition of photosynthesis by a mechanism involving a decrease in cytosolic and stromal free phosphate availability upon soluble sugar accumulation (Herold, 1980; Chapter 2). Starch also accumulated in wheat leaves, but to a much less extent than soluble sugars. However, accumulation of starch in leaves of other species has also been negatively correlated with the rate of photosynthesis at both ambient (Upmeyer and Koller, 1973; Guinn and Mauney, 1980) and at high CO_2 concentrations (Guinn and Mauney, 1980). Cave *et al.* (1981) reported that the large starch accumulation as irregularly shaped grains appeared to disrupt normal chloroplast structure in high CO_2 grown *Trifolium subterraneum* plants.

The evidence is consistent with the notion that carbohydrate accumulated in excess may constitute a *primary stress* (see Levitt, 1980a, for terminology) on leaf physiology. *Carbohydrate stress* appears to be of a reversible nature, at least in wheat leaves under the conditions studied, but its persistent occurrence could substantially limit the potential plant productivity even under apparently optimal conditions. Plants growing at high CO_2 concentrations could be particularly affected by this stress.

Carbohydrates may also accumulate in leaves as a secondary consequence of an environmental stress, such as water stress, low temperature and flooding, presumably because the rate of utilization of carbohydrates for growth is relatively more affected by the primary stress than the rate of synthesis (Levitt, 1980a,b). In some cases there might exist an interaction between the effects of the primary stress and the specific effects of carbohydrate accumulation as, for instance, in plants growing at low temperatures and high CO_2 concentration (Hofstra and Hesketh, 1975), or in some C_4 plants subjected to cold nights (Hilliard and West, 1970; Lush and Evans, 1974). However, the accumulation of carbohydrates may decrease the osmotic potential and the freezing point of cellular solutions, and therefore, may occasionally be advantageous as a mechanism of resistance to water and low temperature stresses (see Levitt, 1980a,b).

Hormonal changes occur under most stresses. Itai and Benzioni (1976) concluded that the balance between cytokinins, growth promoters, and ABA, a growth retardant, decreases in response to several stresses, such as water stress, low and high temperatures, salinity, mineral deprivation, etc. (see also Levitt, 1980a,b). There are indications that variations in ABA levels might also occur in wheat leaves during

carbohydrate stress. Wheat stomata closed and showed greater sensitivity to CO_2 when carbohydrate levels were high (Chapter 2). This response is similar to that produced by either exogenously applied ABA to non-stressed leaves (Raschke, 1975; Dubbe *et al.*, 1978), or by endogenously produced ABA under several stresses (e.g. water stress — Raschke, 1975; chilling — Raschke *et al.*, 1976).

On this basis, one can speculate that changes similar to those occurring in the balance between cytokinins and ABA under several other stresses could also occur under *carbohydrate stress*. These hormonal changes could contribute to the coordination of several processes (e.g. photosynthesis, respiration, translocation, stomata) for adjusting the balance between carbohydrate production and utilization when carbohydrate is in excess.

8.2 REGULATION OF RESPIRATION AND THE ALTERNATIVE PATHWAY IN LEAVES

The rate of respiration in the dark in mature leaves is apparently controlled by substrate availability and adenylate levels. In some species, such as wheat or spinach, carbohydrate levels, and particularly free sugars, may fluctuate diurnally, as a result of the photosynthetic activity, within limits that are regulatory for respiration. However, in peas, for instance, high free sugar levels are maintained during the diurnal cycle in spite of changes in other carbohydrate fractions, and the regulation of respiration by adenylates may predominate. In wheat leaves, whether or not the adenylate control of respiration occurs depends on the substrate levels (Chapters 5 and 6).

Mature leaf respiration usually shows high cyanide resistance, the resistant respiration being sensitive to the alternative path

inhibitor SHAM. This non-phosphorylating pathway is engaged (usually as a small fraction of its total capacity, i.e. $\rho < 0.5$) under normal respiration in the dark in several species. The use of uncouplers of phosphorylation (e.g. FCCP) showed that the alternative path operates in the dark on top of a non-saturated cytochrome path (presumably restricted by adenylate turnover) in wheat and bean leaves (Chapters 5 and 7). The distribution of electrons between the cytochrome and alternative paths is apparently consistent with the overflow model of Bahr and Bonner (1973).

Glycolysis in leaves appears to be less tightly regulated by adenylate levels than oxidative phosphorylation, since the rate of glycolysis can exceed the activity of the cytochrome chain, thus allowing expression of the alternative pathway. The mechanism by which this could be achieved has been discussed in Chapter 5, but whatever the mechanism, it is apparent that in leaves which have an active alternative pathway, the rate of glycolysis is not strictly matched to meet the energy demands of the cell. The activity of the alternative path in leaves in the dark appears to be controlled in the last instance by the availability of substrate for glycolysis (e.g. sugars), which in turn can be determined by the photosynthetic activity during the previous light period. A similar control of this path by sugars also seems to occur in other plant organs (e.g. roots) as discussed by Lambers (1982).

Respiration of bean leaves during growth appears to be mainly regulated by coarse control of the capacities of glycolysis and the cytochrome pathway. In contrast, the capacity of the alternative path remained constant during development suggesting that the rates of synthesis and/or turnover of the components of these pathways are

subjected to different control mechanisms as the leaf ages. On the other hand, the mechanisms of fine control of respiration appear not to greatly differ in young and mature leaves, but they operate within the limits imposed by the coarse control. The cytochrome path appears to be restricted in the dark by adenylates at any age, and the alternative path can operate on top of this path, especially in young leaves (Chapter 7).

The function of the alternative pathway in leaves is not known, but the persistence of this pathway during leaf growth (Chapter 7) suggests that the leaf cell might need to use the alternative path fully under some conditions. The correlation between the capacity of the alternative path and the sugar levels is consistent with the idea that this non-phosphorylating pathway may serve as a means of permitting higher rates of respiration in the face of high cytosolic energy charge (see Laties, 1982). Such respiration could be used either to remove excess carbohydrate from the cell in an energy overflow mechanism (Lambers, 1982), or to facilitate the utilization of carbohydrates in biosynthetic processes independently of their utilization in energy production. It has also been suggested that the alternative pathway could have a role in special cases, as for instance under chilling temperatures or during infections by other organisms (see Laties, 1982).

The hypothesis that the alternative path functions *primarily* as an energy overflow in leaves seems unlikely in my view because the oxidation of excess carbohydrates by the alternative oxidase could be avoided by means of a tighter adenylate control of glycolysis. This situation apparently occurs in slices from storage organs where the alternative path is usually only engaged in the presence of an

uncoupler of phosphorylation (Theologis and Laties, 1978; Laties, 1982).

As I have discussed in Chapter 4, the evidence suggests that an important function of the TCA cycle in the light may be the supply of carbon skeletons for biosynthetic reactions in the cell. Since the alternative path can operate in leaves on top of a restricted cytochrome path in the dark (Chapters 5 and 7), the possibility that the alternative pathway can also operate in the light on top of a possibly more restricted cytochrome path cannot be excluded. In this case, the alternative path could contribute to the reoxidation of the NADH generated in the anaplerotic reactions of the TCA cycle.

It is important to determine if the alternative path participates in either the anabolic or in the catabolic metabolism of the leaf, or in both, because it has implications for the biochemical characterization of growth and maintenance respiration (Penning de Vries, 1972; Thornley, 1977). Growth respiration could include a component due to the alternative path if this path is involved in the anabolic metabolism of the cell. On the other hand, it appears that, at least in wheat leaves, maintenance respiration, which resembles the end-of-night rate of respiration (Chapter 3), does not include the operation of the alternative path (Chapter 5).

There are some indications that glycine has access to different alternative pathway chains than TCA cycle substrates in mature leaf mitochondria, and it was suggested in Chapter 6 that the alternative path may contribute to the reoxidation of NADH produced in the glycine decarboxylation reaction, but mainly acting as an overflow of the cytochrome path and/or metabolite shuttle systems. The very fact that glycine is associated with specific electron transport chains in

itself implies that these operate in the light.

In summary, I favour the view that the alternative pathway in growing and mature leaves of C_3 plants is mainly involved in the synthetic respiratory metabolism, both in the light and in the dark, and that it also participates in photorespiratory metabolism. However, other functions of this path (e.g. energy overflow mechanism) cannot be excluded at this stage, especially when carbohydrates are present in excess of the demands of the plant. Information about the alternative path during senescence, and in leaves of C_4 and CAM plants, would be very useful in order to understand the precise function of this pathway in leaves.

8.3 LEAF RESPIRATION AND PLANT PRODUCTIVITY

Leaves actively evolve CO_2 by respiration not only during the night but also during the day time (Chapter 4). Since these organs constitute a large fraction of plant biomass, total leaf respiration is potentially an important factor affecting plant productivity. The results of Heichel (1971b) and Wilson (1975) confirm this suggestion. These authors found that plants of maize and perennial ryegrass with lower rates of mature leaf respiration consistently produced higher dry matter yields. Furthermore, Wilson (1982) and Wilson and Jones (1982) showed that this characteristic can be genetically selected in perennial ryegrass. These results strongly suggest that some respiration in leaves is apparently *wasteful* in terms of yield.

