Biochemical Basis of the Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis in Mesophyll Protoplasts of Pea *(Pisum sativum* L.)

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Biochemical Basis of the Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis in Mesophyll Protoplasts of Pea *(Pisum sativum)*"** has been carried out by me under the supervision of Professor A.S. Raghavendra, Dept of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad - 500 046, and that this work has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"Biochemical Basis of the Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis in Mesophyll Protoplasts of Pea *(Pisum sativum)*"** is based on the results of the work done by **Ms K. P. M.** S. **V. Padmasree** for the degree **of Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University.

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To

My Parents

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ABBREVIATIONS

AOX	=	alternative oxidase
BPGA	=	1,3-bisphosphoglyceric acid
CCCP	=	carbonyl cyanide *m*-chlorophenylhydrazone
DCMU	=	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DHAP	=	dihydroxyacetone phosphate
F2,6BP	=	fructose 2,6-bisphosphate
FBP	=	fructose 1,6-bisphosphate
FBPase	=	fructose 1,6 bisphoshatase
GAP	=	glyceraldehyde-3-phosphate
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
Glc-6-P	=	glucose-6-phosphate
Glc-6-P-D	=	glucose-6-phosphate dehydrogenase
HK	=	hexokinase
LDH	=	lactate dehydrogenase
LEDR	=	Light enhanced dark respiration
MDH	=	malate dehydrogenase
ME	=	malic enzyme
OAA	=	oxaloacetate
OEC	=	oxygen evolving complex
2-OG	=	2-oxoglutarate
PCRC	=	photosynthetic carbon reduction cycle (or) Calvin cycle
3-PGA	=	3-phosphoglyceric acid (or) 3-phosphoglycerate
PK	=	pyruvate kinase
PRK	=	phosphoribulokinase
Ru5P	=	ribulose-5-phosphate
RuBP	=	ribulose 1,5-bisphosphate
SHAM	=	salicylhydroxamic acid
triose-P	=	triose-phosphate

All remaining abbreviations are according to those found in ***Plant Physiology,*** January 1997, **vol 113, No.1.** pp. xiii-xv.

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Chapter 1

Introduction and Review of Literature

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Introduction and Review of Literature

The requirements of ATP and reducing power (NAD(P)H) for the cells are met by not only photosynthetic reactions in chloroplasts but also oxidative metabolism of mitochondria. Besides mitochondria and cytosol, the reduced equivalents are used up during photorespiration as well as nitrogen metabolism. As a result, in a plant cell, the processes of photosynthesis, respiration, nitrogen metabolism and photorespiration are dependent on each other (Turpin and Weger, 1990; Azcon-Bieto, 1992; Raghavendra et al., 1994; Gardeström and Lernmark, 1995; Kromer. 1995; Padmasree and Raghavendra, 1998). Such strong interaction between the processes of photosynthesis, respiration and photorespiration needs an efficient co-ordination of different cellular compartments of not only chloroplasts and cytosol but also mitochondria and peroxisomes (Husic et al., 1987; Raghavendra et al., 1994; Kromer 1995).

Photosynthesis results in O_2 evolution and the generation of ATP and NADPH, which are then used for the reduction of CO_2 (or other compounds like NO_2^- or SO_4^{2-}). On the other hand, respiration accomplishes oxidation of carbon compounds and evolution of CO_2. NADH produced in these reactions is utilised for ATP production and oxygen consumption. Thus, ATP is generated in both processes, while pyridine nucleotides are reduced during photosynthesis, but are oxidized in respiration. The biochemical nature of photosynthetic and respiratory reactions implies that these two processes are complementary to each other.

Long-Term Interactions

The rate of dark respiration in leaves, is higher after a few hours of illumination than that during the steady-state in prolonged darkness. Such an

increase in dark respiration is often proportional to the period of illumination and depends on temperature. The high rates of respiration decline slowly to reach back the lower level of a steady state, characteristic of the dark period. The increase in respiratory rate is believed to be primarily due to the accumulation of carbohydrates. This effect of photosynthetic activity on respiration should be treated as a long-term effect since the interaction becomes pronounced only after hour(s) of illumination. A long-term (spanning hours or days) illumination increases the carbohydrate content and the respiratory capacity of the tissue. This occurs in several tissues. e.g. in roots with increased carbohydrate flux from the shoots or in cells growing in culture (Azcon Bieto, 1992). Thus, plant respiration responds readily to long-term changes in substrate availability (via photosynthesis).

Short-Term Interactions

Net O_2 evolution during the steady state of photosynthesis is a result of the combined effect of (a) photosynthetic O_2 evolution, (b) O_2 uptake by Mehler reation, (c) respiratory[7] O_2 uptake and (d) O_2 consumption by oxygenase activity of Rubisco and glycolate oxidasc. Similarly, net CO_2 evolution is a result of (a) CO_2 release form tricarboxylic acid cycle (TCA cycle), (b) glycine decarboxylation, (c) photosynthetic CO_2 fixation and (d) phospho*enol*pyruvate carboxylase (PEPC) activity. Thus. if respiration is defined as a process of CO_2 release and/or Cb consumption, several metabolic activities occur in leaves/green cells besides the dark respiration and interact rapidly with photosynthesis. These can be treated as short-term interactions and include photorespiration, chlororespiration, Mehler reaction and the mitochondrial respiration (Raghavendra et al., 1992).

Photorespiration: Photorespiration involves combined cycling of carbon and nitrogen between chloroplasts, mitochondria and cytosol (Husic et al., 1987;

Canvin. 1990; Oliver, 1998). A part of the carbon from photorespiratory glycolate is supplied back to the chloroplast in the form of glycerate. Ammonia released during the conversion of glycine to serine in mitochondria is reassimilated in the cytosol by glutamine synthetase (GS) and further metabolized in the chloroplast by glutamine: 2-oxoglutarate aminotransferase (GOGAT). If reassimilation of ammonia is blocked by methionine sulfoxime, which inhibits GS, then ammonia accumulates in tissues and photosynthesis stops. Nitrogen turnover during photorespiration is much greater than the net nitrogen (e.g. NO_3^-) reduction in the chloroplast.

The photorespiratory pathway requires NADH for hydroxypyruvate reduction. NADH for peroxisomal use is drawn in the form of malate from mitochondria and chloroplasts (Heupel and Heldt. 1992). Such consumption of reductant may be important when photosynthesis is severely restricted, e.g. water-stressed plants with closed stomata, when the electron transport chain is fully reduced and chlorophyll absorbs excess energy forming excited states and damaging products (e.g. superoxide, H_2O_2). Photorespiration, by draining the energy burden, may function together with the carotenoids in quenching excited state of chlorophyll and protecting PS II, which is sensitive to photoinhibition (Lawlor. 1993).

Chloroplast respiration: Although the reactions of dark and photorespiration occur mostly outside the chloroplast. a modified system of respiratory pathway with an O_2 dependent CO_2 efflux occurs in chloroplasts of higher plants and *Chlamydomonas*. This phenomenon, which is termed as chloroplast respiration or chlororespiration, include (i) reactions which oxidize glucose and (ii) electron transport pathway similar to respiratory chain, fed with electrons from reduced plastoquinone. These pathways result in CO_2 efflux and O_2 uptake and may generate limited quantity of reduced equivalents to be either used within the

chloroplast during darkness or exported to cytosol (Garab et al., 1989; Gibbs et al., 1989; Feild et al., 1998).

Mehler reaction: **It** involves light dependent PS I driven O_2 uptake in chloroplasts. Under conditions when the rate of CO_2 fixation is limiting, that is, when the NADPH/NADP ratio is high, a significant amount of the reductant of photosystem I is oxidized by oxygen resulting in the formation of H_2O_2 (Carrier et al., 1989) In the presence of catalase, H_2O_2 undergoes rapid dismutation to water and O_2. Thus uptake as well as evolution of O_2 occurs in the chloroplasts and both these processes require light. Under active photosynthesis, O_2 evolution exceeds O_2 consumption, but, if catalase is removed by washing, or inhibited by azide, the Mehler reaction leads to a net O_2 uptake.

Among these three types of respiration, only photorespiratory rates exceed those of dark respiration. Photorespiration is three to eight times greater than typical dark respiration. On the other hand, the rates of chlororespiration and Mehler reaction are about 20 and 30%, respectively, of dark respiration. Respiration of leaves, measured in darkness, is 5-10% of net photosynthesis in plants of brightly lit environments, but from those of shade it may be of a much larger proportion.

Effects of Photosynthesis on Mitochondrial respiration

The status of dark respiration in illuminated green cells/leaves has been a topic of great interest and constant debate. There was a considerable disagreement in the earlier literature until the last decade on the effect of light on respiration, since dark respiration was reported to be either hardly affected or even stimulated, while in other reports there was an inhibition of up to 100% (Graham and Chapman, 1979; Graham, 1980). Such a large variation is due to several factors: the component of dark respiration being monitored, the

experimental technique being used, and finally the type of plant tissue being studied. For example, it is difficult to assess the operation of photosynthesis or respiration in leaves based on net uptake/evolution of either O_2 or CO_2 since the measurements are compromised by inter- and intra-cellular recycling of the gases. Another problem is the technical difficulty of monitoring precisely the different types of oxidative reactions besides respiration (e.g. photorespiration, Mehler reaction, pseudocyclic electron transport). All these processes result in O_2 uptake and operate concurrently in light. Nevertheless, a promising solution appears to be the technique of mass spectrometry which distinguishes between uptake and efflux of CO_2 or O_2, which occur simultaneously (Carrier et al., 1989; Avelange et al., 1991).

Several recent reports clarify and confirm that the mitochondrial respiration does operate in light in photosynthetic tissues, with some modification. Further, the respiratory activity invariably exhibits an upsurge of activity, after even a short period of illumination (Raghavendra et al., 1994; Krömer, 1995).

Light Enhanced Dark Respiration

The beneficial or stimulatory effect of photosynthesis on mitochondrial respiration is not restricted only to the light period but can also be seen in the successive dark period. There is a marked upsurge in respiratory oxygen uptake even after short periods of illumination (5 to 15 min). This phenomenon termed as "light enhanced dark respiration" (LEDR) is documented in leaves (spinach) and mesophyll protoplasts (pea and barley). Although there is a large variation, the extent of LEDR in mesophyll protoplasts can be quite high, up to eight fold of the rate of dark respiration (Table 1.1).

Table 1.1. *Light enhanced dark respiration* ***{LEDR)*** *observed in different plant tissues and an alga,* Chlamydomonas **reinhardtii.**

LEDR is represented as % stimulation over the control (the rate of respiration before illumination). Adapted from Padmasree and Raghavendra (1998).

Material	O_2 uptake before illumination	O_2 uptake after illumination	Stimulation	Reference
	μmol mg Chl^{-1} h^{-1}		%	
Pea mesophyll protoplasts	7.9	22.8	290	Reddy et al. (1991)
Barley protoplasts	5.4	6.8	130	Gardeström et al. (1992)
Barley protoplasts	3.6	29	800	Hill and Bryce (1992)
Spinach leaf discs	1.2	8.4	700	Stokes et al. (1990)
Dianthus caryophyllus cells	21.6	26.4	120	Avelange et al. (1991)
Chlamydomonas reinhardtii	106*	166*	156	Xue et al. (1996)

* Values are represented as nmol O_2 mL^{-1} h^{-1}.

The respiratory rate is not altered if the protoplasts or leaf discs are kept in continuous darkness for similar periods. The extent of LEDR progressively increases with longer duration of preillumination. The high respiratory rate following illumination persists for only a few min, before subsiding subsequently to reach a lower rate similar to that in the dark-control. The marked stimulation of respirator}' activity within a few minutes of illumination suggests that the interaction between respiration and light is quite rapid and involves early photosynthetic products. The stimulation of LEDR by the presence of bicarbonate, or inhibition by classic photosynthetic inhibitors (e.g. DCMU or DL-glyceraldehyde) during preillumination suggests that the upsurge of respiratory O_2 uptake was dependent on the products of photosynthetic carbon assimilation/electron transport (Stokes et al., 1990; Reddy et al., 1991; Xue et al., 1996). The high rate of LEDR in the presence of even saturating CO_2 and its insensitivity to aminoacetonitrile (AAN), an inhibitor of mitochondrial glycine metabolism, demonstrate that LEDR is distinct from the photorespiratory post-illumination burst of CO_2 (Gardeström et al., 1992).

When photoautotrophic cells of carnation (*Dianthus caryophyllus*) or *Euphorbia characias* were darkened after a few minutes of illumination, the rate of respiratory O_2 uptake in darkness (soon after switching off the light) was enhanced (Avelange et al., 1991). once again confirming the phenomenon of LEDR.

Photorespiratory intermediates such as glycine appear to contribute significantly to respiratory pathway as suggested by the marked inhibition of respiration by both hydroxypyridine methane sulfonate (HPMS) and AAN. However, the suppression of glycine oxidation by AAN resulted in very little inhibition of O_2 uptake even under photorespiratory conditions (Igamberdiev et al., 1997). This could be possibly due to the participation of alternative pathways of glycolate metabolism during inhibition of glycine decarboxylation, such as

direct non-enzymatic oxidation of glyoxylate by hydrogen peroxidase (Grodzinski and Butt, 1976) or the condensation of glyoxylate with succinate (Singh and Naik 1984; Igamberdiev et al., 1997).

During LEDR of barley mesophyll protoplasts, the levels of sucrose, glucose and fructose did not change significantly while malate accumulated during illumination and was metabolised rapidly in subsequent darkness (Hill and Bryce, 1992). Thus, LEDR appears to involve malate oxidation. The enzyme pyruvate dehydrogenase complex (PDC), is known to be inactivated in the light. On transfer to darkness both NAD-malic enzyme (ME) and PDC are reactivated and photosynthetically generated malate is oxidized via both malate dehydrogenasc (MDH) and NAD-ME. Pyruvate produced by the latter is oxidized by PDC and the resulting acetyl-CoA combines with oxaloacetate (OAA) to enhance TCA cycle operation. This rapid malate metabolism leads to the respiratory burst observed as LEDR. A transient increase in the level of pyruvate on transfer to darkness was observed in also leaves of *Elodea* and spinach (Santarius and Heber. 1965). Malate formation during photosynthesis in C3 plants may represent a reserve of reducing power to mediate the transfer from photosynthetically-generated reducing equivalents to the cytosol and later to mitochondria.

Operation of Modified Tricarboxylic Acid Cycle in Light

Dark respiration is comprised of three components: (i) glycolytic reactions. (ii) decarboxylation of carbon compounds to produce CO_2 and reduced nucleotides (NADH and FADH); and (iii) oxidation of NADH/FADH leading to O_2 consumption and ATP production. A strong indication came from observations on carnation cells that the processes of CO_2 efflux and O_2 uptake are not as tightly coupled in light as in darkness. Some of the reactions of TCA cycle, particularly decarboxylation is inhibited in the light, although to varying

extent. A comparison of the effects of two inhibitors of TCA cycle, malonate and fluoroacetate, on carbon metabolism in **mung bean** leaves in light and dark led to the conclusion that on transition from **dark to light,** the TCA cycle was initially inhibited and subsequently recovered (Graham and Chapman, 1979).

Mass spectrometric studies using $^{13/12}CO_2$ and $^{18/16}O_2$ also demonstrate that respirator}' CO_2 efflux is inhibited upon illumination, while O_2 uptake is either unaffected or even stimulated. In photoautotrophic cells of carnation, in presence of high light and saturating levels of CO_2, rate of CO_2 influx represented 75% of the rate of O_2 evolution. After a dark-to-light transition, the rate of CO_2 efflux was inhibited whereas the O_2 uptake was not affected. Thus, TCA cycle activity (decarboxylation of TCA cycle compounds) is inhibited but oxidative electron transport is unaffected by illumination. Such inhibition of CO_2 efflux indicating a significant decrease in TCA cycle activity, has been observed also in different systems: cells of *Euphorbia characias, Commelina communis,* algal cells of *Selenastrum minutum* and *Chlamydomonas reinhardtii* (Peltier and Thiobault, 1985; Gans and Rebeille, 1988; Rebeille et al., 1988; Weger et al., 1988: Avelange and Rebeille, 1991; Gautier et al., 1991).

The adenylate ratio (relative level of ATP to ADP) is an important regulator}' factor of mitochondrial respiration. An increase in the ratio of ATP/ADP could be the reason for the decrease in mitochondrial TCA cycle, as ATP levels in both the chloroplast and cytosol increase during illumination. However, the adenylate levels do not appear to be crucial since the cytosolic ATP/ADP ratios are not much affected by light/dark transitions (compared to the NAD(P)H to NAD(P) ratio, for example) and they are usually not high enough to inhibit mitochondrial oxidative metabolism. The CN-resistant alternative pathway which operates in plant mitochondria helps to maintain oxidative electron transport, independent of ATP formation (Siedow and Umbach, 1995).

The main reason for the decrease in TCA cycle activity appears to be the **marked** inactivation of mitochondrial PDC on illumination, which catalyzes the primary event of oxidative decarboxylation of pyruvate to acetyl CoA. The inactivation of PDC is reversed, when the tissue is returned to the darkness. This reversible inactivation by light was dependent on photosynthetic activity as indicated by its sensitivity to DCMU (an inhibitor of PS II of photosynthesis) and absence of the phenomenon in etiolated seedlings. Not only photosynthesis but also photorespiratory metabolism can stimulate PDC inactivation. Conditions that reduce the photorespiration (i.e. increasing the CO_2 concentration or reducing the O_2 concentration) inhibited the initial light dependent drop in mitochondrial PDC activity.

The biochemical reason for this regulatory mechanism is covalent modification of PDC by phosphorylation (inactivation) in light and dephosphorylation (reactivation) in darkness (Luethy et al., 1996; Randall et al., 1996). Illumination increases the level of ATP in the cytosol or mitochondria and this enhances protein phosphorylation (by a PDC-protein kinase) which inactivates the PDC enzyme. Reactivation of phosphorylated PDC is catalysed by PDC-protein phosphatase.

The reversible phosphorylation of PDC is fine tuned by the inhibition of pyruvate or NADH and stimulation by NH_4^+ of PDC-protein kinase. The inactivation of PDC can be reversed by pyruvate or NADH. If the pH of the incubation media is 7 or less. ME is able to generate sufficient pyruvate to inhibit the kinase and maintain a high level of PDC activity. Further, NADH produced by glycine decarboxylase supports mitochondrial ATP production. This ATP production in combination with the NH_4^+-stimulation of the PDC kinase would result in very rapid phosphorylation and inactivation of PDC.

A major requirement of carbon skeletons is still met by TCA cycle, even in light. Although a few reactions of TCA cycle are inhibited or suppressed, a partial or modified TCA cycle operates in cells during illumination. Mitochondria take up readily OAA from cytosol, because of their highly active OAA-translocator. A major supply of OAA appears to be through the activity of cytosolic PEPC. Within mitochondria, OAA is converted to malate. A part of malate is decarboxylated to form pyruvate and then to acetyl CoA. Although at a subdued rate, acetyl CoA is condensed with OAA to form citrate. As a result, mitochondria can effectively keep up cycling of reduced nucleotides and export citrate to cytosol (Hanning and Heldt, 1993). Citrate is then converted to 2-oxoglutarate (2-OG) which becomes the major source of carbon for amino acid biosynthesis in chloroplasts.

Essentiality of Mitochondrial Metabolism for Photosynthesis

The contribution of mitochondrial oxidative phosphorylation to the ATP demands of a photosynthesizing cell is not in doubt anymore. Recent reports establish that mitochondrial activity is essential for the optimal performance of photosynthesis in green cells (Amthor, 1994; Raghavendra et al., 1994; Gardeström and Lernmark, 1995; Krömer, 1995).

Oligomycin, an inhibitor of mitochondrial oxidative phosphorylation (but not photophosphorylation) suppressed photosynthesis by 30-40% in illuminated barley and pea mesophyll protoplasts at a concentration, as low as 0.05 $\mu g\ ml^{-1}$. Similarly, oligomycin when fed through transpiration stream of barley leaves, inhibits photosynthesis by up to 60%. The inhibition of photosynthesis by oligomycin however is observed only with intact protoplasts. When protoplasts, whose photosynthesis is inhibited by oligomycin are disrupted by forcing them through a 5 urn nylon net, a procedure leaving chloroplasts and mitochondria intact, photosynthesis recovers back to the rate found in those without

oligomycin. These results indicate that the strong inhibition of photosynthesis observed with oligomycin was not due to the effect on chloroplast photosynthesis as such, but interference with reactions outside the chloroplast in the cytosol (Kromer et al., 1988).

In plant cells, most of the photosynthate is converted to sucrose, an ATP consuming process located in the cytosol. The addition of oligomycin caused a drastic decrease in the cellular ATP/ADP ratio, which is mainly due to a decrease in the extra-chloroplastic compartment. A significant part of the ATP requirement for cytoplasmic sucrose synthesis appears to be met by mitochondrial oxidative phosphorylation. The sharp decrease in photosynthesis of intact protoplasts upon addition of oligomycin may be therefore explained partly by a decrease in sucrose synthesis caused by metabolic regulation of fructose-1,6-bisphosphatase (FBPase) and sucrose phosphate synthase (SPS) in response to the decreased availability of cytosolic ATP. The increase in cellular contents of glucose-6-phosphate (Glc-6-P), triose phosphates (triose-P), precursors of sucrose synthesis on addition of oligomycin further confirm this hypothesis. Thus mitochondrial oxidative phosphorylation appears to be an efficient way for providing the cytosol with ATP (Kromer and Heldt, 1991a).

A marked increase in the cellular dihydroxyacetone phosphate/ phosphoglyceric acid (DHAP/PGA) ratios is observed in presence of oligomycin. The increase in the DHAP/PGA ratio on addition of oligomycin in leaves and protoplasts reflects an increased reduction of the stromal NADP-system. Under steady state conditions of photosynthesis, redox equivalents are exported from the chloroplasts by malate-OAA shuttle or DHAP-PGA shuttle to the cytosol where they are oxidized by the external NADPH dehydrogenase system of mitochondrial electron transport chain. The addition of oligomycin may reduce the capacity of mitochondria to oxidize surplus redox equivalents

from the chloroplasts and thus cause an increase in the reductive state of the stromal NADPH/NADP system and of the chloroplast electron transport carriers.

Further experiments with oligomycin and barley leaf protoplasts revealed that the importance of mitochondrial metabolism to photosynthesis may depend on light intensity (Krömer et al., 1993; Kromer, 1995). Mitochondrial ATP synthesis was active under limiting as well as saturating light intensities and under both photorespiratory and non-photorespiratory conditions. A major function of the mitochondrion in a photosynthesizing cell, particularly under low light intensities, seems to be the supply of ATP for cytosolic carbon metabolism, i.e. sucrose synthesis. In high light, mitochondria take on the additional role of oxidizing the excess reducing equivalents generated by photosynthesis, preventing over-reduction of chloroplastic redox carriers and thus maintaining high rates of photosynthesis.

Studies with a starchless mutant of *Nicotiana sylvestris* suggest that a respiratory supply of ATP could affect assimilate partitioning into sucrose and thereby, modulate photosynthesis (Hanson, 1992). The mutant NS 458 contains a defective plastid phosphoglucomutase and accumulates only trace amounts of starch. Treating mutant leaf protoplasts and young leaves with oligomycin reduced photosynthesis by as much as 25% and 40% respectively. The wild type failed to show inhibition by oligomycin, i.e. its effect was masked when starch and sucrose synthesis could interact. Maximal CO_2 assimilation in the mutant thus appears to be fine-tuned by mitochondrial metabolism such that any interruption processes would generate oscillations in photosynthesis.

Interaction in Specialized Cells

The interaction between respiration and photosynthesis is quite pronounced in cells which are deficient in Rubisco/Calvin cycle activity, such as stomatal guard cells and mutants of *Chlamydomonas* (Raghavendra et al., 1994).

Guard cells have high rates of respiratory activity but contain very low levels of Rubisco and consequently limited carbon metabolism through Calvin cycle. Despite the limited CO_2 fixation in guard cells, the reduced equivalents produced by their chloroplasts are exported to the cytosol through OAA-malate or PGA-DHAP shuttles. The reduced pyridine nucleotides (NADH) formed in the cytosol from the oxidation of malate and or DHAP may act as the respiratory substrates for mitochondrial ATP production, needed for K^+ uptake. A very strong interaction between respiration and photosynthesis has been shown in guard cell protoplasts of *Vicia faba* and *Brassica napus* at varying O_2 concentrations. A strong cooperation between chloroplasts and mitochondria appears to be essential for the maintenance of guard-cell bioenergetic processes (Shimazaki et al., 1989; Mawson, 1993).

A similar situation appears to operate in two mutants of *Chlamydomonas reinhardtii*, one devoid of Rubisco and the other lacking functional chloroplast ATP synthase. The *C. reinhardtii* mutant FUD50 lacks β-subunit of chloroplast ATP synthase and cannot produce ATP during photophosphorylation. A modified strain of this mutant FUD50su can grow under photoautotrophic conditions, although it still showed no synthesis of the p-subunit of coupling factor. Photosynthesis in FUD50su mutant was extremely sensitive to antimycin A, a specific inhibitor of mitochondrial electron transport. Photosynthesis in the FUD50su strain is achieved through an unusual interaction between mitochondria and chloroplast. Export of reduced compounds, made in light, from the chloroplast to the mitochondria elicits ATP formation in the latter, and ATP is subsequently imported to the chloroplast (Gans and Rebeille, 1988; Lemaire et al., 1988).

Biochemical basis of mitochondrial influence on to chloroplast photosynthesis

It is intriguing to consider the basis of marked essentiality of mitochondrial metabolism on chloroplast function. Carbon dioxide is assimilated through Calvin cycle, with the help of ATP and NADPH, generated during photochemical reactions. Photosynthetic carbon metabolism requires also optimal levels of several metabolites which are indeed generated during the autocatalytic Calvin cycle.

While there is evidence that the mitochondria could supply the additional ATP required in cytosol for sucrose formation, it is possible that mitochondrial metabolism influences other components of carbon assimilation in chloroplasts, for e.g. relative levels of metabolites and key enzymes.

According to the Farquhar model of photosynthesis, the limits to the rate of photosynthesis are divided among three broad classes. These are (i) CO_2 limited or Rubisco limited (RuBP saturated), (ii) light limited or ribulose-1,5-bisphosphate (RuBP) regeneration limited, and (iii) end product synthesis limited or triose-P use limited. The third limitation will limit the rate of RuBP regeration but it has very different characteristics. One of the most controversial aspects of the Farquhar model is the idea that photosynthesis appears limited by only one process at a time (Farquhar et al., 1980).

Induction

When whole plants, leaves, protoplasts or chloroplasts are taken from the dark and brightly illuminated, photosynthetic activity commences only after a lag period or induction phase (Edwards and Walker, 1983; Walker, 1988). It has also been noticed that usually the phenomenon of O_2 evolution exhibits a longer induction period than that of carbon assimilation during photosynthesis. Such

induction is a fundamental feature of photosynthesis associated with the photosynthetic carbon reduction cycle (PCRC). The autocatalytic PCRC makes its own substrates. For this reason there is often a lag in the capacity for the PCRC when photosynthetic material is first exposed to light.

When Osterhout and Haas (1918) first studied induction in *Ulva* they suggested two possibilities: one was the light-activation (and by implication, dark-deactivation) of catalysts and, the second, depletion (during darkness) of metabolite pools. These explanations are still valid and there is evidence for both. However, light-activation of enzymes is often so rapid, that is unlikely to make such a contribution to long periods of induction and these are probably best explained in terms of the known depletion of metabolites such as RuBP (which often falls to very low concentrations in darkened leaves). Some enzymes. are activated by their substrates, as well as by light, so that there can be complex interactions between the two principal underlying factors which lead to induction. In leaves, stomatal opening may also contribute to lag in gaseous exchange following re-illumination after darkness.

According to Walker and Edwards (1983) induction is associated with two aspects of reductive pentose phosphate pathway. (i) the conversion of PGA to 1,3-bisphosphoglyceric acid (BPGA). which is freely reversible but an unfavourable reaction, /driven in photosynthesis by high concentration of PGA and is particularly susceptiblc to decrease in the [ATP]/[ADP] ratio. The lag in O_2 evolution is therefore primarily related to the increase in PGA which occurs during the first few min of illumination (O_2 evolution involves transfer of electrons from H_2O to $NADP^+$ and this acceptor is regenerated by the reduction of PGA via BPGA to triose phosphate). (ii) Similarly, the lag in CO_2 fixation seems to be primarily associated with an increase in hexose monophosphate.

Although induction is not fully understood (Walker, 1981), the phenomenon appears to involve light activation of enzymes, build-up of dark depleted substrates and the supply of Pi from the cytosol to chloroplast (Walker, 1988). The orthophosphate requirement for photosynthesis by intact chloroplasts has a sharp optimum. Since triose-P is the principal product of the photosynthesizing chloroplast, photosynthesis is severely constrained if it is supplied with inadequate Pi. Conversely, if there is too much Pi in the external medium, triose-P (which is otherwise used to regenerate CO_2 acceptor, RuBP) is "pulled out" of chloroplast and the induction period is prolonged (Walker and Crofts, 1970).

Light activation of enzymes

Photosynthetic rate is regulated markedly also by the patterns of light activation of key enzymes and metabolite status. Due to allosteric interactions, there could be massive interplay between activation of enzymes and the pool sizes of various intermediates. Again both these effects may complement one another (Stitt, 1996).

Marques et al. (1987) reported in pea leaf chloroplasts that enzyme activation preceded the rise in metabolite levels, which in turn appeared to limit photosynthetic CO_2 fixation duirng induction. Thus, the results of Marques et al. (1987) suggest that light activation is required for the build-up of photosynthetic intermediates at least in chloroplasts. Leegood and Walker (1980, 1981) studied the relative contributions of the autocatalytic increase in the level of substrates and the light activation of enzymes in the control of rate of photosynthesis during induction phase or lag in wheat chloroplasts and protoplasts. The light induced reductive activation of ribulose 1,5-bisphosphate carboxylase, NADP-glyceraldehyde-3-P dehydrogenase (GAPDH), phosphoglycerate kinase (PGA-kinase), FBPase and phosphoribulokinase (PRK) raised to levels more than

adequate to support the maximum rate of photosynthesis in wheat chloroplasts. Further the addition of small quantities of intermediates such as DHAP or PGA achieved the maximum rates of photosynthesis within the first minute of illumination.

Stromal GAPDH has some similarities to the respiratory enzyme but it also has two important differences. First, the photosynthetic enzyme can use both NADH and NADPH while the respiratory enzyme uses exclusively NAD. This is consistent with the rule that NAD is involved in degradation while NADPH is involved in synthesis. The second way that photosynthetic GAPDH differs from the respiratory enzyme is that it is light activated. The PGA-kinase and GAPDH reactions consume most of the energy needed for the reduction of carbon. In aerobic respiration, the reducing power generated by GAPDH is converted to ATP in oxidative phosphorylation (Leegood, 1993; Sharkey, 1998).

The fate of the carbon is determined by the activity of stromal and cytosolic FBPase. If fructose-1,6-bisphosphate (FBP) is dephosphorylated by the stromal enzyme, the carbon is committed to starch synthesis or RuBP regeneration. On the other hand if it is dephosphorylated by the cytosolic FBPase, the carbon will be used in sucrose synthesis. Thus regulation of the two FBPases is critical. The stromal FBPase is regulated by dithiol reduction-type light activation. pH and Mg^{2+} (Gardemann et al., 1986). The activation of stromal FBPase also depends upon the presence of FBP. Regulation of the cytosolic FBPase responds both to conditions in the chloroplasts and conditions in the cytosol. The primary regulation of cytosolic FBPase is through the regulatory metabolite fructose-2,6-bisphosphate (F2,6BP). Generally, when measured under optimal conditions for each enzyme, there is about ten times more stromal than cytosolic FBPase activity.

While most of the steps of the PCRC are distributed widely in the biological world, two steps are unique to photosynthesis: the phosphorylation of ribulose-5-phosphate (Ru5P) to RuBP by PRK and the carboxylation of RuBP to produce two molecules of PGA by Rubisco (Sharkey, 1998).

PRK is highly regulated by pH, Mg^{2+}, and dithiol reduction (Gardemann et al., 1983). It uses one ATP, bringing the total ATP required per CO_2 to three and competing with PGA-kinase for ATP. It is inhibited by PGA and 6-phosphogluconate. The PGA sensitivity allows PRK to be slowed if there is insufficient ATP and NADPH to drive the gluconeogenic reactions. This regulation is necessary because PRK has a higher affinity for ATP than does PGA-kinase, so if ATP availabiltiy is low, RuBP synthesis continues while PGA builds up to high levels. This regulation is necessary because RuBP carboxylation is irreversible. so a high ratio of PGA to RuBP would not slow the rate of RuBP carboxylation.

The sensitivity of PRK to changes that occur in the chloroplast upon darkening make this the step, that stops PCRC activity in the dark. It is important to prevent the PCRC before the ATP and NADPH status of the chloroplast becomes too low since this could damage other processes occurring inside chloroplasts, not least of which is synthesis of new proteins from genes encoded in the chloroplasts. While it is true that the PCRC does not occur in darkened chloroplasts, the mechanism is not low ATP/ADP ratios or low NADPH/NADP ratios, but regulation of PRK. In this way ATP/ADP ratios can be kept high at night to allow all of the normal metabolism required in cells.

Metabolite Build-Up

In the gluconeogenic reactions of the PCRC cycle, the triose-P are key intermediates. The glyceraldehyde-3-phosphate (GAP) made initially is

converted by triose-P isomerase to DHAP (Sharkey, 1998). During photosynthesis, triose-P leaves the chloroplast, making triose-P the end product of photosynthesis inside the chloroplast in the light. Some of the triose-P made can be stored temporarily as starch inside the chloroplast, but generally this accounts for half or less of the carbon fixed in photosynthesis. Triose-P exchanges across the chloroplast envelope on the phosphate translocator (Heineke et al., 1991; Raghavendra et al., 1994). The translocator exchanges one triose-P for one inorganic phosphate most of the time. The phosphate translocator can also support PGA in the -2 ionization state and can exchange any of the transported compound in either direction. Photosynthesis begins to oscillate within seconds of changes that alter the balance between triose-P production and triose-P utilization. This is a clear indication of the importance of balancing the rates of all steps involved in photosynthesis.

Thus, while the mechanism of induction and maintenance of photosynthetic activity is in itself interesting, the regulation of photosynthesis in chloroplasts by mitochondrial metabolism adds a new dimension to the intracellular inter-organelle interaction. The present study has therefore, been designed to examine the biochemical components resulting in a dependence of chloroplast photosynthesis on mitochondrial respiration in mesophyll protoplasts.

Chapter 2

Approach and Objectives

Chapter 2

Approach and Objectives

Pea (*Pisum sativum*) is a typical C3 plant, which can be grown easily and the leaf material for experiments can be obtained within 10 to 12 days after sowing the seeds. Further pea plants allow studies on the physiological/biochemical behaviour of a C3 plant at different levels of organization: whole plant, leaves, leaf discs/leaf slices, protoplasts and isolated organelles.

The isolated mesophyll protoplasts offer an excellent experimental system to study the interaction between photosynthesis, respiration and photorespiration as they mimic the *in vivo* conditions in maintaining the coordination between different organelles *viz* chloroplasts, mitochondria and peroxisomes. Moreover they do not have any barrier against diffusion of O_2 and further allow an easy evaluation of the effect of externally added inhibitors or metabolites. As protoplasts can be uniformly suspended the problem of non-uniform photosynthesis can be avoided and the sample can be exposed uniformly to the given treatment. However, some disadvantages of protoplasts are their limited stability at room temperature, fragile nature and tendency of sedimentation. The experiments involving protoplasts should therefore be short and critical of metabolic stability.

Metabolic inhibitors play an important part in advancing our knowledge of biochemistry, frequently being instrumental in the elucidation of metabolic pathways and regulatory steps *in vivo* (Kleczkowski, 1994). We conducted the experiments in the presence of four different mitochondrial inhibitors: oligomycin, antimycin A, SHAM and propyl gallate. 'Oligomycin' is an inhibitor of oxidative phosphorylation and 'antimycin A' is an inhibitor of cytochrome

pathway of oxidative electron transport while 'SHAM' or 'propyl gallate' inhibits oxidative electron transport through AOX (Lambers, 1990).

The electron transport chain in plant mitochondria and the classic inhibitors of oxidative mitochondrial electron transport chain are represented in Figure 2.1. The electron transport chain diverges at the level of ubiquinone to two pathways: CN-sensitive cytochrome pathway and CN-resistant alternative (AOX) pathway (Lambers, 1982; Day et al., 1980; Lance et al., 1985; Moore and Siedow, 1991). Thus when electron are transported through cytochrome pathway, ATP is synthesized at three sites. However, when electrons are transported through AOX pathway, it bypasses two ATP sites.

Further, earlier inhibitor studies indicated that cytochrome pathway is kinetically favoured and that the AOX pathway was engaged only when the electron transport through the cytochrome pathway was either saturated or inhibited (Bahr and Bonner, 1973). Therefore apart from studies with SHAM alone, we have used SHAM, also in presence of antimycin A. This is because in the absence of antimycin A, the addition of SHAM alone is likely to increase the flow of electrons through cytochrome pathway, while the presence of antimycin A would ensure the effective restriction of AOX pathway electron transport by SHAM.

There are a few reports, most of them recent, on the role of mitochondrial oxidative phosphorylation in optimizing photosynthesis in mesophyll protoplasts and leaves (Kromer et al., 1993; Amthor, 1994; Raghavendra et al., 1994; Gardeström and Lernmark, 1995; Kromer, 1995; Padmasree and Raghavendra, 1998). While confirming the concept of essentiality of mitochondrial respiration for photosynthesis, the present project attempts to identify and elucidate further the components of chloroplast photosynthesis benefited by mitochondrial activity'.