It has been discussed in Chapter 3 that, by virtue of the response of leaf respiration to substrate levels, some carbohydrate accumulated in excess could be respired in the leaves without any particular growth, maintenance, or other requirements. Many examples

consistent with this suggestion were given. A similar explanation was suggested by Pearman *et al.* (1981) to account for some apparently wasteful respiration in the stem and ears of wheat plants. The operation of the alternative pathway, which also depends on substrate availability, appears to be *wasteful*, at least in terms of energy production, and it is possible that when the biosynthetic demands of the leaf are already met, the carbohydrates present in excess are removed by the alternative path acting as an energy overflow system.

8.4 COROLLARY

The evidence presented in this thesis suggests that accumulation of carbohydrates in wheat leaves can be an important negative factor contributing to plant yield. On this basis, it is suggested that the efforts of plant physiologists and breeders for increasing plant productivity should not only be destined to improve the efficiency of carbohydrate production, but also to increase the efficiency of carbohydrate utilization at the plant level. Some possible objectives could include the increase in the sink capacity of the plant, and the improvement of the efficiencies of translocation and of the biochemical control of respiration (by decreasing the alternative pathway perhaps?).

APPENDICES

I. GAS EXCHANGE APPARATUS

CO_2 and water exchanges were measured in leaves using an open system gas analysis apparatus, utilizing an IR CO_2 analyzer (Beckman Instruments, model 865, Fullerton, California, U.S.A.) which was operated in both differential and absolute modes, and a dew point hygrometer (Cambridge Systems, model 880, Waltham, Massachusetts, U.S.A.).

One or two attached intact leaves were inserted in a glass and aluminium leaf chamber (total volume 150 ml). A small horizontal fan circulated the air past the leaf and provided rapid mixing within the chamber so as to ensure that the composition of the air is virtually uniform. Illumination was provided by a 2.5 kW water-cooled, high pressure, xenon-arc lamp (Osram, model XBF 2500), the UV and IR components being removed with a Schott KG 2B filter. Quantum flux density (400-700 nm) was 1000 $\mu E.m^{-2}.s^{-1}$ and was measured with a quantum sensor (Lambda Instruments, model LI-190 SR, Lincoln, Nebraska, U.S.A.). Leaf temperature, which was controlled by circulating water through a jacket, was measured with two copper-constantan thermocouples (42 S.W.G., 0.1 mm diameter) in contact with the lower surface.

Air with the desired partial pressure of CO_2 was obtained by injection of 5% CO_2 in air into CO_2-free air through a stainless steel

capillary tubing. A self-venting pressure regulator (Clippard Minimatic, model MAR-IP, Cincinati, Ohio, U.S.A.) and a pressure gauge were used to control the injection rate. CO_2-free air with different oxygen concentrations was obtained by mixing compressed ambient air with nitrogen from a cylinder, and then by passing the resulting gas through two columns of soda lime (Carbosorb, self-indicating, BDH Chemicals Ltd, Poole, England). The oxygen concentration was measured with an oxygen electrode (YSI, model 5331, Yellow Springs, Ohio, U.S.A.). The gas was then humidified in a gas washing bottle with a scintered disc. The dew point of the gas was maintained by passing the gas through a glass condenser, the temperature of the latter being controlled by circulating the water from a temperature controlled water bath. Air flow through the leaf chamber was monitored with a mass flowmeter (Hastings, model AFSC-10K, Hampton, Virginia, U.S.A.). Flowmeters with needle valves and solenoid valves were used to distribute gas flow throughout the system. Copper tubing was used in the circuit. The gas circuit described is shown in Figure A.1.

The outputs of all sensors were registered on a digital voltmeter and the outputs from the CO_2 analyzer and dew point hygrometer were continuously recorded on a two pen potentiometric recorder.

II. CALCULATION OF GAS EXCHANGE PARAMETERS

The equations used to calculate the rate of net CO_2 uptake or efflux, transpiration rate, stomatal conductance, and intercellular CO_2 partial pressure were those given by von Caemmerer and Farquhar (1981). The rate of transpiration of a leaf with surface area of one side a (m^2), is measured as the difference between the rate of water vapour entering the chamber and that leaving the chamber. Thus,

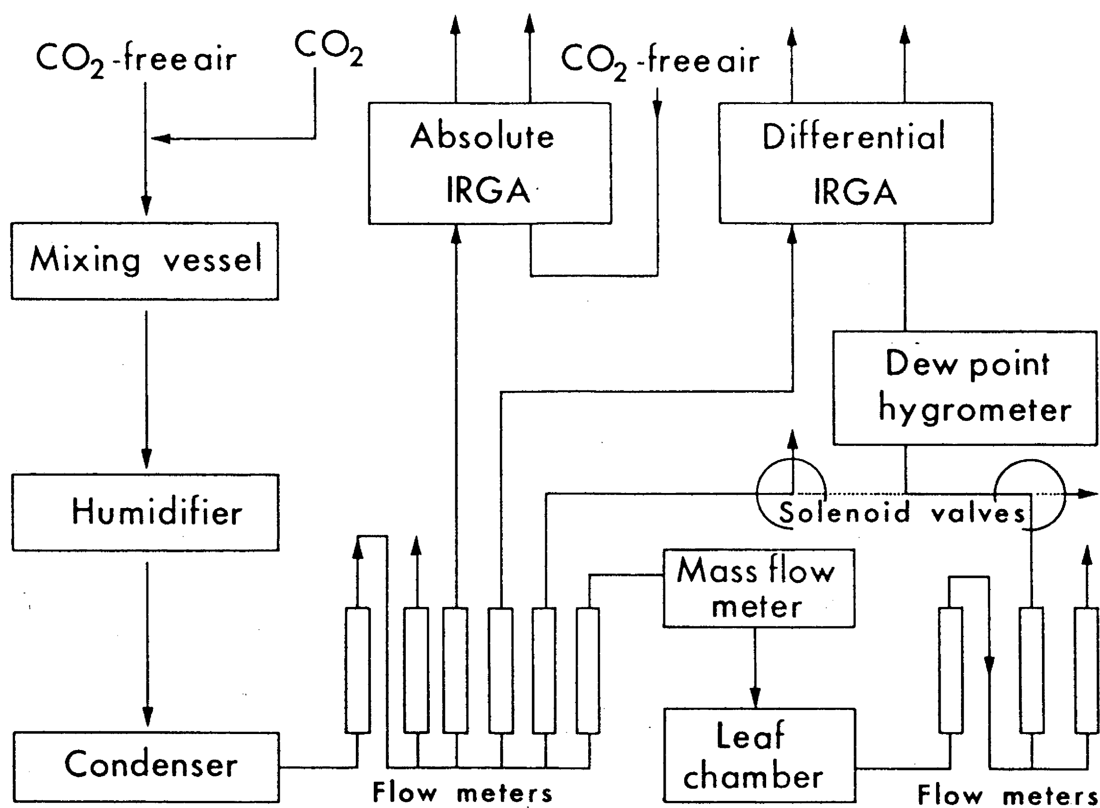

Figure A.1: Gas circuit of the open gas exchange system for whole leaf experiments.

$$E = \frac{u_o w_o - u_e w_e}{a}, \tag{A1}$$

where E is the rate of transpiration per unit leaf area (mol water. $m^{-2}.s^{-1}$); u_e, u_o are the molar flows of air entering and leaving the chamber ($mol.s^{-1}$), and w_e, w_o are the mole fractions of water vapour (bar water.bar^{-1} air) of the incoming and outgoing airstreams, respectively. Similarly,

$$A = \frac{u_e c_e - u_o c_o}{a}, \tag{A2}$$

where A is the rate of CO_2 assimilation per unit leaf area ($mol.m^{-2}.s^{-1}$) and c_e and c_o are the mole fractions of CO_2 (bar CO_2.bar^{-1} air) in the incoming and outcoming airstreams.

The uptake of CO_2 in the chamber is balanced by an efflux of oxygen, but the efflux of water vapour from the leaf increases the flow out of the chamber by the amount aE. Therefore from Eq. (A1)

$$u_o = u_e + aE . \tag{A3}$$

Combining (A1) and (A3) one obtains

$$E = \frac{u_e}{a} \cdot \frac{(w_o - w_e)}{(1 - w_o)} . \tag{A4}$$

Ice traps were used to reduce water vapour concentration in the airstreams entering the IRGA to a standard magnitude. The effect of humidification on the estimation of CO_2 assimilation rate is then eliminated. However the effect of water condensation at the ice traps needs to be considered. If u_{ice} is the flow rate at the ice traps and w_{ice} the mole fraction of water vapour at ice point

$$u_{ice} = u_e \cdot \frac{(1 - w_e)}{(1 - w_{ice})} \tag{A5}$$

and then

$$A = \frac{u_{ice}}{a}(c_e - c_o) = \frac{u_e}{a}\cdot(c_e - c_o)\cdot\frac{(1-w_e)}{(1-w_{ice})} \, . \qquad (A6)$$

Total conductance to water vapour transfer, g, was found as

$$g = \frac{E}{(w_i - w_o)} + E\,\frac{(w_i - w_o)}{2} \, , \qquad (A7)$$

where w_i, the mol fraction of water vapour in the intercellular spaces, is taken as the saturation vapour mol fraction at leaf temperature. The dimensions of g are the same as E, i.e. molar flux density. The term

$$E\,\frac{(w_i - w_o)}{2} \, ,$$

which typically accounts only for 3% of the estimate of E, is introduced to account for the molecular collisions between water vapour and air (see von Caemmerer and Farquhar, 1981).