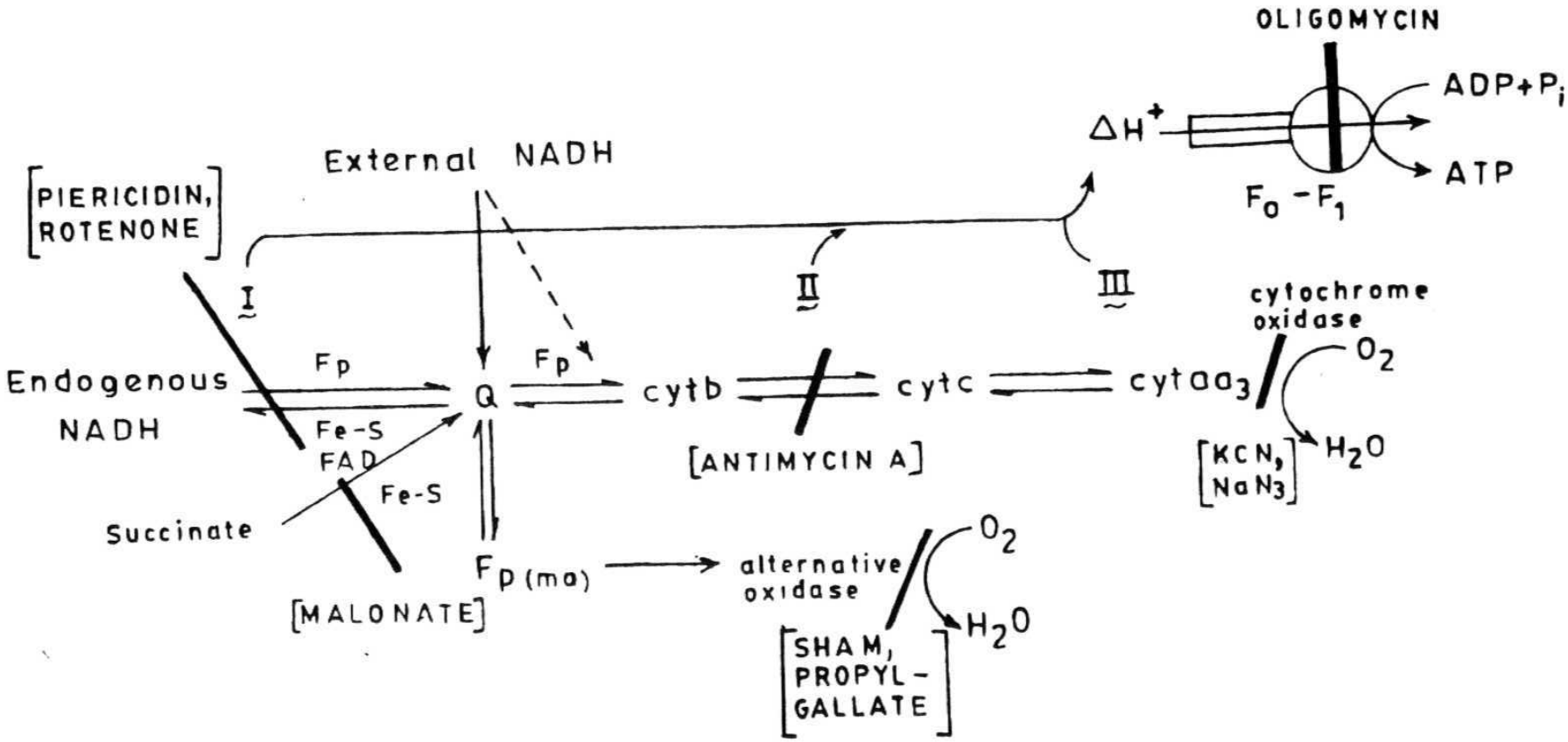

Figure 2.1. The schematic flow of electrons through the components of the oxidative electron transport chain, located in the inner mitochondrial membrane of plant mitochondria. The proton gradient created across the membrane is used to drive ATP synthesis with the help of F_o-F_1 (mitochondrial ATPase). The flow of electrons diverges at the level of ubiquinone to CN-sensitive cytochrome pathway or CN-resistant alternative pathway. Antimycin A inhibits electron flow through cytochrome pathway at site II, SHAM and propyl gallate inhibits electron flow through alternative pathway while oligomycin inhibits ATP formation (oxidative phosphorylation).

In view of the inherent limitations of metabolic inhibitors, we used the test compounds at very low concentrations. Yet, the respiratory inhibitors were quite effective in suppressing photosynthetic activity of protoplasts.

The ultimate objective of the present study is to identify and explain the biochemical basis of the strong interaction between mitochondrial respiration and the photosynthetic activity in the system of mesophyll protoplasts.

The specific objectives of the present study are:

1. To study the essentiality of mitochondrial respiration in sustaining high rates of photosynthetic activity in mesophyll protoplasts of pea (*Pisum sativum*), at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.
2. To assess the effects of antimycin A (inhibitor of cytochrome pathway of oxidative electron transport), SHAM (inhibitor of alternative path) and oligomycin (inhibitor of oxidative phosphorylation) in optimizing photosynthesis.
3. To determine the relative importance of the role of ATP during the mitochondrial interaction with chloroplasts at optimal and limiting CO_2.
4. To examine the consequence of restriction in mitochondrial respiration on the following components of photosynthesis at optimal or limiting CO_2.
   - Lag/induction
   - light activation of photosynthetic enzymes
   - cellular metabolites related to redox state; RuBP & sucrose formation
5. To evaluate the effects of oligomycin, antimycin A, SHAM and propyl gallate (classic inhibitors of AOX pathway) on photochemical activities of chloroplasts.

Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant Material

Plants of pea (*Pisum sativum* L., cv. Arkel) were raised from seeds, procured from Pocha Seeds Company, Pune, India. The seeds were soaked in water overnight and then surface sterilized with 0.1 % (v/v) sodium hypochlorite solution. Big and round seeds were selected and sown in plastic trays filled with soil and farmyard manure (5:1 proportion). The plants were grown outdoors under natural photoperiod of approximately 12 h and average temperature of 30 °C day/20 °C night. The plants were watered daily. The second pair of fully unfolded leaves were picked from 8- to 10 d-old plants (Plate 3.1) and used for experiments.

Experiments were performed with either mesophyll protoplasts or chloroplasts isolated from ruptured protoplasts.

Isolation of Mesophyll Protoplasts

Mesophyll protoplasts were isolated from leaves as described by Saradadevi and Raghavendra (1992). The abaxial epidermis of the pea leaves was stripped off with the help of forceps. The stripped leaves were then cut into pieces of ca. 0.5 cm^2. The portions containing the midrib were discarded. The leaf pieces were floated with naked abaxial surface facing down and in touch with preplasmolysis medium containing 0.3 M scrbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH buffer pH 6.0. After 30 min, the preplasmolysis medium was removed and the digestion medium, containing 2% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Macerozyme R-10, 0.25% BSA, 70 *mM* sodium ascorbate, 0.4 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH buffer pH 5.5, was added.

Plate 3.1. A view of 8 to 10-day-old plants of pea (*Pisum sativum* cv.Arkel) grown in the field (out doors).

The leaf pieces were digested for 30 min at 30 °C under illumination with a tungsten lamp at a light intensity of about 50 μE m^{-2} s^{-1}.

After digestion, the digestion medium was gently removed with help of Pasteur pipette. The washing medium (0.4 M sorbitol and 1 mM $CaCl_2$ in 10 mM Mes-KOH, pH 6.0) was added to the Petri dish containing the digested leaf strips. The Petri dish was swirled and tapped gently, releasing the protoplasts into the medium. The suspension was filtered through nylon filters of 300 and 60 μm and centrifuged at 50*g* for 5 min. The supernatant was discarded and the pellet was washed twice with the washing medium and once with suspension medium of 0.4 M sorbitol, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in 10 mM Hepes-KOH pH 7.0. The protoplast pellet was finally suspended in small aliquot of the above medium and kept on ice.

Purity/Intactness of Protoplasts

The viability and intactness of protoplasts were routinely checked using neutral red and Evans blue. The purity of preparation normally ranged from 90-97%. A typical protoplast preparation is shown in Plate 3.2.

Isolation of **Chloroplasts** from Protoplasts

The protoplasts were ruptured by passing through a 2 mL disposable syringe fitted with a 22.5 μm nylon filter. The chloroplasts were pelleted by centrifuging the broken protoplasts at 250*g* for 2 min (Walker, 1988). The chloroplast pellet was suspended gently by swirling with a cotton swab in a medium containing 50 mM Hepes-KOH, pH 7.6, 0.4 M sorbitol, 0.5 mM $MgCl_2$, 1 mM MnCl2, 10 mM Na2-EDTA, and 0.4% (w/v) BSA. The chloroplast isolation was done at 4 °C. The intactness of chloroplasts, as indicated by ferricyanide dependent O_2 evolution was 80 to 90% (Leegood and Walker, 1985; Walker 1988).

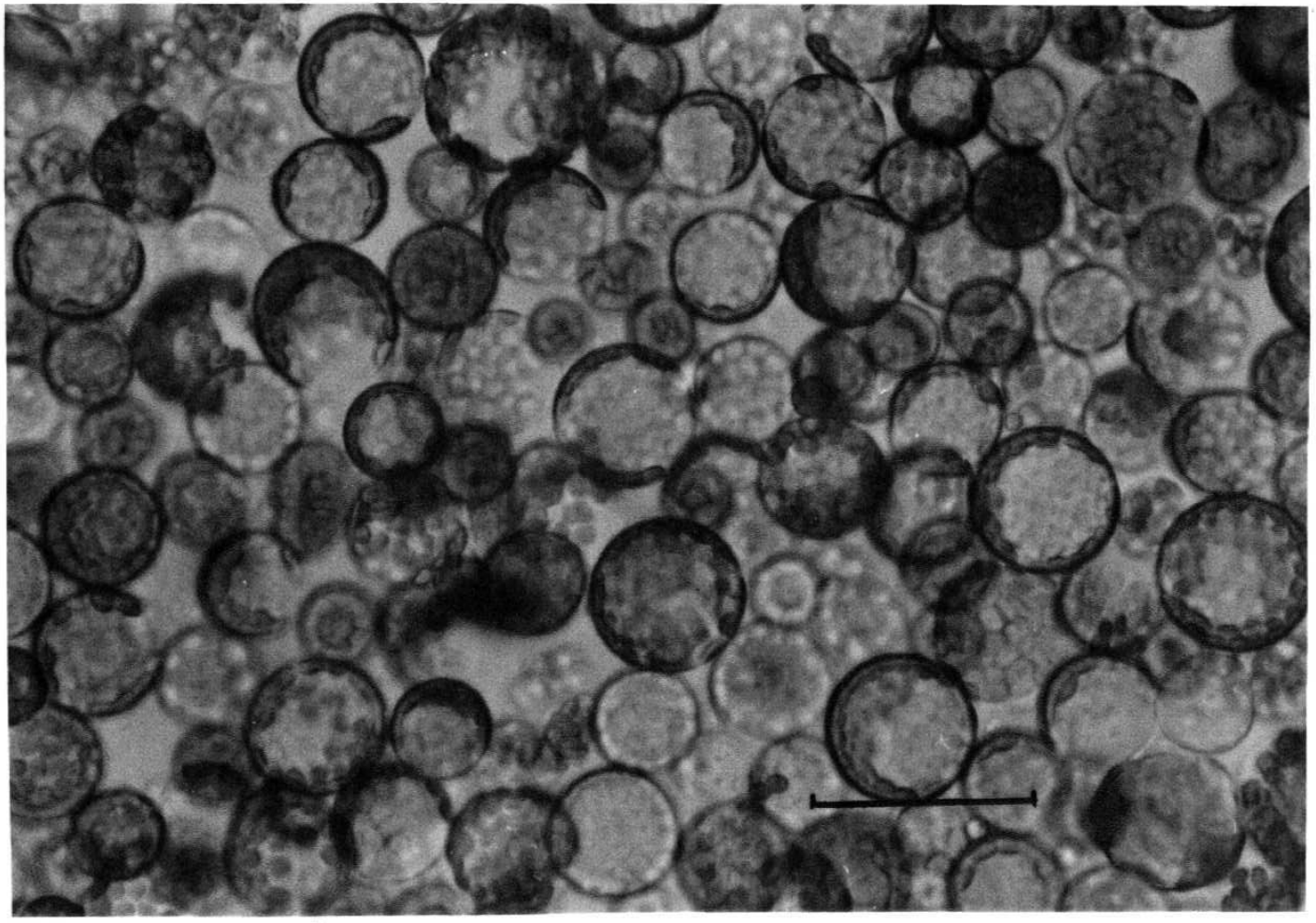

Plate 3.2. Photomicrograph of isolated mesophyll protoplasts suspended in 0.4 M sorbitol (iso-osmotic) containing medium. The Chl concentration of protoplast suspension was 200 μg mL^{-1}. Horizontal bar represents 50 ~~μM.~~ μm

Calibration of oxygen

Calibration of the oxygen content in the electrode chamber was done with air saturated water, assumed to contain 252 nmoles of oxygen mL^{-1} at 25 ^{o}C (Walker, 1988).

Estimation of Chlorophyll

Chlorophyll (Chl) was estimated by extracting into 80% (v/v) acetone (Arnon, 1949).

An aliquot of 25 µl of protoplasts suspension or chloroplasts suspension was added to 10 mL of 80% (v/v) acetone. The absorbance of acetone extract was measured at 652 nm (A_{652} - to determine Chl) and 710 nm (A_{710} - to correct for turbidity), using a spectrophotometer (Shimadzu UV-160A). The Chl concentration was calculated using the following formula

Chl (mg mL^{-1} of protoplasts suspension / Chloroplasts suspension) = $(A_{652} - A_{710}) \times 11.11$

Monitoring Photosynthesis and **Respiration**

Respiratory O_2 uptake in the dark and photosynthetic O_2 evolution in the light (by protoplasts or chloroplasts) were monitored at 25 °C polarographically using a Clark type O_2 electrode (Model DW2, Hansatech Ltd., King's Lynn, UK). The reaction medium of 1 mL for the assay of protoplast photosynthesis/respiration contained 0.4 M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM NaHCO3 for optimal [CO_2] or 0.1 mM $NaHCO_3$ for limiting [CO2] in 10 mM Hepes-KOH, pH 7.5 and protoplasts equivalent to 20 µg (Saradadevi and Raghavendra, 1994).

The reaction medium of 1 mL for the assay of chloroplast photosynthesis at 25 °C contained 0.4 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 25 mM

Na2-EDTA, 10 mM $NaHCO_3$, 150 to 200 units of catalase, 5 mM PPi, 0.5 mM ATP, 50 mM Hepes-KOH, pH 7.6 and chloroplasts equivalent to 20 ug Chl.

Photochemical Activities studied in chloroplasts:

H_2O ⇨ **PSII** ⇨ **PSI** ⇨ MV (O_2 consumption)

DPC ⇨ **PSII** ⇨ **PSI** ⇨ MV (O_2 consumption)

Sodium ascorbate + DCPIP ⇨ **PSI** ⇨ MV (O_2 consumption)

H_2O ⇨ **PSII** ⇨ *p*-BQ (O_2 evolution)

Photosynthetic electron transport measurements were performed in an assay medium containing 25 mM Hepes-KOH pH 7.6, 5 mM $MgCl_2$, 0.1 M sorbitol and chloroplasts equivalent to 5 µg Chl. Whole chain electron transport rates was measured as O_2 uptake when electrons are transferred from H_20 to MV (1 mM) in presence of 0.1 mM NaN_3. PS I electron transport rates was monitored as O_2 uptake when electrons are transferred from Na_2 ascorbate (2 mM) / DCPIP (25 µM) to MV (1 mM) in presence of NaN_3 (0.1 mM) and DCMU (5 µM). PS I rates was measured by blocking PS II with DCMU. PS II electron transport rates was measured as O_2 evolution when electrons are transferred from H_2O to *p*-BQ (1 mM). Whole chain electron transport rate excluding Oxygen evolving complex *(DEC)* was monitored as O_2 uptake when electrons are transferred from DPC (0.5 mM) to MV (1 mM). OEC is blocked by 150 µM NH_2OH. 5 mM NH_4Cl was present in the reaction medium so as to achieve the maximum uncoupled rates of photochemical electron transport.

Water at a constant temperature of 25 °C was circulated through the outer jacket of reaction chamber. Illumination of 700 µmol m^{-2} s^{-1} was provided by a 35-mm slide (Xenophot (halogen) lamp. 24V/150W) projector. Test compounds were incubated in the reaction medium to give the required final concentration,

and protoplasts/chloroplasts were preincubated in darkness at 25 °C for 5 min before switching on the light. During experiments with time course of light activation of various enzymes and estimation of metabolites, an aliquot of protoplasts were retrieved at specific time intervals as soon as the light is switched on and assayed.

Estimation of metabolites

Aliquots (600 μL) of reaction medium containing protoplasts equivalent to 100 μg Chl were withdrawn from protoplast samples (which were either illuminated or dark-adapted at 25 °C) and added to $HClO_4$, to make a final concentration of 3% (v/v). The mixtures were frozen in liquid nitrogen until used (usually the next day). When required, the samples were thawed and centrifuged at 7000*g* for 10 min. The supernatant fraction was neutralized with KOH/triethanolamine and left on ice for 30 min. The neutralized samples were centrifuged at 7000*g* and the cleared supernatant fluid was used for estimation of metabolite concentrations. The enzymatic analysis of metabolites were done using a dual wavelength spectrophotometer (Shimadzu, UV-vis 160A spectrophotometer)

*ATP and **Glc-6-P:*** The cellular levels of ATP and Glc-6-P were monitored by modifying the procedure of Stitt et al. (1989). The levels of Glc-6-P and ATP were measured using enzymatic assays coupled to NADPH formation. The reaction medium (1 mL) for the assay of Glc-6-P contained 150 mM triethanolamine buffer, pH 7.5, 0.5 mM NADP, 10 mM $MgCl_2$, 1.5 U Glc-6-P dehydrogenase (Glc-6-P-DH). After an equilibration period of 3 to 5 min at 25 °C, the reaction was started by the addition of Glc-6-P-DH. The reaction medium for the assay of ATP (1 mL) contained 150 mM Triethanolamine buffer, pH 7.5, 0.5 mM NADP, 10 mM $MgCl_2$, 1.5 U Glc-6-P-DH, 10 mM Glc and 3 U hexokinase (HK). After an equilibration period

of 6 to 8 min at 25 °C, the reaction was started by the sequential addition of Glc and HK. The content of Glc-6-P and ATP was calculated from the net increase in absorbance at 340 nm after the addition of Glc-6-P-DH and HK, respectively.

ADP: The cellular levels of ADP was measured by modifying the procedure of Stitt et al. (1989). The reaction medium for the assay of ADP (1 mL) contained 150 mM Tris-HCl pH 8.1. 7.5 mM $MgCl_2$. 0.08 mM NADH. 2 mM PEP, 3 U Lactate dehydrogenase (LDH) and 4 U Pyruvate kinase (PK). After an equilibration of (2-3 min), the reaction was started by the addition of LDH and PK (after another 5-8 min). The content of ADP was calculated from the net decrease in absorbance at 340 nm after the addition of PK.

RuBP: The cellular levels of RuBP was measured by modifying the procedure of Doncaster et al. (1989). The RuBP levels were monitored by using enzymatic assays coupled to NADH oxidation. The extract was preincubated in the reaction medium (1 mL) containing 100 mM Tris-HCl pH 8.1, 10 mM $MgCl_2$, 10 mM $NaHCO_3$ and 1 mM ATP for 20 min at 25°C before assay. 0.1 mM NADH was then added, followed by 2 U GAPDH and 2 U PGA-kinase. After an equilibration period of 2 to 3 min 50 µg of Rubisco was added to determine RuBP. The amount of RuBP present is proportional to half the decrease in absorbance of NADH at 340 nm since two molecules of PGA are formed from each molecule of RuBP.

PGA: The reaction medium (1 mL) for the assay of PGA contained 100 mM Tris-HCl pH 8.1, 10 mM $MgCl_2$. 1 mM ATP and 0.1 mM NADH. The extract was preincubated in the reaction medium for 20 min at 25 °C. The reaction was initiated by the sequential addition of 2 U GAPDH and 2 U PGA-kinase. The concentration of PGA is proportional to the amount of NADH oxidized at 340 nm (modified procedure of Doncaster et al.. 1989).

Triose-P and FBP: The cellular levels of triose-P and FBP were measured by enzymatic assays coupled to NADH oxidation. The reaction medium (1 mL) for the assay of triose-P contained 200 mM Triethanolamine buffer pH 7.6, 10 mM $MgCl_2$, 0.15 mM NADH. After an equilibration period of 2 to 3 min the reaction for the assay of triose-P was initiated by the addition of an enzymatic mixture of glycerol-3-P dehydrogenase/triose-P isomerase (0.8 U and 2.3 U). The decrease in absorbance of NADH is proportional to the amount of triose-P present. After the reaction is saturated FBP is determined in the same sample by the addition of 0.13 U aldolase. The amount of FBP is proportional to the half the decrease in absorbance of NADH at 340 run since two molecules of triose-P are formed from each molecule of FBP by the action of aldolase (modified procedure of Stitt et al., 1989).

OAA: The level of oxalacetate was calculated from the equation of [(oxoglutarate) x (aspartate)] / [(glutamate) x (6.61)], as suggested by Heineke et al (1991). based on the equilibrium of GOT *(K* = 6.61, Veech et al.. 1969).

The levels of oxoglutarate and asparatate were determined by the decrease in absorbance of NADH at 340 nm. The reaction medium for the assay of oxoglutarate contained 50 mM phosphate buffer pH 7.3, 2 mM $MgSO_4$, 0.1 mM NH_4Cl and 0.4 mM NADH, The extract containing oxoglutarate was incubated in the reaction for 10 min at 25 °C . The reaction is initiated by the addition of 3.5 U glutamate dehydrogenase in $(NH_4)_2SO_4$ suspension. The extract containing aspartate was incubated for 10 min at 25 °C in the reaction medium (50 mM Tris-HCl pH 8.1, 2.5 mM $MgCl_2$, 0.2 mM oxoglutarate, 0.1 mM NADH and 18 U MDH). The reaction is initiated by the addition of 2 U glutamate oxaloacetate transaminase (Bergmeyer, 1983).

The level of glutamate is determined by enzymatic assay coupled to NAD reduction at 340 nm (Bergmeyer, 1983). The assay medium (1 mL) contained

100 mM Tris-HCl, 630 mM Hydrazine sulfate, 1 mM EDTA (pH 9.0), 0.5 mM ADP and 1.5 mM NAD. The extract is incubated for 10 min in the reaction medium at 25 °C. The reaction is initiated by the addition of 24 U glutamate dehydrogenase (solution in glycerine).

Malate: The extract is incubated for 10 min at 25 °C in the reaction medium (100 mM Tris-HCl, 630 mM Hydrazine sulfate. 1 mM EDTA, pH 9.0 and 1.5 mM NAD). The reaction is initiated by the addition of 30 U MDH. The concentration of malate is proportional to the amount of NAD reduced at 340 nm (Heineke et al., 1991).

Enzyme Assays

The protoplast samples after the given mitochondrial inhibitor treatment were examined and compared with their respective controls for the levels of different enzymes as described below. All the enzyme assays were done at 25 °C, with the help of a spectrophotometer (Shimadzu UV-160A). Usually protoplasts were present in both the sample as well as reference cuvettes during the assays, while the substrate was added to only the sample cuvette. 0.02% (v/v) Triton-x was used during the assay so as to solubilise the protoplast membranes.

NADP-Glyceraldehyde-3-P dehydrogenase (EC 1. 2. 1. 13)

NADP-GAPDH was assayed by monitoring NADPH oxidation, according to the method of Shimazaki et al. (1989).

The reaction medium (1 mL) contained 100 mM Tris-HCl (pH 7.8), 1 mM ATP. 10 mM $MgCl_2$. 0.2 mM NADPH. and 1 unit PGA-kinase and protoplasts equivalent to 1 µg Chl. The reaction was initiated by the addition of 5 mM PGA in sample cuvette. The decrease in absorbance at 340 nm was recorded. The reaction was linear at least for 5 min. Enzyme activity was

calculated by taking **into** account the molar extinction coefficient value of NADPH, 6.2 mM^{-1} cm^{-1}.

***Fructose 1,6-bisphosphatase* (EC 3. 1. 3. 11)**

The enzyme activity was assayed by monitoring the reduction of NADP, according to the method of Shimazaki et al. (1989).

Assay mixture of 1 mL contained 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.4 mM NADP, 10 mM $MgCl_2$, 2 U of Glc-6-P-isomerase, 1 U of Glc-6-P-dehydrogenase and 5 µg Chl. The reaction was initiated by the addition of 250 µM FBP. The increase in absorbance was immediately measured at 340 nm. The reaction was linear at least for 5 min. The FBPase activity was expressed as the amount of NADP reduced per min using the molar extinction coefficient of 6.2 mM^{-1} cm^{-1}.

***Phosphoribulokinase* (EC 2. 7. 1. 19)**

The enzyme activity was assayed by determining the oxidation of NADH, with slight modifications of the procedure described by Heber et al. (1982).

One mL of reaction medium contained 100 mM Tris-HCl (pH 7.8), 1 mM ATP, 2 mM PEP. 0.4 mM NADH. 10 mM $MgCl_2$, 0.5 mM EDTA, 4 U PK. 2 U LDH (the activity of endogenous ribose-5-phosphate isomerase was not rate limiting in this assay) and 2 µg Chl. The reaction was initiated by the addition of 1 mM ribose-5-P into sample cuvette. The rate of NADH oxidation was monitored at 340 nm. The reaction was linear at least for 8 min in the case of dark sample and 4 min in the case of light treated sample. Enzyme activity was calculated by taking into account the molar extinction coefficient value of NADH, 6.2 mM^{-1} cm^{-1}.

NADP-Malate dehydrogenase (EC 1. 1. 1. 82)

NADP-MDH was assayed by determining the oxidation of NADPH by slightly modifying the procedure described by Shimazaki et al. (1989).

The assay medium (1 mL) contained 100 mM Tris-HCl (pH 7.8), 0.2 mM NADP, 2 mM EDTA. 5 mM DTT and 1 µg Chl. The reaction was initiated by the addition of 2 mM OAA to the sample cuvette. The decrease in absorbance at 340 nm was monitored quickly and the rate of the reaction in the first min was considered for calculations. Enzyme activity was calculated by taking into account thc molar extinction coefficient value of NADPH, 6.2 mM^{-1} cm^{-1}.

Replications and Statistical Analysis

The data presented are the average values (± SE) of results from three to four experiments conducted on different days. Statistical analysis of data was done using a computerised program written in a basic language.

Materials/Chemicals

Cellulase (Onozuka R-10) and Macerozyme R-10 (pectinase) were procured from Yakult Honsha Co. Ltd.. Nishinomiya, Japan. Antimycin A, *p*-BQ, methyl viologen. NADPH, oligomycin, propyl gallate, SHAM, sodium azide and all enzymes were from Sigma Chemical Company, USA and Boehringer Mannheim, Germany. All other chemicals were of Sisco Research Laboratories, E. Merck (India) Ltd., Spectrochem Pvt Ltd.. Loba Chemie, HiMedia Laboratories Pvt Ltd., or Ranbaxv Laboratories Ltd., all from India.

Chapter 4

Importance of both Cytochrome and Alternative pathways of Oxidative Electron Transport in Optimizing Photosynthesis

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Introduction

Photosynthesis is traditionally considered to be an autonomous process since carbon can be assimilated by isolated intact chloroplasts. However, in recent years it has become evident that photosynthetic carbon assimilation is dependent strongly on mitochondrial metabolism (Kromer et al., 1988; Raghavendra et al., 1994; Gardestrom and Lernmark, 1995; Kromer, 1995).

In the present study, we tried to examine which part of the mitochondrial oxidation plays a major role in optimizing photosynthesis, either oxidative electron transport or oxidative phosphorylation. Two modes of oxidative electron transport operate in plant mitochondria: the conventional CN-sensitive cytochrome path and CN-resistant AOX pathway (Lambers, 1985; Siedow and Berthold, 1986; Mclntosh, 1994). The cyanide-insensitive AOX pathway is mediated by an alternative oxidase (AOX) (Siedow and Umbach, 1995). There is a marked variation in the amount of AOX protein and relative activity among plant species besides the dynamic regulation of AOX pathway (Breidenbach et al., 1997). Yet the role of AOX in plant metabolism is not completely understood, except for the rapid oxidation of substrates and generation of heat in tissues such as aroid spadices (Seymour and Shultze-Motel, 1996).

We have therefore attempted to examine the role of cyanide-insensitive AOX pathway as well as the cyanide-sensitive cytochrome pathway in benefiting photosynthesis in mesophyll protoplasts of pea. The AOX pathway is quite active

and is reported to be completely engaged in mesophyll protoplasts of pea (Vani **and Raghavendra,** 1994).

Furthermore, the response of photosynthesis in protoplasts to respiratory inhibitors was studied at limiting or optimal CO_2 levels so as to assess the relative importance of the role of ATP during the mitochondrial interaction with chloroplasts. At optimal CCb, the photosynthetic demand for ATP is expected to be very high, while such a need for ATP would be low at limiting CCb. Our results indicate that mitochondrial metabolism is essential for photosynthesis at both optimal and limiting CO_2. We demonstrate for the first time that not only the cytochrome pathway but also the AOX pathway is essential for protoplast photosynthesis.

Results

The effect of mitochondrial inhibitors was ascertained by determining the respiratory O_2 uptake and intracellular ATP levels. During most of the experiments, low concentrations of oligomycin (100 ng mL^{-1}), antimycin A (100 nM) or SHAM (100 uM) were used so as to minimize the possible perturbation of the metabolic system.

The effects of oligomycin (an inhibitor of oxidative phosphorylation), and antimycin A (an inhibitor of cytochrome pathway of oxidative electron transport) on respiratory O_2 uptake and intracellular ATP levels were quite different. Oligomycin decreased primarily the ATP levels (by 65%) in protoplasts while inhibiting respiration marginally (<10%). On the other hand, antimycin A caused a marked decrease in both ATP levels and O_2 uptake (Fig. 4.1, A and B).

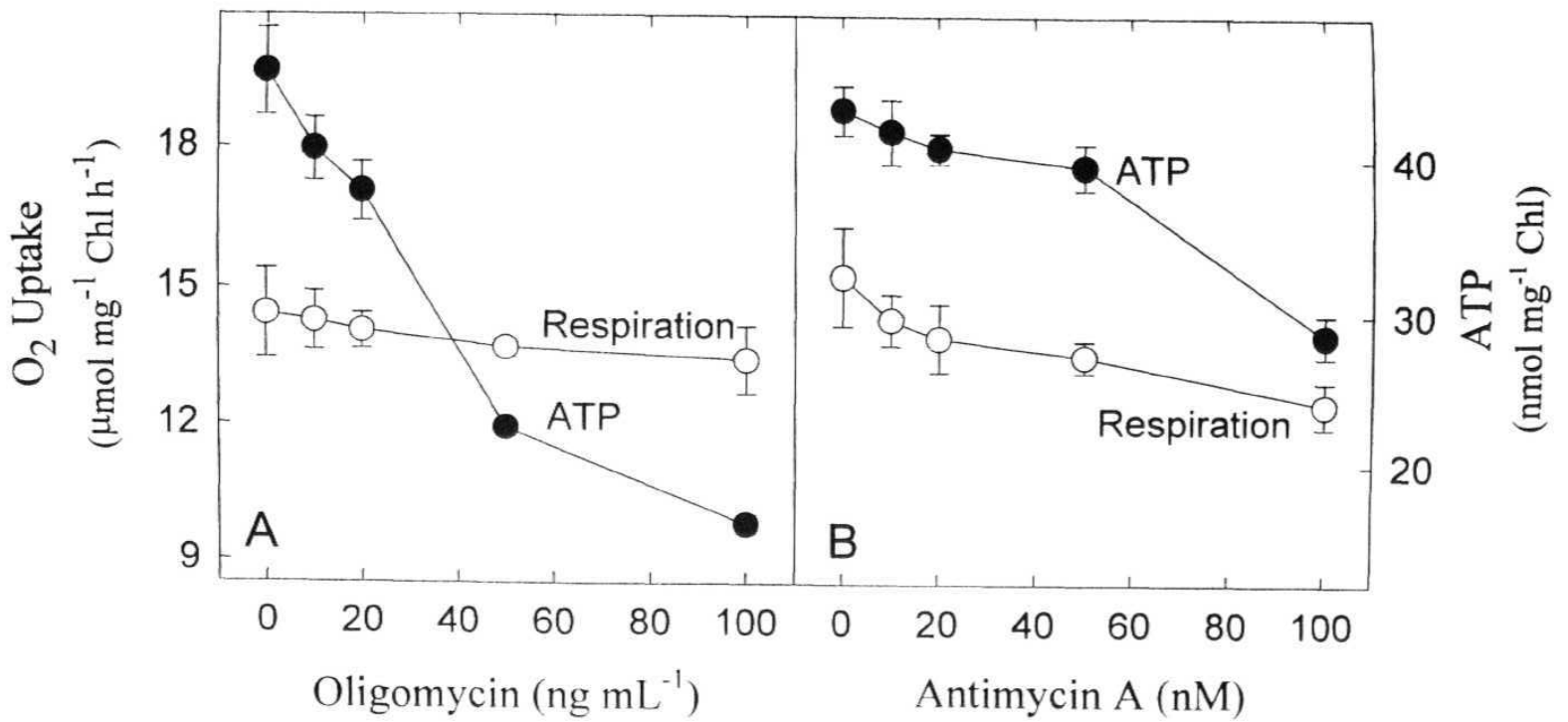

Figure 4.1. Effects of oligomycin (inhibitor of oxidative phosphorylation) and antimycin A (inhibitor of oxidative electron transport through cytochrome pathway) on dark respiration and cellular ATP in mesophyll protoplasts of pea. The test compounds were included at the indicated concentrations in the incubation medium of protoplasts and left for 5 min in darkness at 25 ^{o}C. The average values for control sets (without inhibitors) were: dark respiration, 14 $\pm$ 1 μmol mg^{-1} Chl h^{-1}; ATP content, 45 $\pm$ 1 nmol mg^{-1} Chl.

Most of the studies on mitochondrial inhibitors indicate that the AOX pathway is engaged only when the electron flow through the cytochrome pathway is either saturated or inhibited (Bahr and Bonner, 1973; Møller et al., 1988; Millar et al., 1995). In agreement with these earlier observations, the presence of SHAM alone increased the respiratory O_2 uptake (Fig. 4.2A). On the other hand, the inhibition of respiratory O_2 uptake by SHAM can be noticed clearly in presence of 100 nM antimycin A (Fig. 4.2B). The small but consistent decrease in ATP levels (8 to 16%) by SHAM occurred both in the absence and presence of antimycin A. We have therefore studied the effect of SHAM, both in presence or absence of 100 nM antimycin A. At this concentration, antimycin A, alone caused 18% decrease in respiratory rate and lowered the ATP levels by 34 % (Fig. 4.1B).

However, the decrease in ATP levels by antimycin A (<35% inhibition) was less than that caused by oligomycin. In contrast. 100 μM SHAM caused an inhibition of 13% in respirator}' rate, while decreasing the ATP levels by about 16 %, over and above the effect of antimycin A (Fig. 4.2B).

The effect of these mitochondrial inhibitors on photosynthetic O_2 evolution was assessed at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2. The extent *of* inhibition of photosynthesis by oligornycin or antimycin A was more at optimal CCb than at limiting CCb (Fig. 4.3, A and B). There was a decrease of 25 to 35% in the rate of photosynthesis at optimal CO_2 while there was an inhibition of 15 to 25% at limiting CO_2. SHAM and propyl gallate also restricted photosynthetic activity, the suppression again being stronger at optimal CO_2 than that at limiting CCb (Fig. 4.4, A and C). However, the inhibition due to SHAM in presence of antimycin A was marginally higher at limiting CO_2 than that at optimal CCb (Fig. 4.4B).

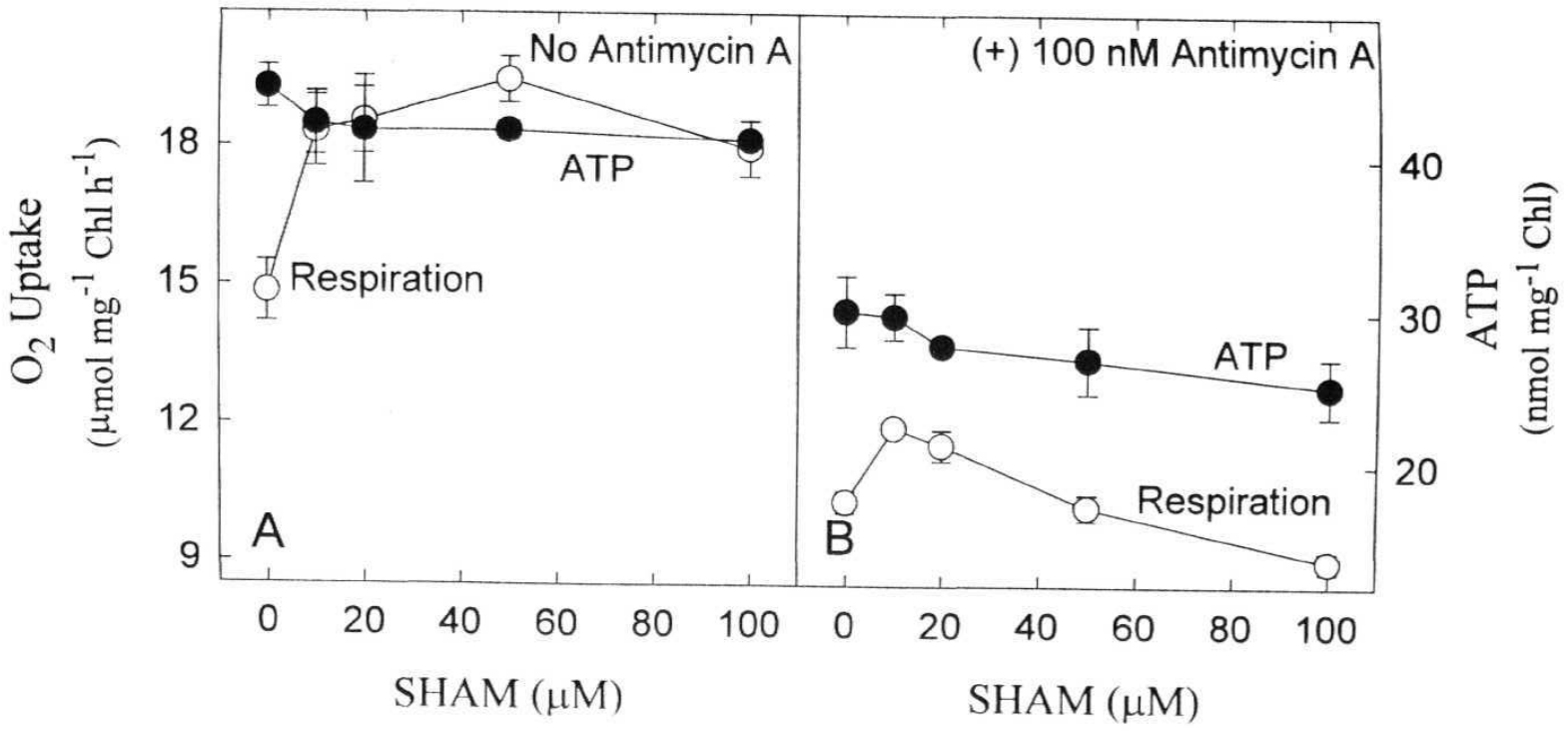

Figure 4.2. Effect of SHAM, an inhibitor of alternative pathway (± 100 nM antimycin A) on dark respiration and cellular ATP in mesophyll protoplasts of pea. Further details were as in Figure 4.1. Same scales are used in Figures 4.1 and 4.2 for an easy comparison of the effects of three test compounds. The use of 100 nM antimycin A is to restrict cytochrome pathway so as to ensure maximal effect of SHAM on AOX pathway.