The boundary layer conductance to water vapour, g_b, was 2.2 mol. $m^{-2}.s^{-1}$. Stomatal conductance to water vapour, g_s, is calculated from

$$\frac{1}{g} = \frac{1}{g_b} + \frac{1}{g_s} \, . \qquad (A8)$$

Stomatal (and cuticular) conductance to CO_2 can be calculated as $g_s/1.6$, and boundary layer conductance to CO_2 can be calculated as $g_b/1.37$, where 1.6 is the ratio of diffusivities of CO_2 and water in air, and 1.37 is the ratio of diffusivities of CO_2 and water vapour in the boundary layer. Total diffusive conductance to CO_2, g^c, is calculated analogously to that to water (see Eq. A8).

Intercellular CO_2 mol fraction, c_i, can be calculated from the equation

$$A = g^c(c_o - c_i) - E\,\frac{(c_o + c_i)}{2} \, . \qquad (A9)$$

The term

$$E\,\frac{(c_o + c_i)}{2}$$

is introduced to account for the interference produced by the molar flux of water leaving the stomatal pore to the entry of CO_2.

Rearranging Eq. (A9),

$$c_i = \frac{(g^c - E/2)\ c_o - A}{g^c + E/2} \quad \text{(A10)}$$

The intercellular CO_2 partial pressure, p_i, is then given by the product of c_i and the total pressure.

III. RESPIRATORY CONTROL IN ISOLATED MITOCHONDRIA

Isolated mitochondria show several states of oxygen consumption (Chance and Williams, 1956) (see Fig. A.2). In the presence of an oxidizable substrate and ADP (P_i is always present in excess) the rate of electron transport is rapid and ATP is synthesized, resulting in what is termed state 3 oxygen consumption. When ADP is depleted the rate of oxygen consumption abruptly decreases to what is known as state 4 rate. This phenomenon is called respiratory control. The ratio of state 3 to state 4, the respiratory control ratio (RCR), gives an indication of the degree to which the level of phosphate acceptor can control the rate of oxidation, and it is used to determine the degree of intactness of isolated mitochondria. State 4 rates can be substantial if the proton motive force is partly dissipated by metabolite ion transport or leakage of protons (due to membrane damage), if ATP is hydrolyzed by ATPase activity of disrupted mitochondria or from other origin (Wiskich, 1980), or if electron transport is diverted to the non-phosphorylating alternative pathway (Day *et al.*, 1980). The state 4 respiration rate also depends on the

nature of the substrate used; it is slow with malate and faster with succinate or glycine (in leaf mitochondria). It is unlikely that state 4 respiration is realized *in vivo* since ADP content is unlikely to be zero (Atkinson, 1977).

In the presence of an uncoupling agent (e.g., FCCP), which eliminates the obligatory dependence of electron flow on phosphorylation, the rate of respiration in the absence of ADP is as high as the state 3 rate (Fig. A.2); this suggests that state 3 rates are not limited by phosphorylation (Wiskich, 1980). Mitochondria in intact tissues probably operate somewhere between state 3 and state 4 rates, since uncouplers stimulate the rate of respiration of intact tissues in most cases (Day *et al.*, 1980; Wiskich, 1980).

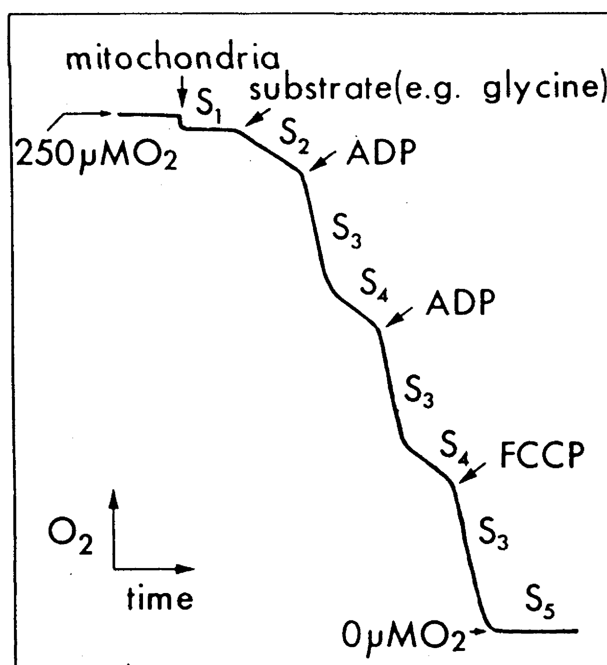

Figure A.2: Idealized oxygen electrode trace of isolated leaf mitochondria showing different states (s) of activity.

IV. MEASUREMENT OF THE ACTIVITY OF THE ALTERNATIVE PATHWAY USING A TITRATION TECHNIQUE

The contribution of the alternative path to respiration (v_{alt}) can be estimated by titration with inhibitors of the alternative pathway. This technique was introduced by Bahr (see Bahr and Bonner, 1973), and has been reviewed in detail by Solomos (1977), Day *et al.* (1980) and Laties (1982). The capacity of the alternative path (V_{alt}) is established by measuring the rate of O_2 uptake in the presence of an inhibitor of the cytochrome chain (e.g. KCN, antimycin A). The alternative path is then titrated with an inhibitor of this path (e.g. SHAM). The titration values obtained are represented by $g(i)_{alt}$ and denote the maximal possible alternative path activity in the presence of a given SHAM concentration. The titration is then repeated in the absence of cyanide (or antimycin A) to obtain V_T, the value of respiration at each SHAM concentration (see Fig. 2 in Chapter 5). V_T is then plotted against $g(i)_{alt}$ (Fig. A.3). If such a plot yields a straight line, which is usually the case (Day *et al.*, 1980), it indicates that the alternative path is superimposed on a constant contribution of the cytochrome path, v_{cyt}*. That is, varying the rate of alternative path does not affect the rate of the cytochrome path. Thus $V_T = v_{cyt} + \rho g(i)_{alt}$, where the intercept respresents the activity of the cytochrome path, and ρ, the slope of the line, represents the fraction of the maximum capacity of the alternative pathway that is expressed. The rate of the alternative path in the absence of inhibitors is $\rho g(i)_{alt}$. When $\rho = 1$ the full capacity of the alternative path is realized, and when $\rho = 0$ there is no contribution by the alternative path to V_T, and in a plot of V_T against $g(i)_{alt}$ the line will be horizontal (Fig. A.3).

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* v_{cyt} does not necessarily denote the maximum capacity of the cytochrome pathway.

If the cytochrome path were operating less than maximally and SHAM inhibition of the alternative path diverted electrons to the cytochrome path, then v_{cyt} would not be constant and a plot of V_T vs $g(i)_{alt}$ would not give a straight line. A similar result would also be obtained if SHAM inhibited the cytochrome path in addition to the alternative path. SHAM concentrations higher than 2 mM inhibit the cytochrome path in isolated mitochondria (Schonbaum *et al.*, 1971).

In cases where respiration is not completely inhibited by the simultaneous addition of SHAM and cyanide (or antimycin A) at appropriate concentrations, the expression above should be modified as follows: $V_T = \rho.g(i)_{alt} + v_{cyt} + V_{res}$, where V_{res} represents the so-called residual respiration, a value that is subtracted in graphical analyses to give V_T' which is plotted against $g(i)_{alt}$ to yield a rectilinear curve with slope ρ (Theologis and Laties, 1978).

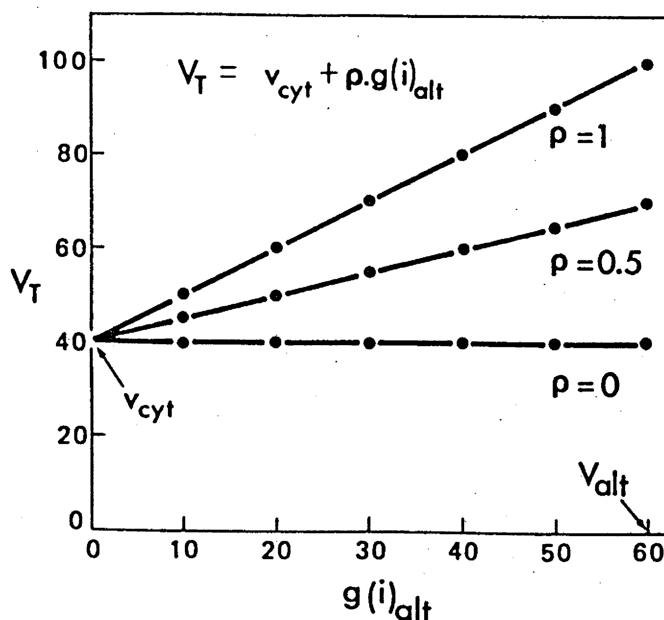

Fig. A.3: Plot of the rate of O_2 uptake obtained at several concentrations of an inhibitor of the alternative path, V_T, against a similar set of values obtained in the presence of an inhibitor of the cytochrome path, $g(i)_{alt}$. In the example shown, the capacity of the alternative path is 60% of the total electron chain capacity.

REFERENCES

Alberda, T.H., 1968. The influence of carbohydrate reserves on respiration, photosynthesis, and dry matter production of intact plants. *Proc. XI Int. Grassld. Congr.*, pp.517-522.

Allen, P.J., D.R. Goddard, 1938. A respiratory study of powdery mildew of wheat. *Amer. J. Bot.* 25:613-621.

Amir, J., J. Preiss, 1982. Kinetic characterization of spinach leaf sucrose-phosphate synthase. *Plant Physiol.* 69:1027-1030.

ap Rees, T., 1980a. Assessment of the contributions of metabolic pathways to plant respiration. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.1-30, Academic Press, New York.

ap Rees, T., 1980b. Integration of pathways of synthesis and degradation of hexose phosphates. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 3, *Carbohydrates: Structure and Function*, J. Preiss (ed.), pp.1-42, Academic Press, New York.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts; polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24:1-5.

Aslam, M., R.C. Huffaker, D.W. Rains, K.P. Rao, 1979. Influence of light and ambient carbon dioxide concentration on nitrate assimilation by intact barley seedlings. *Plant Physiol.* 63: 1205-1209.

Atkinson, D.E., 1977. *Cellular Energy Metabolism and its Regulation.* Academic Press, New York, San Francisco, London.

Austin, R.B., J. Edrich, 1975. Effects of ear removal on photosynthesis, carbohydrate accumulation and on the distribution of assimilated ^{14}C in wheat. *Ann. Bot.* 39:141-152.

Avery, D.J., C.A. Priestley, K.J. Treharne, 1979. Integration of assimilation and carbohydrate utilization in apple. In *Photosynthesis and Plant Development*, R. Marcelle, H. Clijsters and M. van Poucke (eds.), pp.221-231, Dr. W. Junk, The Hague, Boston, London.

Azcón-Bieto, J., G.D. Farquhar, A. Caballero, 1981. Effects of temperature, oxygen concentration, leaf age and seasonal variations on the CO_2 compensation point of *Lolium perenne* L. Comparison with a mathematical model including non-photorespiratory CO_2 production in the light. *Planta* 152:497-504.

Badger, M.R., G.H. Lorimer, 1981. Interaction of sugar phosphates with the catalytic site of ribulose-1,5-bisphosphate carboxylase. *Biochemistry* 20:2219-2225.

Badger, M.R., T.D. Sharkey, S. von Caemmerer, 1983. Factors limiting photosynthesis as determined by levels of photosynthetic intermediates in rapidly killed leaves of *Phaseolus vulgaris* L. related to gas exchange characteristics of the intact leaf. *Planta*, in press.

Bahr, J.T., W.D. Bonner, Jr., 1973. Cyanide-insensitive respiration. II. Control of the alternate pathway. *J. Biol. Chem.* 248: 3446-3450.

Ball, M.C., 1981. Physiological ecology of seedling establishment by *Avicennia marina*. Ph.D thesis, Australian National University, Canberra, 300 pp.

Bassham, J.A., P.O. Larsen, A.L. Lawyer, K.L. Cornwell, 1981. Relationships between nitrogen metabolism and photosynthesis. In *Nitrogen and Carbon Metabolism*, J.D. Bewley (ed.), pp.135-163, Martinus Nijhoff/Dr. W. Junk, Publishers, The Hague, Boston, London.

Bate, G.C., D.T. Canvin, 1971. The effect of some environmental factors on the growth of young aspen trees (*Populus tremuloides*) in controlled environments. *Can. J. Bot.* 49:1443-1453.

Beevers, H., 1953. 2,4-Dinitrophenol and plant respiration. *Amer. J. Bot.* 40:91-96.

Beevers, H., 1961. *Respiratory Metabolism in Plants*. Row Peterson, Evanston, Illinois.

Beevers, H., 1970. Respiration in plants and its regulation. In *Prediction and Measurement of Photosynthetic Productivity*, I. Setlik (ed.), pp.209-214, Proc. IBP/PP Technical Meeting, Trebon, 14-21 September 1969. Centre for Agricultural Publishing and Documentation (PUDOC), Wageningen.

Beevers, H., 1974. Conceptual developments in metabolic control, 1924-1974. *Plant Physiol.* 54:437-442.

Bhagsari, A.S., D.A. Ashley, R.H. Brown, H.R. Boerma, 1977. Leaf photosynthetic characteristics of determinate soybean cultivars. *Crop Sci.* 17:929-932.

Bidwell, R.G.S., 1963. Pathways leading to the formation of amino acids and amides in leaves. *Can. J. Bot.* 41:1623-1638.

Bidwell, R.G.S., G. Krotkov, G.B. Reed, 1955. The influence of light and darkness on the metabolism of radioactive glucose and glutamine in wheat leaves. *Can. J. Bot.* 33:189-196.

Birecka, H., 1968. Translocation and redistribution of ^{14}C-assimilates in cereal plants deprived of the ear. I. Spring wheat. *Bull. Acad. Polon. Sci., Sér. sci. biol.* 16:455-460.

Birecka, H., L. Dakić-Wlodkowska, 1963. Photosynthesis, translocation, and accumulation of assimilates in cereals during grain development. III. Spring wheat-photosynthesis and the daily accumulation of photosynthates in the grain. *Acta Soc. Bot. Pol.* 32:631-650.

Birecka, H., T. Skiba, Z. Kozlowska, 1969. Translocation and redistribution of ^{14}C-assimilates in cereal plants deprived of the ear. III. Assimilate distribution in root and respiration of culm in wheat plants. *Bull. Acad. Polon. Sci., Sér. sci. biol.* 17: 121-127.

Bown, A.W., T. Aung, 1974. The influence of 0.03% carbon dioxide on protein metabolism of etiolated *Avena sativa* coleoptiles. *Plant Physiol.* 54:19-22.

Bown, A.W., I.J. Dymock, T. Aung, 1974. A synergistic stimulation of *Avena sativa* coleoptile elongation by indoleacetic acid and carbon dioxide. *Plant Physiol.* 54:15-18.

Breeze, V., J. Elston, 1978. Some effects of temperature and substrate content upon respiration and the carbon balance of field beans (*Vicia faba* L.). *Ann. Bot.* 42:863-876.

Buchanan, B.B., 1980. Role of light in the regulation of chloroplast enzymes. *Ann. Rev. Plant Physiol.* 31:341-374.

Caemmerer, S. von, 1981. On the relationship between chloroplast biochemistry and gas exchange of leaves. Ph.D thesis, Australian National University, Canberra, 191 pp.

Caemmerer, S. von, G.D. Farquhar, 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153:376-387.

Canvin, D.T., 1978. Photorespiration and the effect of oxygen on photosynthesis. In *Photosynthetic Carbon Assimilation*, H.W. Siegelman, G. Hind (eds.), pp.61-76, Plenum Press, New York.

Canvin, D.T., N.D.H. Lloyd, H. Fock, K. Przybylla, 1976. Glycine and serine metabolism and photorespiration. In *CO_2 Metabolism and Plant Productivity*, R.H. Burris, C.C. Black (eds.), pp.161-176, Univ. Park Press, Baltimore, Maryland.

Čatský, J., I. Tichá, 1979. CO_2 Compensation concentration in bean leaves: Effect of photon flux density and leaf age. *Biol. Plant.* 21:361-364.

Čatský, J., I. Tichá, J. Solárová, 1976. Ontogenetic changes in the internal limitations to bean-leaf photosynthesis. 1. Carbon dioxide exchange and conductances for carbon dioxide transfer. *Photosynthetica* 10:394-402.

Cave, G., L.C. Tolley, B.R. Strain, 1981. Effect of carbon dioxide enrichment on chlorophyll content, starch content and starch grain structure in *Trifolium subterraneoum* leaves. *Physiol. Plant.* 51: 171-174.

Challa, H., 1976. An analysis of the diurnal course of growth, carbon dioxide exchange and carbohydrate reserve content of cucumber. *Agric. Res. Reports* No. 861, PUDOC, Wageningen.

Chance, B., G.R. Williams, 1956. The respiratory chain and oxidative phosphorylation. *Advan. Enzymol.* 17:65-134.

Charles-Edwards, D.A., 1978. Leaf carbon dioxide compensation points at high light flux densities. *Ann. Bot.* 42:733-739.

Chatterton, N.J., 1973. Product inhibition of photosynthesis in alfalfa leaves as related to specific leaf weight. *Crop. Sci.* 13: 284-285.

Chatterton, N.J., G.E. Carlson, W.E. Hungerford, D.R. Lee, 1972. Effect of tillering and cool nights on photosynthesis and chloroplasts starch in *Pangola*. *Crop Sci.* 12:206-208.

Clough, J.M., M.M. Peet, P.J. Kramer, 1981. Effects of high atmospheric CO_2 and sink size on rates of photosynthesis of a soybean cultivar. *Plant Physiol.* 67:1007-1010.