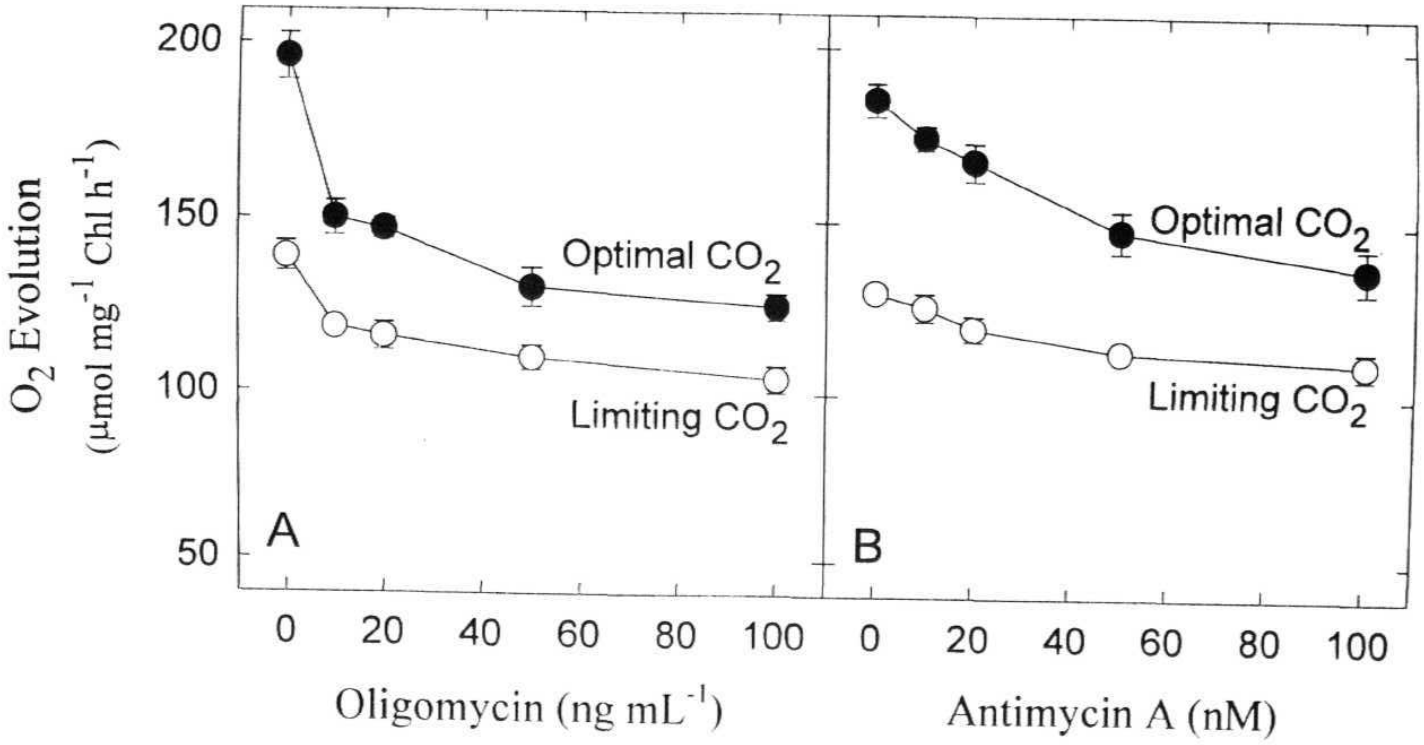

Figure 4.3. Effect of oligomycin or antimycin A on photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2 in mesophyll protoplasts of pea. Protoplasts were kept in darkness for 5 min before switching on the light. The reaction medium contained the test compounds at the indicated concentrations. The photosynthetic rates were measured at 10 min after illumination. The average rates of photosynthetic O_2 evolution in control sets (without any inhibitor) at optimal CO_2 and limiting CO_2 were 190 ± 5 and 135 ± 4 μmol mg^{-1} Chl h^{-1}.

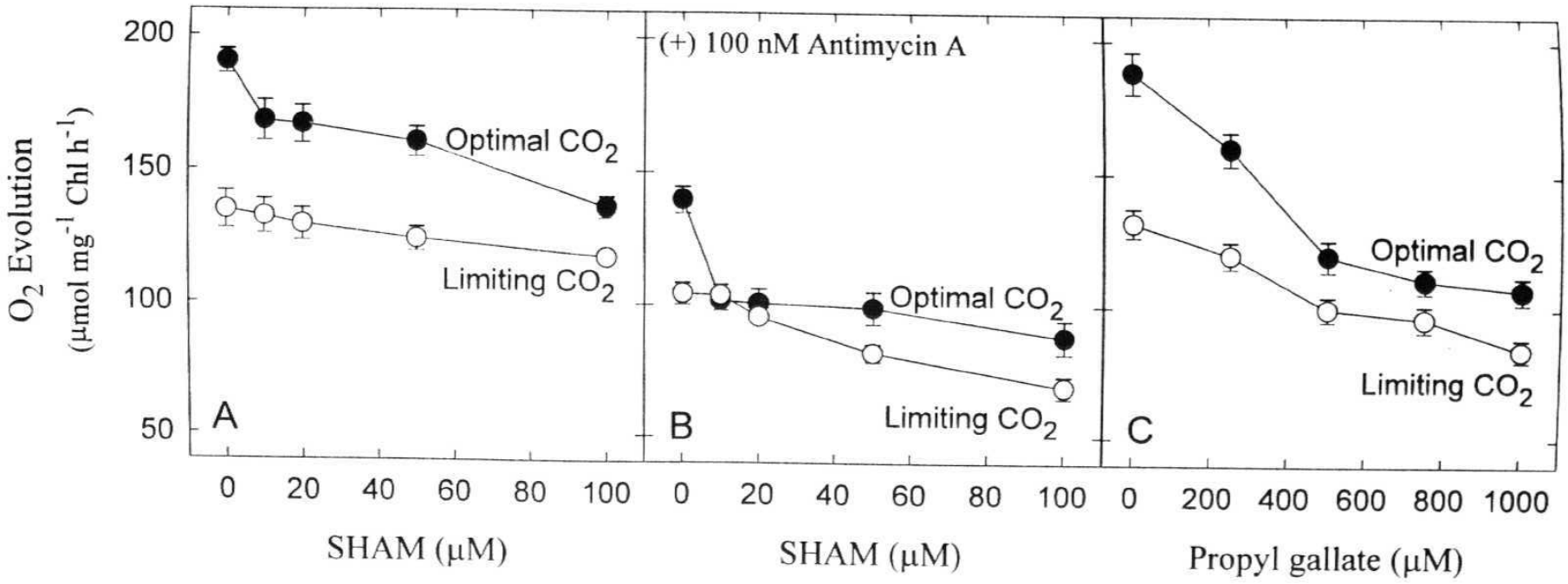

Figure 4.4. Effect of SHAM in the absence or presence of 100 nM antimycin A and propyl gallate on photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2 in mesophyll protoplasts of pea. The photosynthetic rates were measured at 10 min after illumination. Other details were as shown in Figure 4.3.

The effects of these inhibitors were evaluated at a wider range of concentrations so as to ensure their direct effects on chloroplasts (Fig. 4.5; Fig. 4.6). For example, in presence of 1 ug mL^{-1} oligomycin, there was nearly a 40% decrease in the rate of photosynthesis while the decrease in the rate of respiration was <25% (Fig. 4.5A). Similarly, there was a large depression in photosynthetic activity (60%) compared to respiration (25%), in the presence of 1 μM antimycin A (Fig. 4.5B). However, even at these high inhibitor concentrations, bicarbonate dependent O_2 evolution by intact chloroplasts was not affected.

The rate of respiration was stimulated with SHAM as shown earlier (Fig. 4.2B; Fig. 4.6A), but decreased in presence of antimycin A (Fig. 4.2B; Fig. 4.6B). The rate of photosynthesis was significantly decreased by 60% in presence of SHAM. The effects of SHAM (in presence of 100 nM antimycin A) on respiration and photosynthesis of protoplasts were stronger than that of oligomycin or antimycin A. At 1 mM SHAM (in presence of 100 nM antimycin A), respiration was reduced by 40% and photosynthesis by 72% (Fig. 4.6B). The rate of photosynthesis was affected more seriously (47%) than respiration (30%) even in presence of propyl gallate (Fig. 4.6C).

There was a biphasic correlation between the decrease in the rate of respiration and the corresponding decrease in the rate of photosynthesis during the experiments in presence of oligomycin (Fig. 4.7A; Fig. 4.8A). At optimal CO_2, in the first phase, a small change (of about 5%) in the rate of respiration inhibited photosynthesis to a much larger extent (nearly 35%). In the second phase, there was not much effect on photosynthesis, despite the decrease in the rate of respiration. In the presence of antimycin A or SHAM (+antimycin A), the decrease in the rate of photosynthesis was associated with a nearly linear

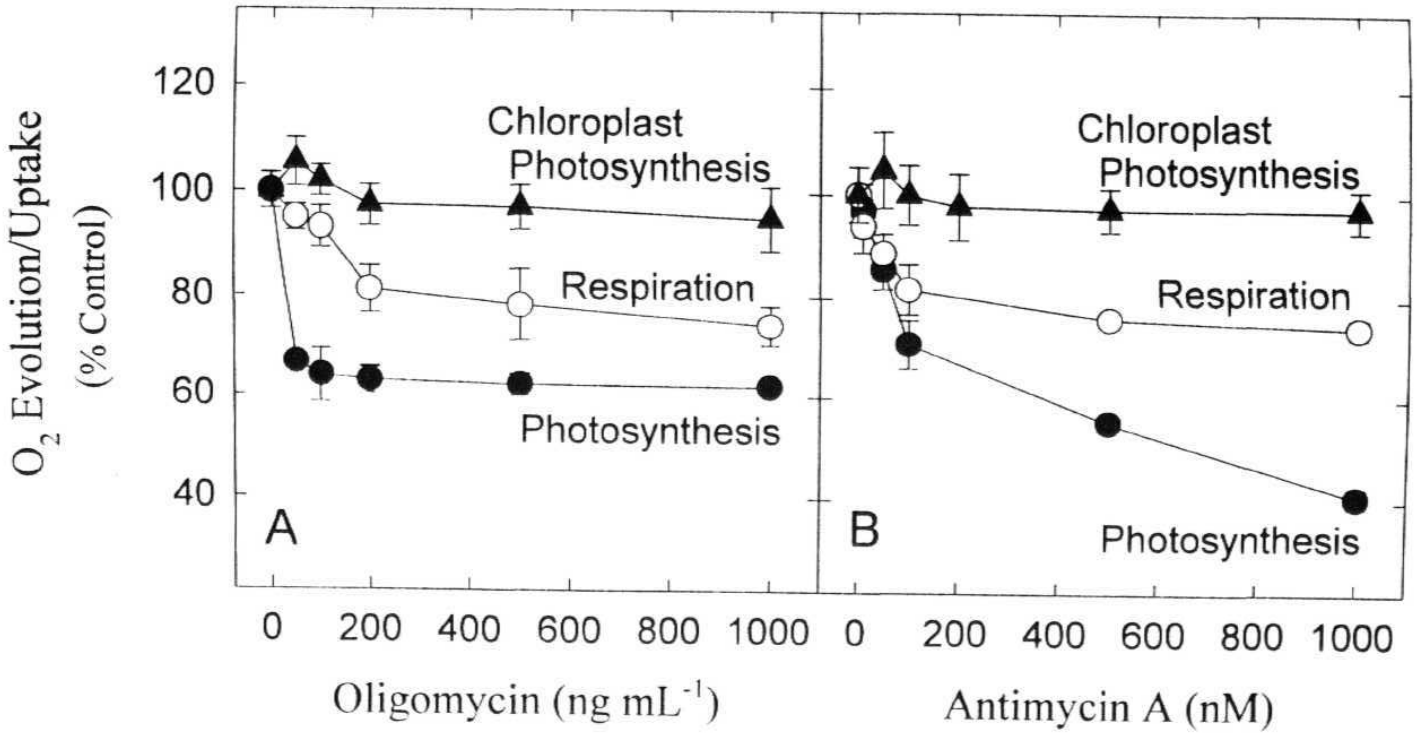

Figure 4.5. Effect of oligomycin and antimycin A on photosynthetic O_2 evolution in the light and respiratory O_2 uptake in darkness by mesophyll protoplasts of pea at optimal CO_2 (1.0 mM $NaHCO_3$). The pattern of photosynthetic O_2 evolution by intact chloroplasts is also represented. The average rates of photosynthetic O_2 evolution by protoplasts (O) and chloroplasts (Δ) in the absence of inhibitor (control at 100%) were 175 ± 9 μmol mg^{-1} Chl h^{-1} and 100 ± 6 μmol mg^{-1} Chl h^{-1}, respectively. The rate of respiratory O_2 uptake by mesophyll protoplasts (O) was 18 ± 2 μmol mg^{-1} Chl h^{-1} in the control. Further details were as in Figures 4.1 and 4.3.

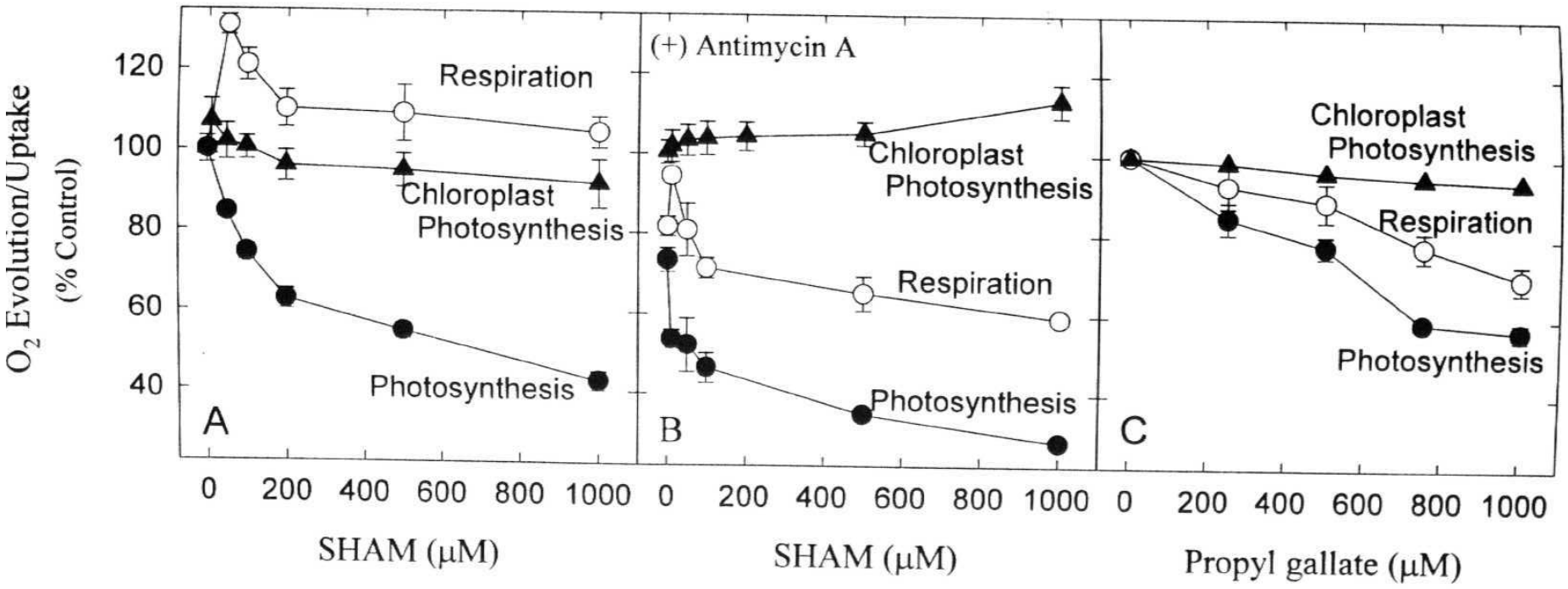

Figure 4.6. Effect of SHAM in the absence or presence of 100 nM antimycin A and propyl gallate on photosynthetic O_2 evolution in the light and respiratory O_2 uptake in darkness by mesophyll protoplasts of pea at optimal CO_2 (1.0 mM $NaHCO_3$). The pattern of photosynthetic O_2 evolution by intact chloroplasts is also represented. Other details are as in Figure 4.5.

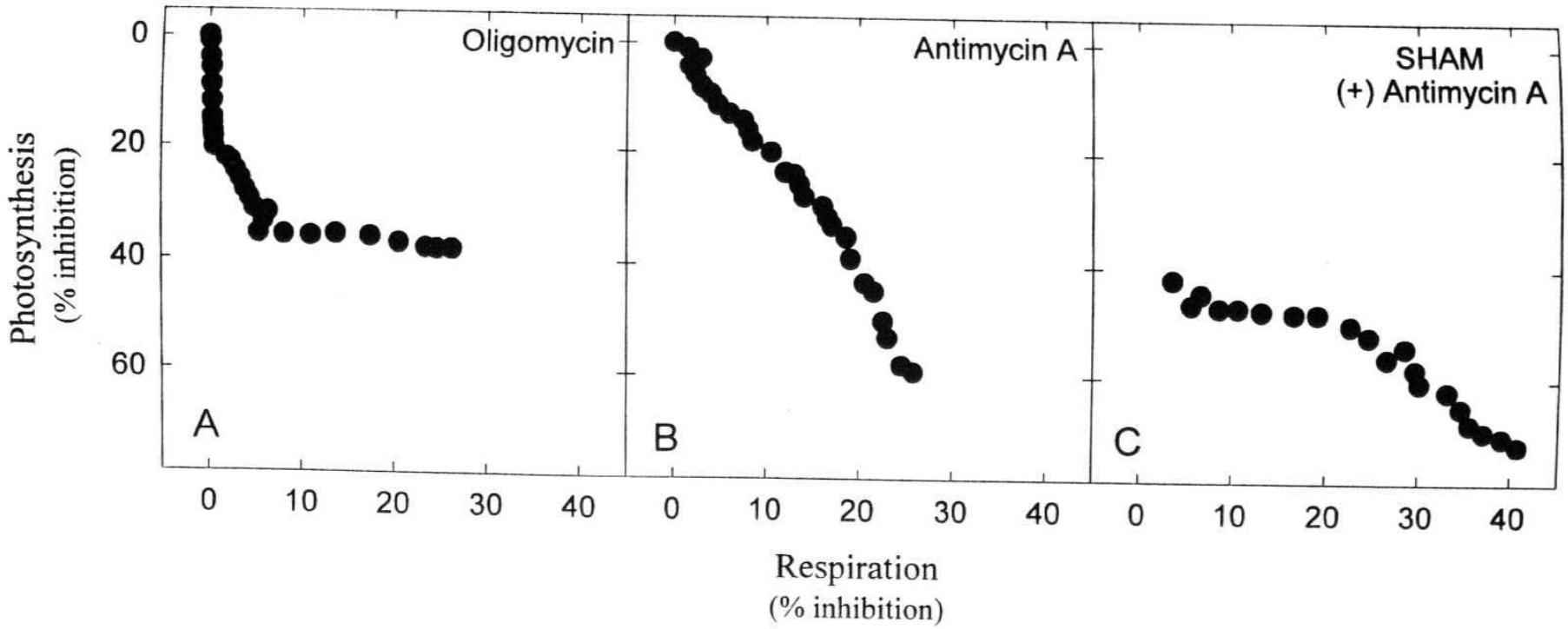

Figure 4.7. Correlation between photosynthesis and respiration (as % inhibition) at optimal CO_2 (1.0 mM $NaHCO_3$) in mesophyll protoplasts of pea upon incubation with oligomycin, antimycin A or SHAM (+ 100 nM antimycin A). These are collective data obtained with different concentrations of metabolic inhibitors (0 to 1 μg mL^{-1} of oligomycin; 0 to 1 μM of antimycin A; 0 to 1 mM of SHAM), as shown in Figures 4.1 - 4.6.

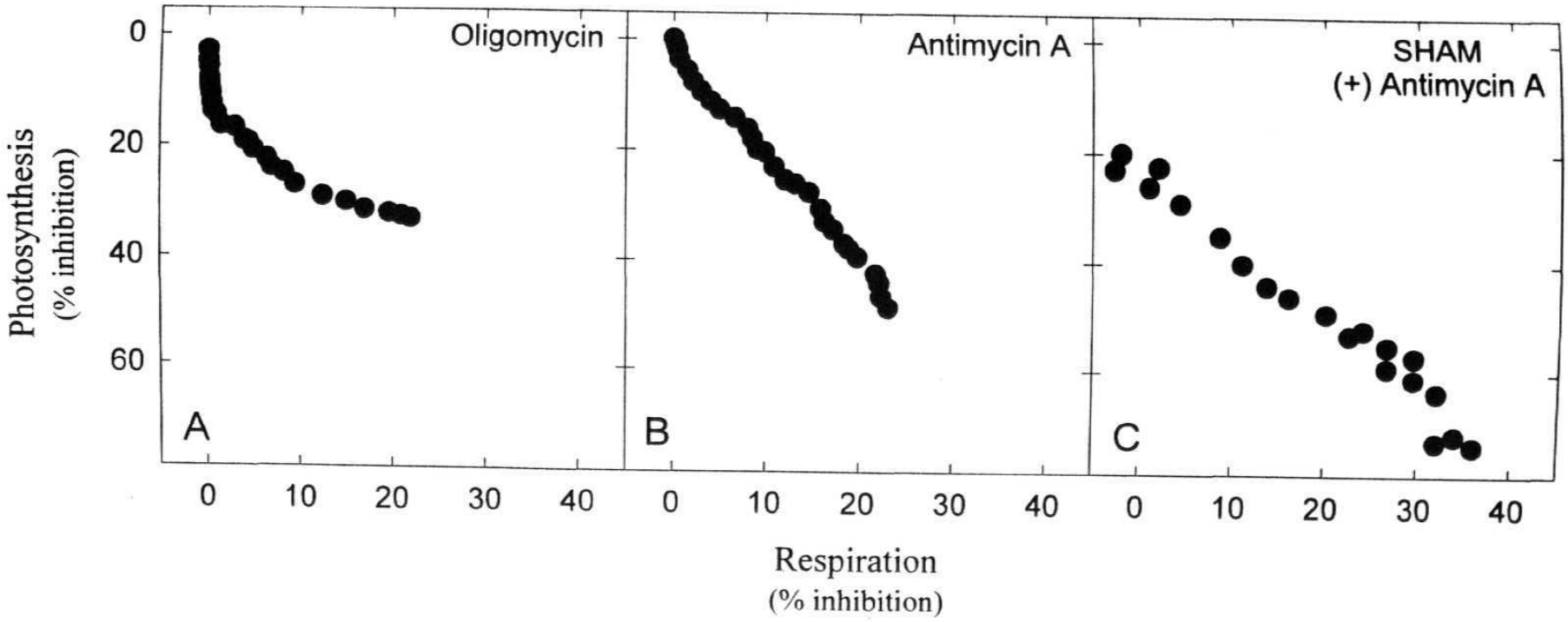

Figure 4.8. Correlation between photosynthesis and respiration (as % inhibition) at limiting CO_2 (0.1 mM $NaHCO_3$) in mesophyll protoplasts of pea upon incubation with three mitochondrial inhibitors These are collective data obtained with different concentrations of inhibitor-compounds (0 to 1 μg mL^{-1} of oligomycin; 0 to 1 μM of antimycin A; 0 to 1 mM of SHAM), as shown in Figures 4.1 - 4.6.

decrease in respiration at both optimal (Fig. 4.7, B and C) and limiting CO_2 (Fig. 4.8, B and C).

The change in the levels of intracellular ATP during illumination caused by mitochondrial inhibitors at limiting CO_2 was in contrast to that of photosynthesis. There was a significant decrease in intracellular ATP and increase in ADP levels in presence of oligomycin or antimycin at both optimal and limiting CO_2 (Fig. 4.9; Fig. 4.10). The decrease in the level of ATP at limiting CO_2 (Fig. 4.10A) was much more than that under optimal CO_2 (Fig. 4.9A), particularly in the presence of oligomycin. The level of ATP decreased by about 20% and 30% at optimal and limiting CO_2 respectively, in presence of 100 nM antimycin A (Fig. 4.9B; Fig. 4.10B). The presence of 100 ng mL^{-1} oligomycin caused a decrease of about 25% and 55% at optimal and limiting CO_2, respectively (Fig. 4.9A; Fig. 4.10A). On the other hand, the ADP levels increased by <20% in presence of oligomycin or antimycin A.

There was a small decrease in ATP levels (3 to 11%) in presence of SHAM or propyl gallate at both optimal and limiting CCb (Fig. 4.11, A and C; Fig. 4.12, A and C). However the ATP levels decreased by <20% with SHAM in presence of antimycin A (Fig. 4.11B; Fig. 4.12B). The ADP levels decreased by <15% in presence of SHAM or propyl gallate at either CO_2 concentrations. However the ADP levels raised to 17 and 35% at both optimal and limiting CO_2 respectively with SHAM in presence of antimycin A (Fig. 4.11; Fig. 4.12).

There was a drastic decrease in ATP/ADP ratio in presence of oligomycin or antimycin A at both optimal and limiting CO_2 (Fig. 4.13). The decrease in ATP/ADP ratio was more pronounced in presence of oligomycin than that of antimycin A, particularly at limiting *CO2.* However there was a small, but clear

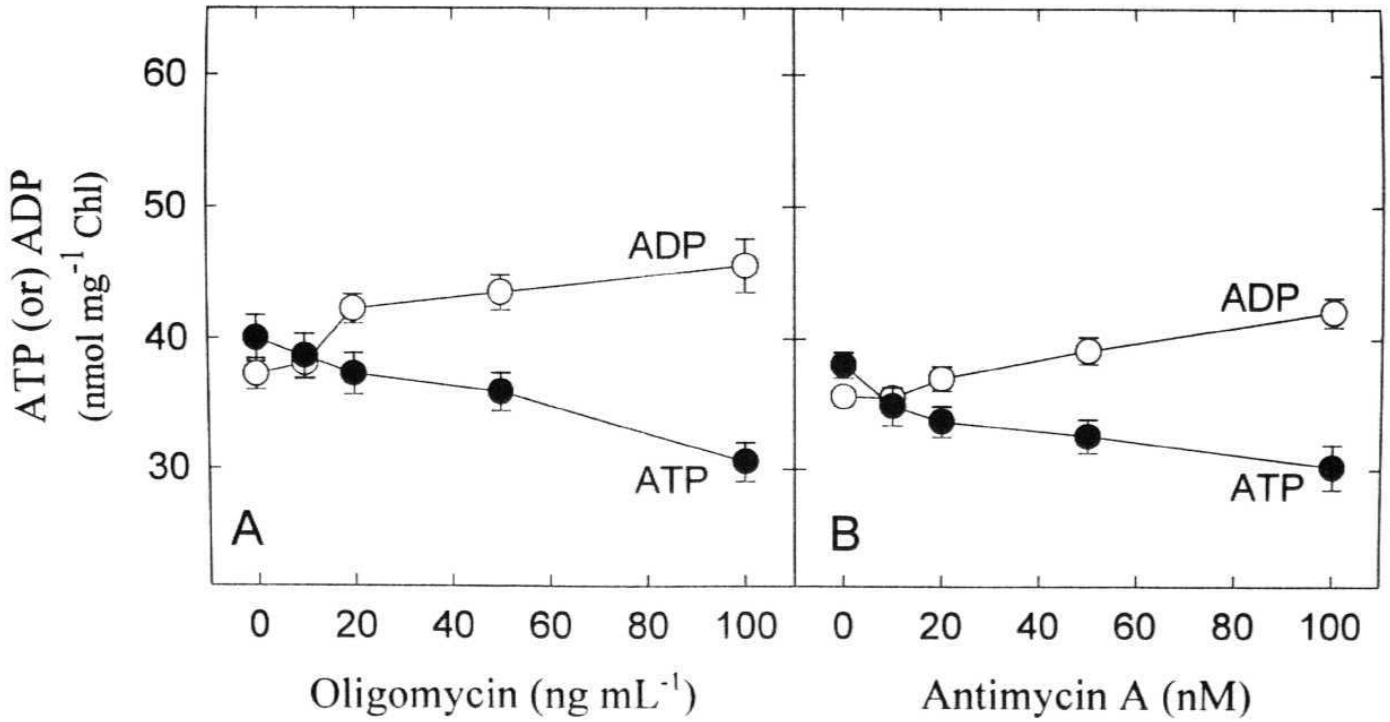

Figure 4.9. Effect of oligomycin and antimycin A on intracellular ATP and ADP levels in pea mesophyll protoplasts at optimal (1.0 mM $NaHCO_3$) CO_2. The mesophyll protoplasts were incubated with test inhibitors in dark for 5 min at 25 °C before illumination. After 10 min of illumination, aliquots of protoplast samples were collected, frozen dry in liquid N_2 and were later analyzed for ATP and ADP as described in 'Materials and Methods'. The ATP and ADP levels in the control sets (without inhibitor) were 39 ± 1.1 and 36 ± 0.8 nmol mg^{-1} Chl.

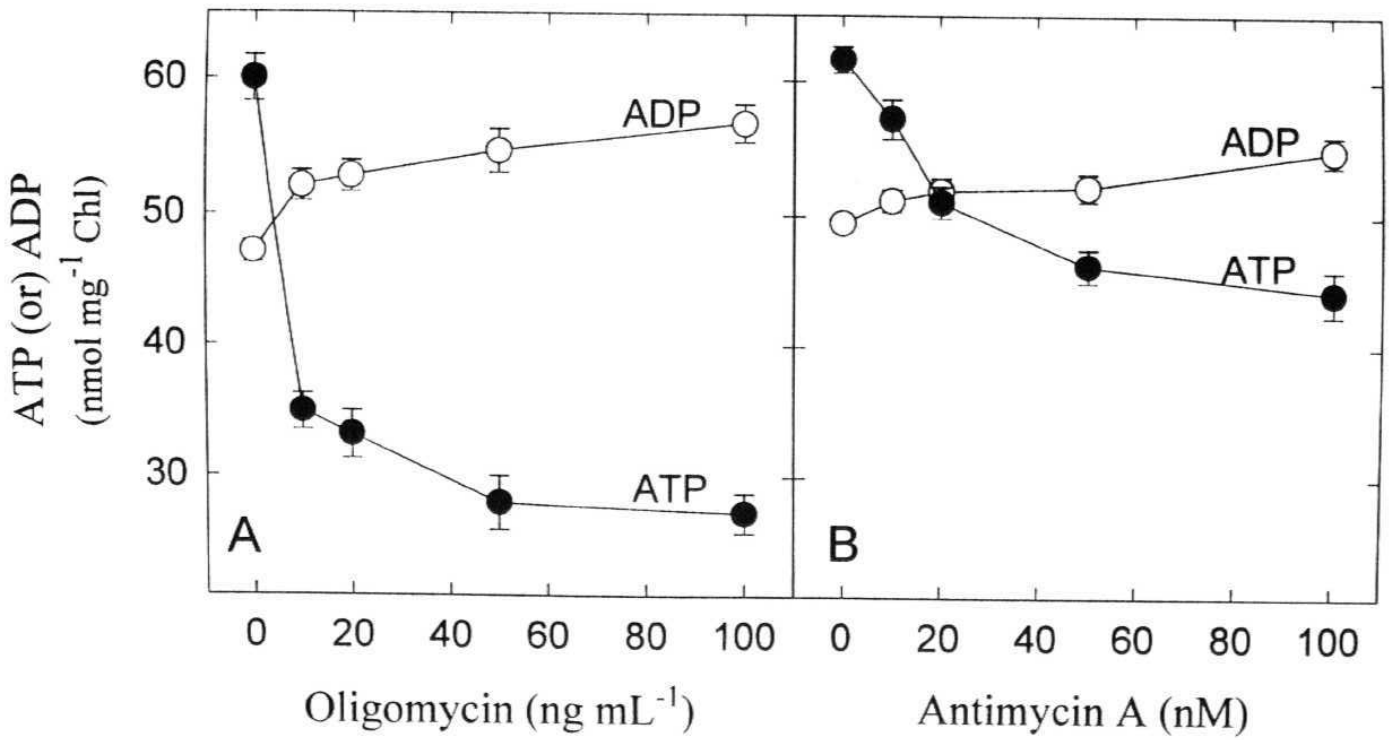

Figure 4.10. Effect of oligomycin and antimycin A on intracellular ATP and ADP levels in pea mesophyll protoplasts at limiting (0.1 mM $NaHCO_3$) CO_2. Other details were as described in Figure 4.9. The ATP and ADP levels in the control sets (without inhibitor) were 61 ± 1.0 and 48 ± 0.9 nmol mg^{-1} Chl.

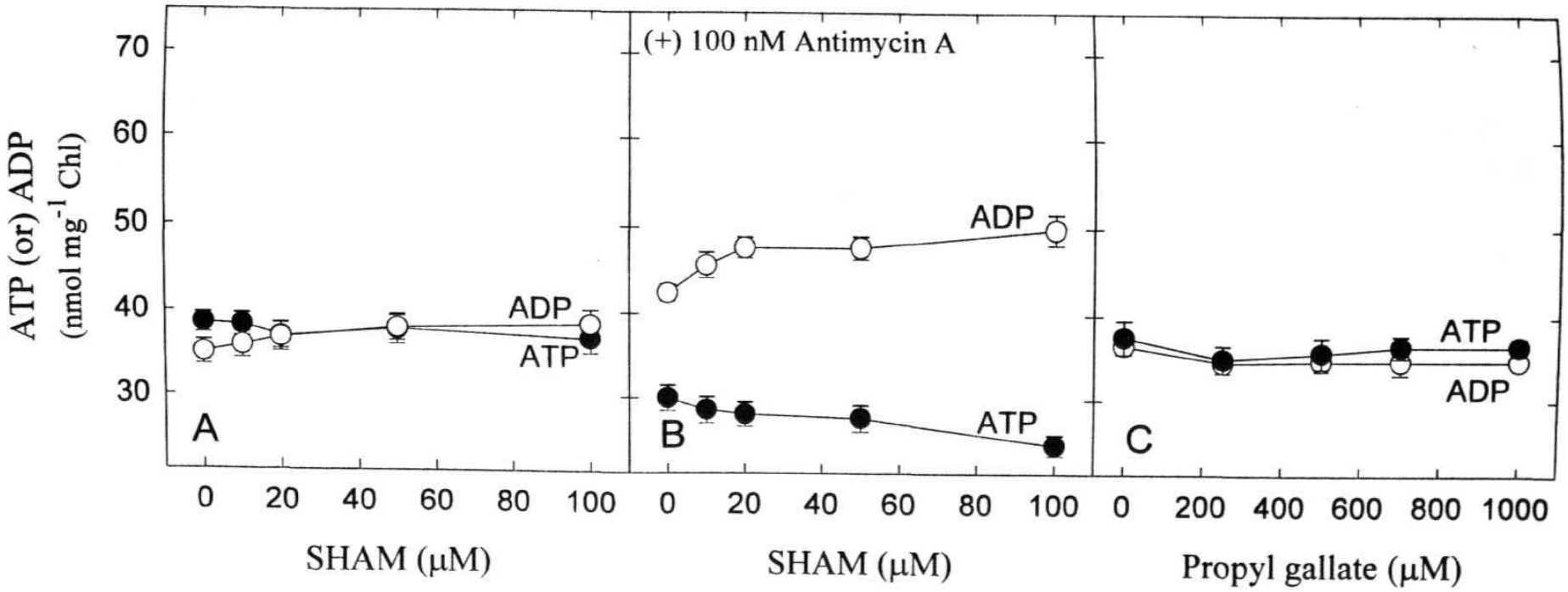

Figure 4.11. Effect of SHAM in the absence or presence of 100 nM antimycin A and propyl gallate on intracellular ATP and ADP levels in pea mesophyll protoplasts at optimal (1.0 mM $NaHCO_3$) CO_2. Other details were as described in Figure 4.9.