Coggeshall, B.M., H.F. Hodges, 1980. The effect of carbohydrate concentration on the respiration rate of soybean. *Crop Sci.* 20: 86-90.

Coudret, A., F. Ferron, J.P. Gaudillère, 1981. Photosynthates formation in wheat under different partial oxygen pressures and temperatures. *Photosynthetica* 15:21-27.

Coulson, C.L., A.L. Christy, D.A. Cataldo, C.A. Swanson, 1972. Carbohydrate translocation in sugar beet petioles in relation to petiolar respiration and adenosine 5'-triphosphate. *Plant Physiol.* 49:919-923.

Csêke, C., N.F. Weeden, B.B. Buchanan, K. Uyeda, 1982a. A special fructose bisphosphate functions as a cytoplasmic regulatory metabolite in green leaves. *Proc. Natl. Acad. Sci. USA* 79: 4322-4326.

Csêke, C., A.N. Nishizawa, B.B. Buchanan, 1982b. Modulation of chloroplast phosphofructokinase by NADPH. A mechanism for linking light to the regulation of glycolysis. *Plant Physiol.* 70:658-661.

Cunningham, G.L., J.P. Syvertsen, 1977. The effect of nonstructural carbohydrate levels on dark CO_2 release in creosotebush. *Photosynthetica* 11:291-295.

Day, D.A., 1980. Malate decarboxylation by *Kalanchoe daigremontiana* mitochondria, and its role in Crassulacean acid metabolism. *Plant Physiol.* 65:675-679.

Day, D.A., J.B. Hanson, 1977. Pyruvate and malate transport and oxidation in corn mitochondria. *Plant Physiol.* 59:630-635.

Day, D.A., J.T. Wiskich, 1977. Factors limiting respiration by isolated cauliflower mitochondria. *Phytochemistry* 16:1499-1502.

Day, D.A., J.T. Wiskich, 1981. Glycine metabolism and oxalacetate transport by pea leaf mitochondria. *Plant Physiol.* 68:425-429.

Day, D.A., G.P. Arron, G.G. Laties, 1980. Nature and control of respiratory pathways in plants: the interaction of cyanide-resistant respiration with the cyanide-sensitive pathway. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.197-241, Academic Press, New York.

Delrot, S., J.L. Bonnemain, 1981. Involvement of protons as a substrate for the sucrose carrier during phloem loading in *Vicia faba* leaves. *Plant Physiol.* 67:560-564.

Dennis, D.T., J.A. Miernyk, 1982. Compartmentation of non-photosynthetic carbohydrate metabolism. *Ann. Rev. Plant Physiol.* 33: 27-50.

De Troostembergh, J.C., E.J. Nyns, 1978. Kinetics of the respiration of cyanide-insensitive mitochondria from the yeast *Saccharomycopsis lipolytica. Eur. J. Biochem.* 85:423-432.

Dickmann, D.I., D.H. Gjerstad, J.C. Gordon, 1975. Developmental patterns of CO_2 exchange, diffusion resistance and protein synthesis in leaves of *Populus × euramericana*. In *Environmental and Biological Control of Photosynthesis*, R. Marcelle (ed.), pp.171-181, Dr. W. Junk, The Hague.

Dittrich, P., K. Raschke, 1977. Uptake and metabolism of carbohydrates by epidermal tissue. *Planta* 134:83-90.

Dixon, M., E.C. Webb, 1979. In *Enzymes*, third edition, Academic Press, New York, San Francisco, p.174.

Dizengremel, P., M. Chaveau, J. Roussaux, 1982. Inhibition by adenine derivatives of the cyanide-insensitive electron transport pathway of plant mitochondria. *Plant Physiol.* 70:585-589.

Doehlert, D.C., M.S.B. Ku, G.E. Edwards, 1979. Dependence of the post-illumination burst of CO_2 on temperature, light, CO_2 and O_2 concentrations in wheat (*Triticum aestivum*). *Physiol. Plant.* 46: 299-306.

Douillard, R., 1981. Hypothèses sur la localisation et le rôle des lipoxygénases des cellules végétales. *Physiol. Vég.* 19:533-542.

Drake, B., K. Raschke, 1974. Prechilling of *Xanthium strumarium* L. reduces net photosynthesis and independently, stomatal conductance while sensitizing stomata to CO_2. *Plant Physiol.* 53:808-812.

Dry, I.B., J.T. Wiskich, 1982. Role of the external adenosine triphosphate/adenosine diphosphate ratio in the control of plant mitochondrial respiration. *Arch. Biochem. Biophys.* 217:72-79.

Dubbe, D.R., G.D. Farquhar, K. Raschke, 1978. Effect of abscisic acid on the gain of the feedback loop involving carbon dioxide and stomata. *Plant Physiol.* 62:413-417.

Ducet, G., A.J. Rosenberg, 1962. Leaf respiration. *Ann. Rev. Plant Physiol.* 13:171-200.

Evans, L.T., I.F. Wardlaw, R.A. Fischer, 1975. Wheat. In *Crop Physiology. Some Case Histories*, L.T. Evans (ed.), pp.101-149. Cambridge University Press, Cambridge.

Farquhar, G.D., S. von Caemmerer, J.A. Berry, 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* 149:78-90.

Farquhar, G.D., S. von Caemmerer, 1982. Modelling of photosynthetic response to environmental conditions. In *Physiological Plant Ecology*, Vol. B, *Water Relations and Photosynthetic Productivity*, O.L. Lange, P.S. Nobel, C.B. Osmond, H. Ziegler (eds.), pp.549-587, Springer-Verlag, Heidelberg.

Farquhar, G.D., T.D. Sharkey, 1982. Stomatal conductance and photosynthesis. *Ann. Rev. Plant Physiol.* 33:317-345.

Farrar, J.F., 1980. The pattern of respiration rate in the vegetative barley plant. *Ann. Bot.* 46:71-76.

Ferrar, P.J., 1980. Environmental control of gas exchange in some savanna woody species. I. Controlled environment studies of *Terminalia sericea* and *Grewia flavescens*. *Oecologia* 47:204-212.

Geiger, D.R., 1976. Effects of translocation and assimilate demand on photosynthesis. *Can. J. Bot.* 54:2337-2345.

Geiger, D.R., 1979. Control of partitioning and export of carbon in leaves of higher plants. *Bot. Gaz.* 140:241-248.

Geiger, D.R., S.A. Sovonik, 1975. Effects of temperature, anoxia and other metabolic inhibitors on translocation. In *Encyclopedia of Plant Physiology, New Series*, Vol. 1, *Phloem Transport*, M. Zimmermann, J.A. Milburn (eds.), pp.256-286. Springer-Verlag, Berlin, Heidelberg, New York.

Geronimo, J., H. Beevers, 1964. Effects of aging and temperature on respiratory metabolism of green leaves. *Plant Physiol.* 39:786-793.

Giaquinta, R.T., 1980. Translocation of sucrose and oligosaccharides. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 3, *Carbohydrates: Structure and Function*, J. Preiss (ed.), pp.271-320, Academic Press, New York.

Goldstein, A.H., J.O. Anderson, R.G. McDaniel, 1980. Cyanide-insensitive and cyanide-sensitive O_2 uptake in wheat. I. Gradient-purified mitochondria. *Plant Physiol.* 66:488-493.

Goldstein, A.H., R.G. McDaniel, J.O. Anderson, 1982. Lipid oxygenation may account for alternative respiration. *Suppl. Plant Physiol.* 69:61.

Goldthwaite, J., 1974. Energy metabolism of *Rumex* leaf tissue in the presence of senescence-regulating hormones and sucrose. *Plant Physiol.* 54:399-403.

Gordon, A.J., G.J.A. Ryle, C.E. Powell, D. Mitchell, 1980a. Export, mobilization, and respiration of assimilates in uniculm barley during light and darkness. *J. Exp. Bot.* 31:461-473.

Gordon, A.J., G.J.A., Ryle, G. Webb, 1980b. The relationship between sucrose and starch during "dark" export from leaves of uniculm barley. *J. Exp. Bot.* 31:845-850.

Graham, D., 1980. Effects of light and "dark" respiration. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.525-579, Academic Press, New York.

Grodzinsky, B., L. Woodrow, 1981. Serine synthesis and CO_2 release from intermediates of the glycolate pathway. *Proc. 5th Intern. Congr. Photosynthesis*, Vol. 4, *Regulation of Carbon Metabolism*, G. Akoyunoglou (ed.), pp.551-559, Balaban Int. Sci. Services, Philadelphia.

Grover, S.D., G.G. Laties, 1981. Disulfiram inhibition of the alternative respiratory pathway in plant mitochondria. *Plant Physiol.* 68:393-400.

Guinn, G., J.R. Mauney, 1980. Analysis of CO_2 exchange assumptions: feedback control. In *Predicting Photosynthesis for Ecosystem Models*, Vol. 2, J.D. Hesketh and J.W. Jones (eds.), pp.1-16, CRC Press, Boca Ratôn, Florida.

Hampp, R., M. Goller, H. Ziegler, 1982. Adenylate levels, energy charge, and phosphorylation potential during dark-light and light-dark transition in chloroplasts, mitochondria, and cytosol of mesophyll protoplasts from *Avena sativa* L. *Plant Physiol.* 69: 448-455.