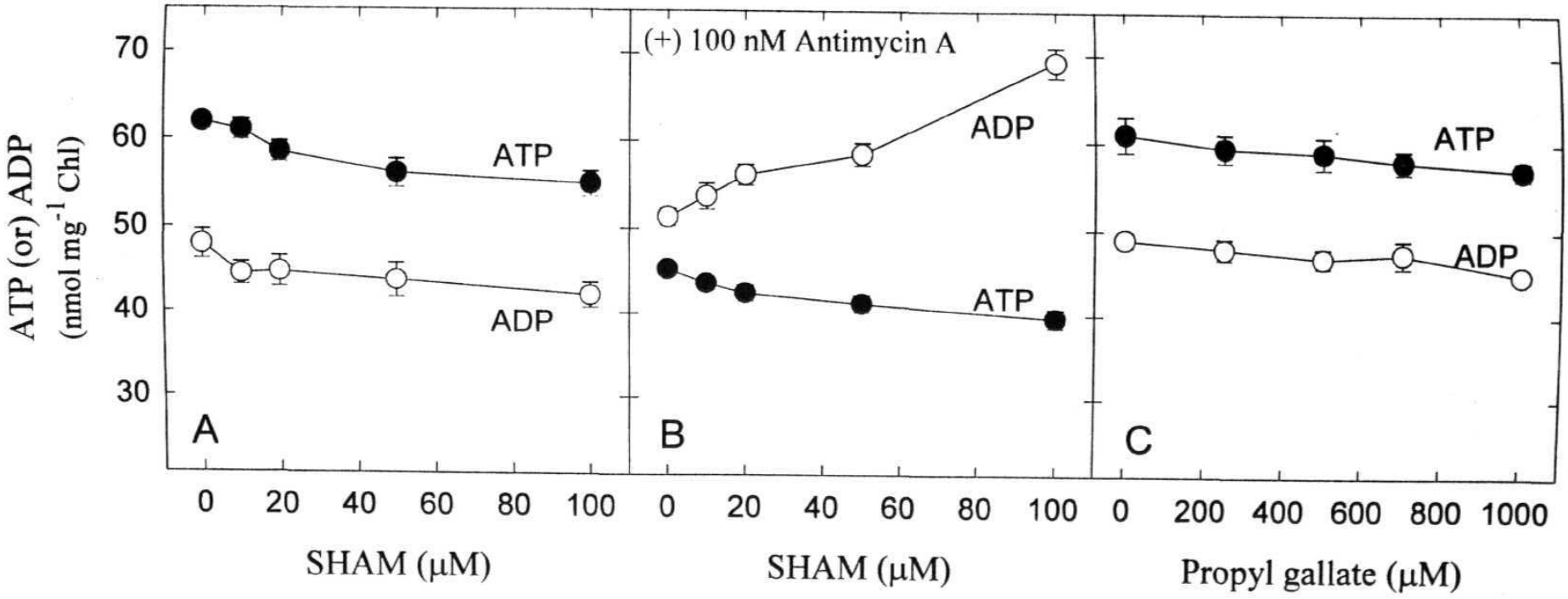

Figure 4.12. Effect of SHAM in the absence or presence of 100 nM antimycin A and propyl gallate on intracellular ATP and ADP levels in pea mesophyll protoplasts at limiting (0.1 mM $NaHCO_3$) CO_2. Other details were as described in Figure 4.10.

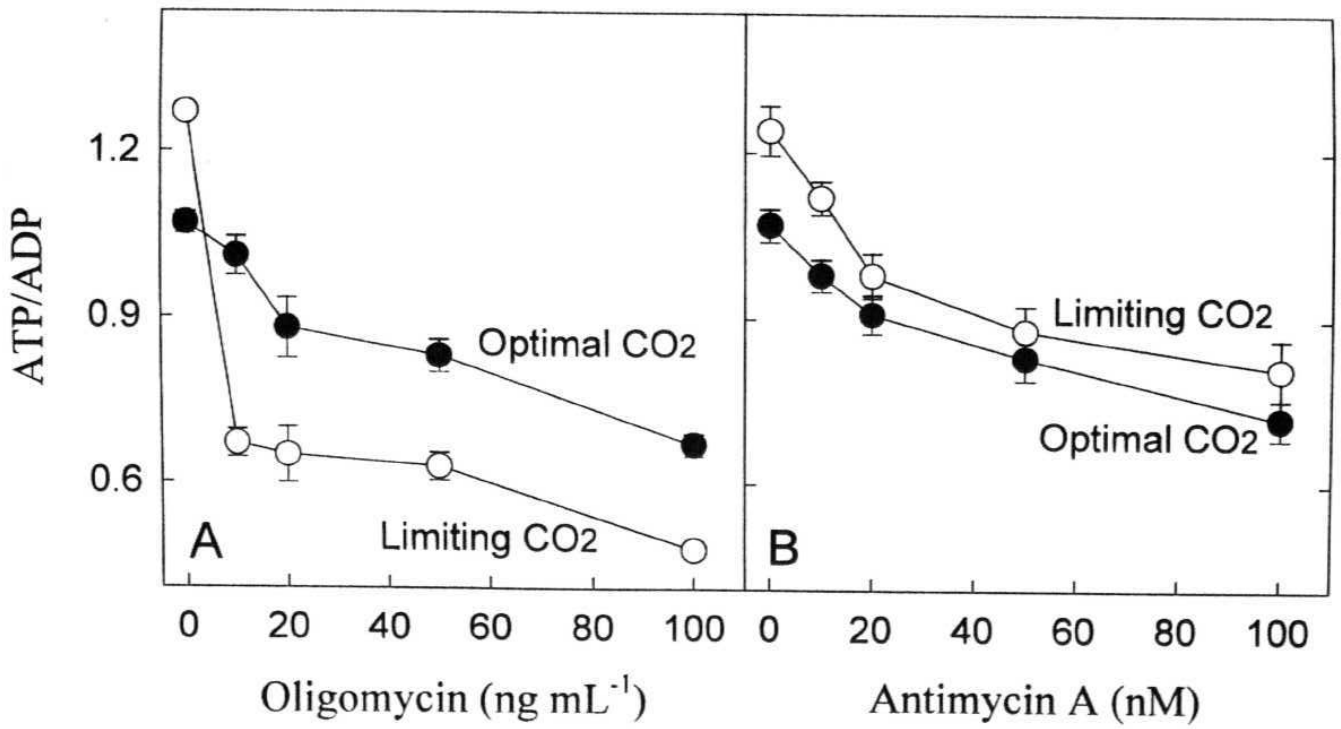

Figure 4.13. The ratio of intracellular ATP to ADP in pea mesophyll protoplasts, during photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2. These ratios were calculated from the data in Figures 4.9 and 4.10. The ratio of ATP to ADP in control sets (in the absence of inhibitors) at optimal and limiting CO_2 were 1.07 and 1.27, respectively.

distinct decrease in ATP/ADP ratio with **SHAM** in presence **of antimycin A compared to that** of SHAM and propyl gallate (Fig. 4.14).

The relation between ATP/ADP ratio in protoplasts and their photosynthesis at optimal CO_2 (Fig. 4.15) or limiting CO_2 (Fig. 4.16) was assessed. Despite the expectation that ATP demands would be low at limiting CO_2, there was a steep positive correlation between the rates of photosynthesis and ratios of ATP to ADP in protoplasts in the presence of oligomycin (Fig. 4.16A) or antimycin A (Fig. 4.16B). On the other hand, there was a biphasic correlation in presence of oligomycin (Fig. 4.15A) or antimycin A (Fig. 4.15B) at optimal CO_2. In contrast, there was no correlation with the ratios of ATP to ADP, in spite of the marked decrease in the photosynthetic rate of protoplasts by SHAM at optimal (Fig. 4.15C) or limiting CO_2 (Fig. 4.16C). Nevertheless, a positive correlation could again be seen between ATP/ADP ratio and the rate of photosynthesis when antimycin A was present along with SHAM (Fig.4.15D;Fig. 4.16D).

The Glc-6-P level increased by 19-30% in the presence of both oligomycin and antimycin A at optimal CO_2 conditions. However, at limiting CO_2, there was no change in Glc-6-P levels in presence of oligomycin but there was about a 30% decrease in presence of antimycin A (Table 4.1). On the other hand, SHAM decreased marginally (20% to 40%) the Glc-6-P levels, both at optimal and limiting CO_2 in absence or presence of antimycin A. However the Glc-6-P levels increased to <10% in presence of propyl gallate (Table 4.1).

Discussion

The initial experiments established that the effect of oligomycin or antimycin A or SHAM on dark respiration and cellular ATP levels was quite

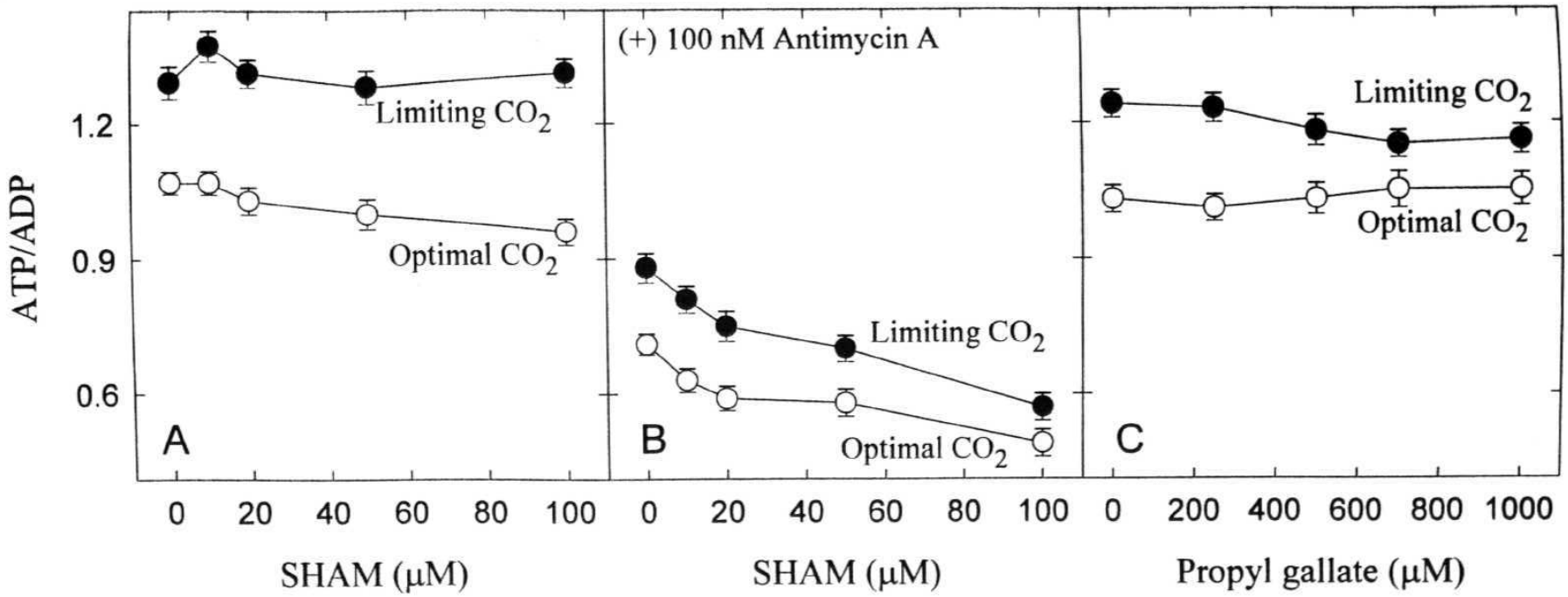

Figure 4.14. Effect of SHAM (± 100 nM antimycin A) and propyl gallate on the intracelluar ATP to ADP ratio in pea mesophyll protoplasts during photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2. These ratios were calculated from the data in Fig. 4.11 and 4.12. Other details were as described in Figure 4.13.

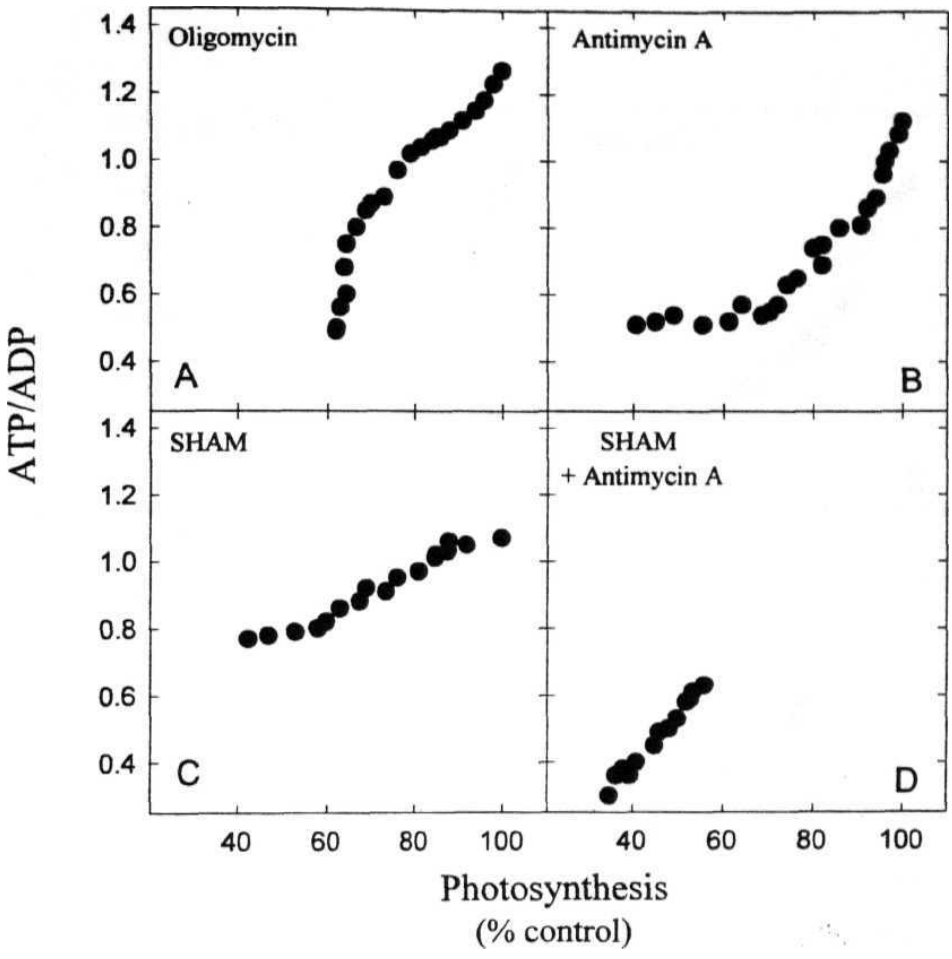

Figure 4.15. Correlation between the relative rates of photosynthesis (as % of control) and the ratios of ATP/ADP in mesophyll protoplasts after 10 min of illumination at optimal CO_2 (1.0 mM $NaHCO_3$) in presence of oligomycin or antimycin A or SHAM (± 100 nM antimycin A). These are collective data obtained with different concentrations of mitochondrial inhibitors (0 to 1 μg mL^{-1} of oligomycin; 0 to 1 μM of antimycin A; 0 to 1 mM of SHAM).

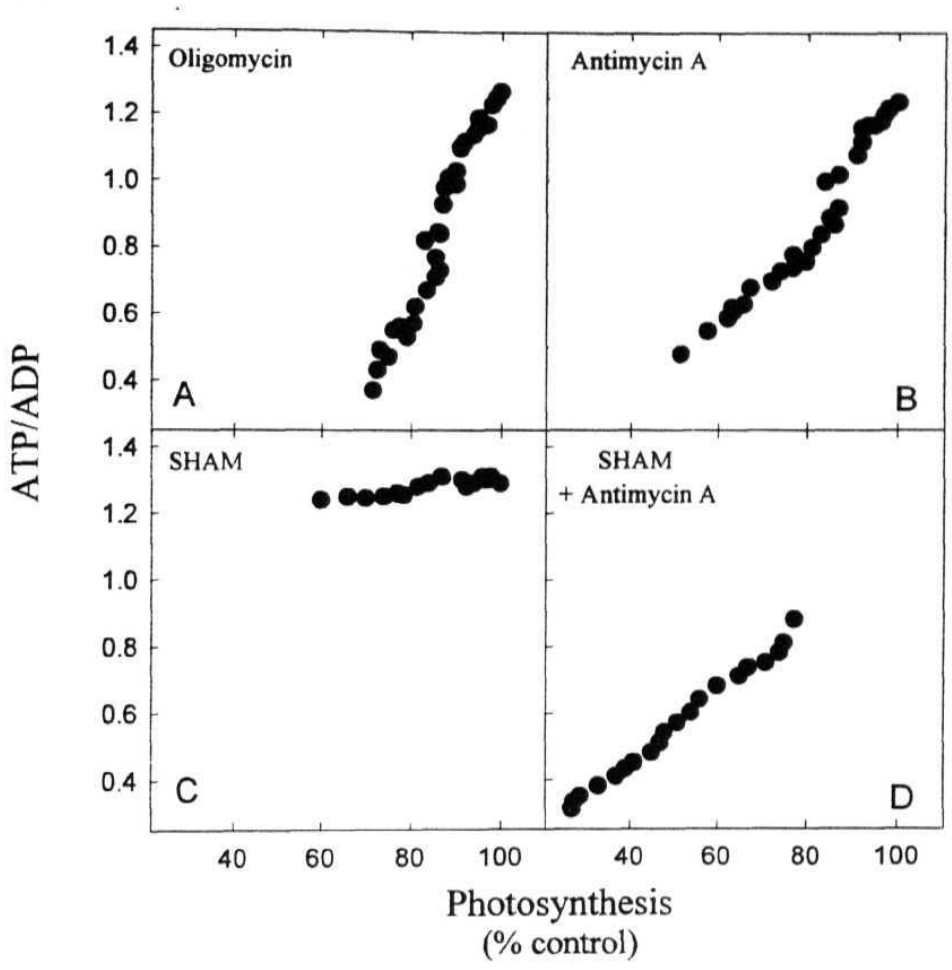

Figure 4.16. Correlation between the relative rates of photosynthesis (as % of control) and the ratios of ATP/ADP in mesophyll protoplasts after 10 min of illumination at limiting CO_2 (0.1 mM $NaHCO_3$) in presence of oligomycin or antimycin A or SHAM (± 100 nM antimycin A). These are collective data obtained with different concentrations of mitochondrial inhibitors (0 to 1 μg mL^{-1} of oligomycin; 0 to 1 μM of antimycin A; 0 to 1 mM of SHAM).

Table 4.1. *Response to different metabolic inhibitors of Glc-6-P levels in mesophyll protoplasts of pea during photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

Mesophyll protoplasts were incubated in darkness for 5 min at 25 °C with and without (control) test inhibitors. Protoplast samples were collected after 10 min illumination (corresponding to activated phase of photosynthesis), frozen and analysed for Glc-6-P.