Hanson, J.B., D.A. Day, 1980. Plant mitochondria. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 1, *The Plant Cell*, N.E. Tolbert (ed.), pp.315-358, Academic Press, New York.

Harris, G.C., J.K. Cheesbrough, D.A. Walker, 1981. The effects of mannose on spinach leaf discs. *Suppl. Plant Physiol.* 67:147.

Hawker, J.S., 1967. Inhibition of sucrose phosphatase by sucrose. *Biochem. J.* 102:401-406.

Heber, U., 1974. Metabolite exchange between chloroplasts and cytoplasm. *Ann. Rev. Plant Physiol.* 25:393-421.

Heber, U., H.W. Heldt, 1981. The chloroplast envelope: structure, function and role in leaf metabolism. *Ann. Rev. Plant Physiol.* 32:139-168.

Heichel, G.H., 1970. Prior illumination and the respiration of maize leaves in the dark. *Plant Physiol.* 46:359-362.

Heichel, G.H., 1971a. Response of respiration of tobacco leaves in light and darkness and the CO_2 compensation concentration to prior illumination and oxygen. *Plant Physiol.* 48:178-182.

Heichel, G.H., 1971b. Confirming measurements of respiration and photosynthesis with dry matter accumulation. *Photosynthetica* 5: 93-98.

Henry, M.F., E.J. Nyns, 1975. Cyanide-insensitive respiration. An alternative mitochondrial pathway. *Sub-Cell. Biochem.* 4:1-65.

Herold, A., 1980. Regulation of photosynthesis by sink activity — the missing link. *New Phytol.* 86:131-144.

Hilliard, J.H., S.H. West, 1970. Starch accumulation associated with growth reduction at low temperatures in a tropical plant. *Science* 168:494-496.

Ho, L.C., 1979. Partitioning of ^{14}C-assimilate within individual tomato leaves in relation to the rate of export. In *Photosynthesis and Plant Development*, R. Marcelle, H. Clijsters, M. van Poucke (eds.), pp.243-250, Dr. W. Junk, The Hague, Boston, London.

Ho, L.C., J.H.M. Thornley, 1978. Energy requirements for assimilate translocation from mature tomato leaves. *Ann. Bot.* 42:481-483.

Hofstra, G., J.D. Hesketh, 1975. The effects of temperature and CO_2 enrichment on photosynthesis in soybean. In *Environmental and Biological Control of Photosynthesis*, R. Marcelle (ed.), pp.71-80, Dr. W. Junk, The Hague.

Holmgren, P., P.G. Jarvis, 1967. Carbon dioxide efflux from leaves in light and darkness. *Physiol. Plant.* 20:1045-1051.

Hopkinson, J.M., 1964. Studies on the expansion of the leaf surface. IV. The carbon and phosphorus economy of a leaf. *J. Exp. Bot.* 15: 125-137.

Huber, S.C., 1981a. Inter- and intra-specific variation in photosynthetic formation of starch and sucrose. *Z. Pflanzenphysiol.* Bd. 101.S:49-54.

Huber, S.C., 1981b. Interspecific variation in activity and regulation of leaf sucrose phosphate synthetase. *Z. Pflanzenphysiol.* Bd. 102.S:443-450.

Huber, S.C., D.W. Israel, 1982. Biochemical basis for partitioning of photosynthetically fixed carbon between starch and sucrose in soybean (*Glycine max* Merr.) leaves. *Plant Physiol.* 69:691-696.

Hunt, W.F., R.S. Loomis, 1979. Respiration modelling and hypothesis testing with a dynamic model of sugar-beet growth. *Ann. Bot.* 44: 5-17.

Huq, S., J.M. Palmer, 1978. Isolation of a cyanide-resistant duroquinol oxidase from *Arum maculatum* mitochondria. *FEBS Lett.* 95:217-220.

Ishii, R., G.H. Schmid, 1981. The Kok effect and its relationship to photorespiration in tobacco. *Z. Naturforsch.* 36c:450-454.

Itai, C., A. Benzioni, 1976. Water stress and hormonal response. In *Water and Plant Life. Problems and Modern Approaches*, O.L. Lange, L. Kappen, E.D. Schulze (eds.), pp.225-242, Springer-Verlag, Berlin, Heidelberg, New York.

James, W.O., 1953. *Plant Respiration.* Clarendon, Oxford.

Jones, M.B., E.L. Leafe, W. Stiles, B. Collett, 1978. Pattern of respiration of a perennial ryegrass crop in the field. *Ann. Bot.* 42:693-703.

Journet, E.P., M. Neuburger, R. Douce, 1981. Role of glutamate-oxaloacetate transaminase and malate dehydrogenase in the regeneration of NAD^+ for glycine oxidation by spinach leaf mitochondria. *Plant Physiol.* 67:467-469.

Kaiser, G., E. Martinoia, A. Wiemken, 1982. Rapid appearance of photosynthetic products in the vacuoles isolated from barley mesophyll protoplasts by a new fast method. *Z. Pflanzenphysiol.* Bd. 107.S:103-113.

Kandler, O., H. Hopf, 1980. Occurrence, metabolism, and function of oligosaccharides. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 3, *Carbohydrates: Structure and Function*, J. Preiss (ed.), pp.221-270, Academic Press, New York.

Kano, H., M. Kageyama, 1977. Effects of cyanide on the respiration of musk melon (*Cucumis melo* L.) roots. *Plant & Cell Physiol.* 18: 1149-1153.

Kelly, G.J., E. Latzko, 1980. The cytosol. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 1, *The Plant Cell*, N.E. Tolbert (ed.), pp.183-208, Academic Press, New York.

Kent, S.S., 1979. Photosynthesis in the higher plant *Vicia faba*. V. Role of malate as a precursor of the tricarboxylic acid cycle. *Plant Physiol.* 64:159-161.

Keys, A.J., 1980. Synthesis and interconversion of glycine and serine. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 5, *Amino Acids and Derivatives*, B.J. Miflin (ed.), pp.359-374, Academic Press, New York.

Kidd, F., G.E. Briggs, C. West, 1921. A quantitative analysis of the growth of *Helianthus annuus*. Part I. The respiration of the plant and its parts throughout the life cycle. *Proc. Roy. Soc.* B.92: 368-384.

King, R.W., I.F. Wardlaw, L.T. Evans, 1967. Effect of assimilate utilization on photosynthetic rate in wheat. *Planta* 77:261-276.

Kinraide, T.B., L.F. Marek, 1980. Wounding stimulates cyanide-sensitive respiration in the highly cyanide-resistant leaves of *Bryophyllum tubiflorum* Harv. *Plant Physiol.* 65:409-410.

Klingenberg, M., 1980. The ADP-ATP translocation in mitochondria, a membrane potential controlled transport. *J. Membrane Biol.* 56: 97-105.

Kok, B., 1949. On the interrelation of respiration and photosynthesis in green plants. *Biochim. Biophys. Acta* 3:625-631.

Kowallik, W., 1982. Blue light effects on respiration. *Ann. Rev. Plant Physiol.* 33:51-72.

Kramer, P.J., 1981. Carbon dioxide concentration, photosynthesis, and dry matter production. *BioScience* 31:29-33.

Ku, S.B., G.E. Edwards, D. Smith, 1978. Photosynthesis and nonstructural carbohydrate concentration in leaf blades of *Panicum virgatum* as affected by night temperature. *Can. J. Bot.* 56:63-68.

Lambers, H., 1982. Cyanide-resistant respiration: a nonphosphorylating electron transport pathway acting as an energy overflow. *Physiol. Plant.* 55:478-485.

Lambers, H., R. Noord, F. Posthumus, 1979. Respiration of *Senecio* shoots: inhibition during photosynthesis, resistance to cyanide and relation to growth and maintenance. *Physiol. Plant.* 45: 351-356.

Lambers, H., D.A. Day, J. Azcón-Bieto, 1983. Cyanide-resistant respiration in roots and leaves. Measurements with intact tissues and isolated mitochondria. *Physiol. Plant.*, in press.

Larsson, C., 1979. $^{14}CO_2$ fixation and compartmentation of carbon metabolism in a recombined chloroplast-'cytoplasm' system. *Physiol. Plant.* 46:221-226.

Laties, G.G., 1982. The cyanide-resistant, alternative path in higher plant respiration. *Ann. Rev. Plant Physiol.* 33:519-555.

Levitt, J., 1980a. *Responses of Plants to Environmental Stresses*, Vol. 1, *Chilling, Freezing and High Temperature Stresses*, Second Edition, Academic Press, New York.

Levitt, J., 1980b. *Responses of Plants to Environmental Stresses*, Vol. 2, *Water, Radiation, Salt, and Other Stresses*, Second Edition, Academic Press, New York.

Liedvogel, B., P.K. Stumpf, 1982. Origin of acetate in spinach leaf cell. *Plant Physiol.* 69:897-903.

Lorimer, G.H., T.J. Andrews, 1981. The C_2 chemo- and photorespiratory carbon oxidation cycle. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 8, *Photosynthesis*, M.D. Hatch, N.K. Boardman (eds.), pp.329-374, Academic Press, New York.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

Ludwig, L.J., T. Saeki, L.T. Evans, 1965. Photosynthesis in artificial communities of cotton plants in relation to leaf area. I. Experiments with progressive defoliation of mature plants. *Aust. J. Biol. Sci.* 18:1103-1118.

Ludwig, L.J., D.T. Canvin, 1971. The rate of photorespiration during photosynthesis and the relationship of the substrate of light respiration to the products of photosynthesis in sunflower leaves. *Plant Physiol.* 48:712-719.

Ludwig, L.J., D.A. Charles-Edwards, A.C. Withers, 1975. Tomato leaf photosynthesis and respiration in various light and carbon dioxide environments. In *Environmental and Biological Control of Photosynthesis*, R. Marcelle (ed.), pp.29-36, Dr. W. Junk, The Hague.

Lush, W.M., L.T. Evans, 1974. Translocation of photosynthetic assimilate from grass leaves, as influenced by environment and species. *Aust. J. Plant Physiol.* 1:417-431.

MacDonald, I.R., P.C. De Kok, 1958. The stimulation of leaf respiration by respiratory inhibitors. *Physiol. Plant.* 11:464-477.

Mangat, B.S., W.B. Levin, R.G.S. Bidwell, 1974. The extent of dark respiration in illuminated leaves and its control by ATP levels. *Can. J. Bot.* 52:673-681.

Marsh, P.B., D.R. Goddard, 1939. Respiration and fermentation in the carrot, *Daucus carota*. I. Respiration. *Amer. J. Bot.* 26:724-728.

Meeuse, B.J.D., 1975. Thermogenic respiration in aroids. *Ann. Rev. Plant Physiol.* 26:117-126.

Meidner, H., 1970. Precise measurements of carbon dioxide exchange by illuminated leaves near the compensation point. *J. Exp. Bot.* 21: 1067-1075.

Miflin, B.J., P.J. Lea, 1977. Amino acid metabolism. *Ann. Rev. Plant Physiol.* 28:299-329.

Miller, M.G., R.L. Obendorf, 1981. Use of tetraethylthiurum disulfide to discriminate between alternative respiration and lipoxygenase. *Plant Physiol.* 67:962-964.

Milner, H.W., W.M. Hiesey, 1964. Photosynthesis in climatic races of *Mimulus*. II. Effect of time and CO_2 concentration on rate. *Plant Physiol.* 39:746-750.

Mitchell, P., 1966. *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation.* Glynn Research, Bodmin, Cornwall, England.

Møller, I.M., J.M. Palmer, 1982. Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria. *Physiol. Plant.* 54: 267-274.

Moore, A.L., C. Jackson, B. Haliwell, J.E. Dench, D.O. Hall, 1977. Intramitochondrial localisation of glycine decarboxylase in spinach leaves. *Biochem. Biophys. Res. Commun.* 78:483-491.

Moser, L.E., J.J. Volenec, C.J. Nelson, 1982. Respiration, carbohydrate content, and leaf growth of tall fescue. *Crop. Sci.* 22: 781-786.

Moutot, F., E. Jolivet, 1981. Photosynthesis and photorespiratory metabolisms of fiber and linseed flax (*Linum usitatissimum* L.), *Proc. 5th Intern. Congr. Photosynthesis*, Vol. 6, *Photosynthesis and Productivity, Photosynthesis and Environment*, G. Akoyunoglou (ed.), pp.87-96, Balaban Int. Sci. Services, Philadelphia.

McCaig, T.N., R.D. Hill, 1977. Cyanide-insensitive respiration in wheat: cultivar differences and effects of temperature, carbon dioxide, and oxygen. *Can. J. Bot.* 55:549-555.

McCree, K.J., 1970. An equation for the rate of respiration of white clover plants grown under controlled conditions. In *Prediction and Measurement of Photosynthetic Productivity*, I. Setlik (ed.), pp.221-229, Proc. IBP/PP Technical Meeting, Trebon, 14-21 September 1969, Centre for Agricultural Publishing and Documentation (PUDOC), Wageningen.

McCree, K.J., 1974. Equations for the rate of dark respiration of white clover and grain sorghum, as functions of dry weight, photosynthetic rate, and temperature. *Crop. Sci.* 14:509-514.

McCree, K.J., J.H. Troughton, 1966. Prediction of growth rate at different light levels from measured photosynthesis and respiration rates. *Plant Physiol.* 41:559-566.

Neales, T.F., L.D. Incoll, 1968. The control of leaf photosynthesis rate by the level of assimilate concentration in the leaf: a review of the hypothesis. *Bot. Rev.* 34:107-125.

Neuburger, M., E.P. Journet, R. Bligny, J.P. Carde, R. Douce, 1982. Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. *Arch. Biochem. Biophys.* 217:312-323.

Osmond, C.B., 1981. Photorespiration and photoinhibition. Some implications for the energetics of photosynthesis. *Biochim. Biophys. Acta* 639:77-98.

Osmond, C.B., J.A.M. Holtum, 1981. Crassulacean acid metabolism. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 8, *Photosynthesis*, M.D. Hatch, N.K. Boardman (eds.), pp.283-328, Academic Press, New York.

O'Toole, J.C., P.M. Ludford, J.L. Ozbun, 1977. Gas exchange and enzyme activity during leaf expansion in *Phaseolus vulgaris*. *New Phytol.* 78:565-571.

Palmer, J.M., 1976. The organization and regulation of electron transport chain in plant mitochondria. *Ann. Rev. Plant Physiol.* 27:133-157.

Palmer, J.M., I.M. Møller, 1982. Regulation of NAD(P)H dehydrogenases in plant mitochondria. *Trends Biochem. Sci.* 7:258-261.

Parrish, D.J., A.C. Leopold, 1978. Confounding of alternate respiration by lipoxygenase activity. *Plant Physiol.* 62:470-472.

Pearman, I., S.M. Thomas, G.N. Thorne, 1981. Dark respiration of several varieties of winter wheat given different amounts of nitrogen fertilizer. *Ann. Bot.* 47:535-546.

Peisker, M., P. Apel, 1980. Dark respiration and the effect of oxygen on CO_2 compensation concentration in wheat leaves. *Z. Pflanzenphysiol.* Bd. 100.S:389-395.

Peisker, M., I. Tichá, J. Čatský, 1981. Ontogenetic changes in the internal limitations to bean-leaf photosynthesis. 7. Interpretation of the linear correlation between CO_2 compensation concentration and CO_2 evolution in darkness. *Photosynthetica* 15:161-168.

Penning de Vries, F.W.T., 1972. Respiration and growth. In *Crop Processes in Controlled Environments*, A.R. Rees, K.E. Cockshull, D.W. Hand, R.G. Hurd (eds.), pp.327-347, Academic Press, London, New York.

Penning de Vries, F.W.T., 1975. The cost of maintenance processes in plant cells. *Ann. Bot.* 39:77-92.

Penning de Vries, F.W.T., A.H.M. Brunsting, H.H. Van Laar, 1974. Products, requirements and efficiency of biosynthesis. *J. theor. Biol.* 45:339-377.

Penning de Vries, F.W.T., J.M. Witlage, D. Kremer, 1979. Rates of respiration and of increase in structural dry matter in young wheat, ryegrass, and maize plants in relation to temperature, to water stress and to their sugar content. *Ann. Bot.* 44:595-609.

Platt, S.G., Z. Plaut, J.A. Bassham, 1977. Steady-state photosynthesis in alfalfa leaflets. Effects of carbon dioxide concentration. *Plant Physiol.* 60:230-234.

Porter, H.K., V.C. Runeckles, 1956. Some effects of 2,4-dinitrophenol on the metabolism of tobacco leaf disks. *Biochim. Biophys. Acta* 20:100-108.

Potter, J.R., P.J. Breen, 1980. Maintenance of high photosynthetic rates during the accumulation of high leaf starch levels in sunflower and soybean. *Plant Physiol.* 66:528-531.

Pradet, A., P. Raymond, 1982. Adenylate energy charge, an indicator of energy metabolism. In *Physiology and Biochemistry of Plant Respiration*, J.M. Palmer (ed.), Cambridge Univ. Press, in press.

Preiss, J., 1982. Regulation of the biosynthesis and degradation of starch. *Ann. Rev. Plant Physiol.* 33:431-454.

Raison, J.K., 1980. Effect of low temperature on respiration. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.613-626, Academic Press, New York.

Randall, D.D., C.V. Givan, 1981. Subcellular location of $NADP^+$-isocitrate dehydrogenase in *Pisum sativum* leaves. *Plant Physiol.* 68:70-73.

Raschke, K., 1970. Stomatal responses to pressure changes and interruptions in the water supply of detached leaves of *Zea mays* L. *Plant Physiol.* 45:415-423.

Raschke, K., 1975. Simultaneous requirement of carbon dioxide and abscisic acid for stomatal closing in *Xanthium strumarium* L. *Planta* 125:243-259.

Raschke, K., M. Pierce, C.C. Popiela, 1976. Abscisic acid content and stomatal sensitivity to CO_2 in leaves of *Xanthium strumarium* L. after pretreatments in warm and cold growth chambers. *Plant Physiol.* 57:115-121.

Rich, P.R., 1978. Quinol oxidation in *Arum maculatum* mitochondria and its application to the assay, solubilisation and partial purification of the alternative oxidase. *FEBS Lett.* 96:252-256.

Robinson, S.P., D.A. Walker, 1981. Photosynthetic carbon reduction cycle. In *The Biochemistry of Plants. A Comprehensive Treatise* Vol. 8, *Photosynthesis*, M.D. Hatch, N.K. Boardman (eds.), pp.193-236, Academic Press, New York.

Rook, D.A., 1969. The influence of growing temperature on photosynthesis and respiration of *Pinus radiata* seedlings. *N.Z. J. Bot.* 7:43-55.

Ryle, G.J.A., J.M. Cobby, C.E. Powell, 1976. Synthetic and maintenance losses of $^{14}CO_2$ in uniculm barley and maize. *Ann. Bot.* 40:571-586.

Saglio, P.H., A. Pradet, 1980. Soluble sugars, respiration, and energy charge during ageing of excised maize root tips. *Plant Physiol.* 66:516-519.

Schonbaum, G.R., W.D. Bonner, Jr., B.T. Storey, J.T. Bahr, 1971. Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiol.* 47: 124-128.

Secor, J., D.M. Ford, R. Shibles, 1982. Ontogenetic changes in ribulose-1,5-bisphosphate carboxylase-oxygenase activity in soybean leaves. *Plant Sci. Lett.* 27:147-154.

Setter, T.L., W.A. Brun, M.L. Brenner, 1980. Effect of obstructed translocation in leaf abscisic acid, and associated stomatal closure and photosynthesis decline. *Plant Physiol.* 65:1111-1115.

Shingles, R.M., G.P. Arron, R.D. Hill, 1982. Alternative pathway respiration and lipoxygenase activity in aged potato slice mitochondria. *Plant Physiol.* 69:1435-1438.

Sicher, R.C., W.G. Harris, D.F. Kremer, N.J. Chatterton, 1982. Effects of shortened day length upon translocation and starch accumulation by maize, wheat, and pangola grass leaves. *Can. J. Bot.* 69:1304-1309.

Silvius, J.E., D.F. Kremer, D.R. Lee, 1978. Carbon assimilation and translocation in soybean leaves at different stages of development. *Plant Physiol.* 62:54-58.

Smillie, R.M., 1962. Photosynthetic and respiratory activities of growing pea leaves. *Plant Physiol.* 37:716-721.

Smith, E.W., N.E. Tolbert, H.S. Ku, 1976. Variables affecting the CO_2 compensation point. *Plant Physiol.* 58:143-146.

Solomos, T., 1977. Cyanide-resistant respiration in higher plants. *Ann. Rev. Plant Physiol.* 28:279-297.

Splittstoesser, W.E., 1966. Dark CO_2 fixation and its role in the growth of plant tissue. *Plant Physiol.* 41:755-759.

Stitt, M., T. ap Rees, 1978. Pathways of carbohydrate oxidation in leaves of *Pisum sativum* and *Triticum aestivum*. *Phytochemistry* 17: 1251-1256.

Stitt, M., H.W. Heldt, 1981. Simultaneous synthesis and degradation of starch in spinach chloroplasts in the light. *Biochim. Biophys. Acta* 638:1-11.

Stitt, M., R. McC. Lilley, H.W. Heldt, 1982. *Plant Physiol.* 70: 971-977.

Storey, B.T., 1976. Respiratory chain of plant mitochondria. XVIII. Point of interaction of the alternate oxidase with the respiratory chain. *Plant Physiol.* 58:521-525.

Storey, B.T., 1980. Electron transport and energy coupling in plant mitochondria. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.125-195, Academic Press, New York.

Stoy, V., 1965. Photosynthesis, respiration, and carbohydrate accumulation in spring wheat in relation to yield. *Physiol. Plant. Suppl.* 4, 125 pp.

Stumpf, P.K., 1980. Biosynthesis of saturated and unsaturated fatty acids. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 4, *Lipids: Structure and Function*, P.K. Stumpf (ed.), pp.177-204, Academic Press, New York.

Tanaka, A., 1977. Photosynthesis and respiration in relation to productivity of crops. In *Biological Solar Energy Conversion*, A. Mitsui, S. Miyachi, A. San Pietro, S. Tamura (eds.), pp.213-229, Academic Press, New York, London, San Francisco.

Tenhunen, J.D., J.D. Hesketh, P.C. Harley, 1980. Modelling C_3 respiration in the light. In *Predicting Photosynthesis for Ecosystem Models*, Vol. 2, J.D. Hesketh, J.W. Jones (eds.), pp.17-47, CRC Press, Boca Ratón, Florida.

Tetley, R.M., K.V. Thimann, 1974. The metabolism of oat leaves during senescence. I. Respiration, carbohydrate metabolism, and the action of cytokinins. *Plant Physiol.* 54:294-303.

Theologis, A., G.G. Laties, 1978. Relative contribution of cytochrome mediated and cyanide-resistant electron transport in fresh and aged potato slices. *Plant Physiol.* 62:232-237.

Thornley, J.H.M., 1977. Growth, maintenance and respiration: A re-interpretation. *Ann. Bot.* 41:1191-1203.

Thorpe, N., 1980. Accumulation of carbon compounds in the epidermis of five species with either different photosynthetic systems or stomatal structure. *Plant, Cell Environ.* 3:451-460.

Tichá, I., J. Čatský, 1981. Photosynthetic characteristics during ontogenesis of leaves. 5. Carbon dioxide compensation concentration. *Photosynthetica* 15:401-428.

Tolbert, N.E., 1980. Photorespiration. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.487-523, Academic Press, New York.

Troughton, J.H., B.G. Currie, F.H. Chang, 1977. Relations between light level, sucrose concentration, and translocation of carbon 11 in *Zea mays* leaves. *Plant Physiol.* 59:808-820.

Turner, J.F., D.H. Turner, 1980. The regulation of glycolysis and the pentose phosphate pathway. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.279-316, Academic Press, New York.

Upmeyer, D.J., H.R. Koller, 1973. Diurnal trends in net photosynthetic rate and carbohydrate levels of soybean leaves. *Plant Physiol.* 51:871-874.

Usuda, H., G.E. Edwards, 1982. Influence of varying CO_2 and orthophosphate concentrations on rates of photosynthesis, and synthesis of glycolate and dihydroxyacetone phosphate by wheat chloroplasts. *Plant Physiol.* 69:469-473.

Walton, D.C., 1980. Biochemistry and physiology of abscisic acid. *Ann. Rev. Plant Physiol.* 31:453-489.

Wardlaw, I.F., 1968. The control and pattern of movement of carbohydrates in plants. *Bot. Rev.* 34:79-105.

Wilson, D., 1975. Variation in leaf respiration in relation to growth and photosynthesis of *Lolium*. *Ann. Appl. Biol.* 80:323-338.

Wilson, D., 1982. Response to selection for dark respiration rate of mature leaves in *Lolium perenne* and its effects on growth of young plants and simulated swards. *Ann. Bot.* 49:303-312.

Wilson, D., J.G. Jones, 1982. Effect of selection for dark respiration rate of mature leaves on crop yields of *Lolium perenne* cv. S23. *Ann. Bot.* 49:313-320.

Winzeler, H., L. Hunt, J.D. Mahon, 1976. Ontogenetic changes in respiration and photosynthesis in a uniculm barley. *Crop Sci.* 16: 786-790.

Wiskich, J.T., 1977. Mitochondrial metabolite transport. *Ann. Rev. Plant Physiol.* 28:45-69.

Wiskich, J.T., 1980. Control of the Krebs cycle. In *The Biochemistry of Plants. A Comprehensive Treatise*, Vol. 2, *Metabolism and Respiration*, D.D. Davies (ed.), pp.243-278, Academic Press, New York.

Wiskich, J.T., D.A. Day, 1982. Malate oxidation, rotenone-resistance, and alternative path activity in plant mitochondria. *Plant Physiol.*, in press.

Wong, S.C., I.R. Cowan, G.D. Farquhar, 1979. Stomatal conductance correlates with photosynthetic capacity. *Nature* 282:424-426.

Woo, K.C., C.B. Osmond, 1976. Glycine decarboxylation in mitochondria isolated from spinach leaves. *Aust. J. Plant Physiol.* 3:771-785.

Woo, K.C., D.T. Canvin, 1980. The role of malate in nitrate reduction in spinach leaves. *Can. J. Bot.* 58:517-521.

Woo, K.C., M. Jokinen, D.T. Canvin, 1980. Reduction of nitrate via a dicarboxylate shuttle in a reconstituted system of supernatant and mitochondria from spinach leaves. *Plant Physiol.* 65:433-436.

Yoshida, S., Tagawa, F., 1979. Alteration of the respiratory function in chill-sensitive callus due to low temperature stress. I. Involvement of the alternate pathway. *Plant and Cell Physiol.* 20:1243-1250.

Yoshino, M., K. Murakami, 1982. AMP deaminase reaction as a control system of glycolysis in yeast. Activation of phosphofructokinase and pyruvate kinase by the AMP deaminase-ammonia system. *J. Biol. Chem.* 257:2822-2828.