Treatment	Optimal CO_2	Limiting CO_2
	nmol mg $^{-1}$ Chl	
Control	37 ± 1.5	51 ± 4.0
100 ng mL^{-1} Oligomycin	44 ± 1.0	52 ± 3.5
100 nM Antimycin A	48 ± 3.5	37 ± 2.0
500 µM SHAM	33 ± 2.0	44 ± 2.8
1 mM Propyl gallate	39 ± 3.0	56 ± 6.0
500 µM SHAM + 100 nM Antimycin A	31 ± 2.5	41 ± 3.2

different. Oligomycin affected primarily ATP synthesis while antimycin A (an inhibitor of mitochondrial electron transport through cytochrome pathway) or SHAM (an inhibitor of AOX pathway) affected both dark respiration as well as ATP synthesis, the latter marginally (Fig. 4.1; Fig. 4.2).

The significant decrease in the rate of photosynthesis under both limiting and optimal CO_2 in presence of these mitochondrial inhibitors suggests that mitochondrial metabolism is essential for maximal photosynthesis under limiting CO_2 (photorespiratory conditions) as well as at optimal CO_2 (Fig. 4.3; Fig. 4.4). We also emphasise that even a small decrease in the rate of respiration causes a big drop in photosynthesis. For example, a decrease of <10% in the rate of respiration resulted in a 35% decrease in the rate of photosynthesis (under optimal CO_2) in the presence of oligomycin (Fig. 4.7A). Similarly, a decrease of 25% in respiration caused an inhibition as high as 60% in photosynthesis in the presence of antimycin A (Fig. 4.7B). The decrease in the rate of photosynthesis to a greater extent at optimal CO_2 as compared to that under photorespiratory conditions (limiting CO_2) is in agreement with the results obtained with barley protoplasts (Kromer et al., 1993).

In plant cells. most of the photosynthate is converted to Suc, an ATP consuming process located in the cytosol. The decrease in photosynthesis of protoplasts upon addition of oligomycin or antimycin A may be, therefore, explained partly by a decrease in Suc synthesis caused by the decreased availability of cytoplasmic ATP leading to down-regulation of cytosolic FBPase and SPS. The increase in the cellular content of Glc-6-P (Table 4.1) and triose-P (Kromer et al., 1988; Kromer and Heldt, 1991a; Kromer et al., 1993; also see Chapter 7), precursors of Suc synthesis, upon addition of oligomycin and antimycin A supports this hypothesis at optimal CO_2. However, at limiting CO2,

there is either no change in the level of Glc-6-P (in presence of oligomycin) or a decrease in Glc-6-P (in presence of antimycin A), indicating that SPS activity is not limiting photosynthesis under photorespiratory conditions. Further, the presence of SHAM, an inhibitor of AOX, which leads to only a limited ATP production (Fig. 4.11, A and B; Fig. 4.12, A and B) had negligible effect on Glc-6-P (Table 4.1), while affecting markedly photosynthesis in mesophyll protoplasts (Fig. 4.4, A and B; Fig. 4.6, A and B). Thus, the mitochondrial supply of ATP for Suc synthesis may be only a secondary factor during the interaction with photosynthesis at limiting CO_2

A cursory look at the relationship between the rates of photosynthesis and ATP/ADP levels (Fig. 4.15; Fig. 4.16) gives an impression that ATP levels are positively correlated with photosynthetic capacity. The requirement of ATP for photosynthesis is expected to be much higher at optimal CO_2 than that under limiting CO_2. However, a critical examination indicates that the positive correlation between ATP/ADP ratio and photosynthesis in the presence of oligomycin is much stronger under optimal CO_2 than that under limiting CO_2. For example, there was no further decrease in photosynthesis, beyond 40% inhibition, although the ATP/ADP ratio decreased from 1.3 to 0.5 in the presence of oligomycin (Fig. 4.15A). On the other hand, the inhibition of photosynthesis reached 60%, as the ATP/ADP ratio decreased from 1.15 to 0.5 in the presence of antimycin A at optimal CO_2 (Fig. 4.15B). Thus, restriction of ATP/ADP ratio in the presence of oligomycin caused limited inhibition of photosynthesis (Fig. 4.15A; Fig. 4.16A), while the inhibition increased further in presence of antimycin A, presumably due to the restriction of oxidative electron transport (Figs. 4.15B; Fig. 4.16B).

The correlation between photosynthesis and ATP/ADP ratio seen in the presence of even antimycin A can be interpreted by the fact that a decrease in the extent of electron transport leads to a decrease in ATP content (e.g., Fig. 4.1B; 4.9B; 4.10B) and thereby ATP/ADP ratio (Fig. 4.13B). The weak relation or lack of any correlation between the marked decrease in photosynthesis (< 40%) and ATP/ADP ratio of protoplasts in presence of SHAM (Fig. 4.15C; Fig. 4.16C) suggests that the effect of SHAM is independent of oxidative phosphorylation. Since antimycin A is an inhibitor of oxidative electron transport, we suggest that the mitochondrial electron transport system is more crucial than that of oxidative phosphorylation in optimizing photosynthesis.

Cellular respiration is flexible and the partitioning of electrons between cytochrome and AOX pathways is modulated by several factors. For example, AOX activity is increased by chilling, presence of pyruvate and a redox pool (Vanlerberghe and McIntosh, 1996). Recent reports suggest that AOX can support respiratory carbon metabolism (Vanlerberghe et al., 1997), limits superoxide production in mitochondria (Purvis, 1997), besides mediating the classic role of heat-generation (Ordentlich et al., 1991; Breidenbach et al., 1997). The present observations suggest that the AOX pathway is essential for the optimization of photosynthesis in mesophyll protoplasts, both at limiting and optimal CO_2 (Fig. 4). Incidentally, the content of AOX in green tissues is more than that of etiolated ones (Ribas-Carbo et al., 1997).

Two possible objections against the use of SHAM are that (i) the compound may not enter the protoplasts and (ii) the inhibition may be unspecific. The marked inhibition of photosynthesis (as well as respiration) by even low concentrations (Fig. 4.2; Fig. 4.4, A and B) suggest that SHAM is presumably entering the protoplasts. Similar observations have been made recently by other

workers (Lynnes and Weger, 1996; Igamberdiev et al., 1997). The presence of SHAM did not affect chloroplast activity, while inhibiting protoplast photosynthesis (Fig. 4.6, A and B). We are therefore confident that at the low concentrations used in the present work, SHAM affects specifically mitochondrial electron transport via AOX. Oligomycin or antimycin A also had no direct effect on chloroplast photosynthesis (Fig. 4.5; also Krömer et al., 1988).

The importance of oxidative electron transport (through both cytochrome and AOX pathways) in optimizing photosynthesis is supported by three observations: (a) the stronger reduction in photosynthesis at optimal CO_2, than that at limiting CO_2 (Fig. 4.3; Fig. 4.4), while an opposite trend exists in the response of cellular ATP levels (Figs. 4.9 - 4.12). Although the mitochondrial contribution to cellular ATP is high, photosynthesis remains less sensitive to oligomycin or antimycin A at limiting CO_2 than that at optimal CO_2; (b) a much stronger inhibition of photosynthesis, at both limiting and optimal CO_2, by antimycin A or SHAM than by oligomycin, particularly at a wide range of concentrations (Figs. 4.5 - 4.8); (c) the extreme sensitivity of photosynthesis in protoplasts (but not chloroplasts) to SHAM, an inhibitor of AOX, mediating the non-phosphorylating alternative path of mitochondrial electron transport (Fig. 4.6).

In view of the inherent limitations of metabolic inhibitors, we used the test compounds at very low concentrations. Yet, the respiratory inhibitors were quite effective in suppressing photosynthetic activity of protoplasts. Antimycin A is known to inhibit cyclic photophosphorylation at a concentration as high as 5 μM (Izawa et al., 1967; Drechsler et al.. 1969; Izawa and Good, 1972; Moss and Bendall, 1984). At the low concentrations used during most of our experiments (< 0.1 μM), antimycin A is expected not to affect even cyclic photophosphory-

lation. Furthermore, antimycin A may even stimulate photosynthetic CO_2 fixation in chloroplasts (Schacter and Bassham, 1972; Miginiac-Maslow and Champigny, 1974). The extent of inhibition of photosynthesis by oligomycin in our experiments with pea mesophyll protoplasts (about 35-40% at 50 ng mL^{-1}) is similar to that reported for barley protoplasts (Kromer and Heldt, 1991a).

Oligomycin affects primarily ATP formation in mitochondria (Lambers, 1990). As ATP synthesis is restricted, electron transport would decrease due to the absence of proper coupling. This might be the reason for the biphasic relationship between the extent of inhibition of respiration and that of photosynthesis in the presence of oligomycin (Fig. 4.7A; Fig. 4.8 A). On the other hand, the single phase of correlation between respiration and photosynthesis in the presence of antimycin A indicates that a single factor is involved in this interaction, presumably electron transport (Fig. 4.7B; Fig. 4.8B).

The maximal capacity of mitochondrial ATP synthesis in a leaf was estimated as about 25% of the rate of noncyclic photophosphorylation at maximal photosynthesis (Kromer and Heldt, 1991a). The decrease in the total cellular ATP (Fig. 4.9; Fig. 4.10) as well as ATP/ADP ratio (Fig. 4.13) upon addition of oligomycin or antimycin A would be likely due to a decrease in the ATP/ADP ratio in the extra-chloroplastic compartment, as observed in barley protoplasts in the presence of oligomycin (Kromer et al., 1993). Gardeström (1987) has shown that the ATP/ADP ratio in protoplast extracts increases as CO_2 becomes a limiting factor for photosynthesis.

The ATP levels are higher under photorespiratory conditions (>60 nmol mg^{-1} Chl) than those under optimal CO_2 (< 40 nmol mg^{-1} Chl). Oligomycin or antimycin A caused a significant decrease in intracellular ATP

levels and increase in ADP levels of protoplasts (Fig. 4.9; Fig. 4.10) suggesting that mitochondria have a high potential for ATP production in the light. Unlike the sensitivity of photosynthesis, the decrease in intracellular ATP as well as ATP/ADP ratio caused by these two respiratory inhibitors was higher in limiting CO_2 than that under optimal CO_2. This observation suggests that the mitochondrial contribution to total cellular ATP is much higher (up to 50%) under photorespiratory conditions than that under optimal CO_2. This is expected, because Gly is the predominant substrate for mitochondrial oxidation under photorespiratory conditions (Bergman and Ericson, 1983; Dry et al., 1983).

Although the existence and operation of AOX pathway are well established, the role of this pathway is not yet established (Siedow and Umbach, 1995; Breidenbach et al., 1997), nor its interaction with photosynthesis is known. Our results demonstrate for the first time that the AOX pathway of mitochondria is essential for optimising photosynthesis in mesophyll protoplasts.

AOX pathway is often described as a non-phosphorylating pathway (Lambers, 1982; Ribas-Carbo et al., 1997). The consistent decrease in ATP levels due to SHAM (Fig. 4.2; Fig. 4.11. A and B; Fig. 4.12, A and B) indicate that there is a small but significant contribution to ATP formation by the AOX pathway. This is not unexpected, because there is possibility of limited ATP formation, as electrons flow from complex I/II to ubiquinone, prior to their branching off to AOX (Siedow and Umbach, 1995; Vianello et al. 1997).

Our results illustrate that mitochondrial metabolism is essential for optimal photosynthesis in pea mesophyll protoplasts at both limiting as well as optimal CO_2. Even a small drop in the rate of respiration (e.g., 10%) caused a significant decrease in the photosynthetic rate (>35%) of mesophyll protoplasts. The

inhibition of photosynthesis by mitochondrial inhibitors was stronger at optimal CO_2 than that at limiting CO_2. On the other hand, the decrease in total cellular ATP and ATP/ADP ratio by mitochondrial inhibitors was more pronounced at limiting CO_2 than that at optimal CO_2. The sensitivity of protoplast photosynthesis to mitochondrial inhibitors even at limiting CO_2, when the ATP requirement for photosynthesis is low suggests that under photorespiratory conditions mitochondrial electron transport is more crucial than oxidative phosphorylation. Further, the marked sensitivity of photosynthesis in protoplasts (but not chloroplasts) to SHAM confirms that the AOX pathway of electron transport is also essential for optimal photosynthesis.

Chapter 5

Prolongation of Photosynthetic Induction by Mitochondrial Inhibitors

Chapter 5

Prolongation of Photosynthetic Induction by Mitochondrial Inhibitors

Introduction

When leaves, protoplasts or chloroplasts are illuminated after a period of darkness, photosynthetic CO_2 fixation or CO_2 dependent O_2 evolution does not reach a maximum until after a few minutes and this initial lag is called as 'induction'. This phenomenon of induction is a common feature of photosynthesis (Edwards and Walker, 1983; Walker, 1988). This lag is due to the building of Calvin cycle intermediates depleted in the preceding dark period and the delay in light activation of photosynthetic enzymes (Walker, 1976; Leegood and Walker, 1980, 1981). During the experiments designed to study the effect of mitochondrial inhibitors on the activity of photosynthesis (See Chapter 4), we observed that there was always an increase in the 'lag' or 'induction'. Even a marginal interference with photosynthetic metabolism by respiratory inhibitors increased the lag period significantly.

Since RuBP is the acceptor of CO_2 as well as the end product in the regenerative phase of PCRC cycle, changes in RuBP pools are important during the induction of photosynthesis. Studies with transgenic plants suggested that a specific reduction of even GAPDH activity in chloroplasts causes dramatic alterations to RuBP levels, and that low levels of RuBP appears to be the main reason for decrease in the assimilation rate (Price et al., 1995). Further, analysis of metabolite pools following cold hardening in winter rye suggested that oligomycin, an inhibitor of mitochondrial oxidative phosphorylation inhibited photosynthesis by limiting regeneration of RuBP (Hurry et al., 1995).

One of the most important enzymes involved in the regeneration of RuBP is PRK. We therefore chose to study the changes in RuBP pool size and PRK activity brought about by mitochondrial inhibitors in the mesophyll protoplasts. A recent study on the effects of reduction in PRK activity on the operation of carbon reduction cycle, in tobacco plants transformed with antisense PRK constructs, has shown that adjustments are made in metabolite concentrations to accommodate the loss in PRK activity (Paul et al., 1995).

This chapter describes an attempt to characterize the following processes involved in PCRC on exposure of mesophyll protoplasts to mitochondrial inhibitors. These were examined in relation with induction or steady state of photosynthesis at optimal or limiting CO_2.

a) Quantitative estimation of lag period
b) Time course of photosynthetic O_2 evolution demonstrating the induction and steady state phases
c) Changes in RuBP levels
d) Pattern of PRK activity

Results

Figure 5.1 presents the typical recorder traces of photosynthetic O_2 evolution by mesophyll protoplasts at optimal CO_2 in presence or absence of 100 nM antimycin A and 100 ng mL^{-1} oligomycin. There was a significant increase in the lag up to 4 min and nearly 5 min in presence of antimycin A and oligomycin respectively, compared to control (<3 min). However, the linearity of the photosynthetic rate after the initial lag period was not affected in presence of oligomycin or antimycin A-

The mitochondrial inhibitors prolonged the lag period of photosynthesis at both optimal and limiting CO_2 (Fig. 5.2; Fig. 5.3). In the control (without any

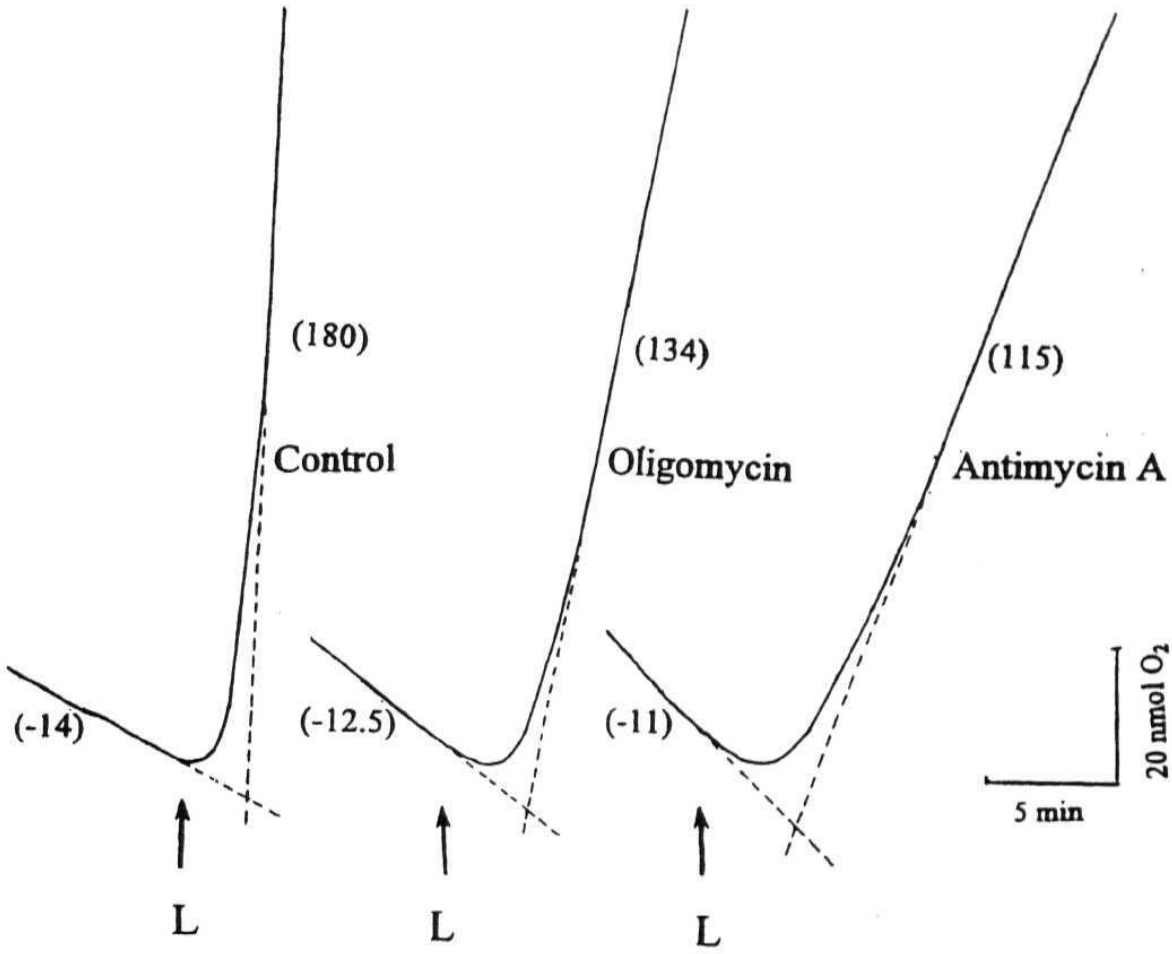

Figure 5.1. Typical examples of recorder traces of photosynthetic CO_2 dependent O_2 evolution by protoplasts indicating the prolongation of photosynthetic induction in presence of mitochondrial inhibitors. The evolution of O_2 does not start immediately after light is switched as indicated by 'L'. The induction period was <3 min in control, but extended to >4 min and nearly 5 min in presence of 100 nM antimycin A and 100 ng mL^{-1} oligomycin, respectively. The figures on the curves represent the rates of photosynthesis or respiration (negative values) in $\mu mol\ O_2$ evolution/uptake mg~ Chl h^{-1}. The photosynthetic rates were reduced by 25% and 36% of control in presence of oligomycin and antimycin A at optimal CO_2.

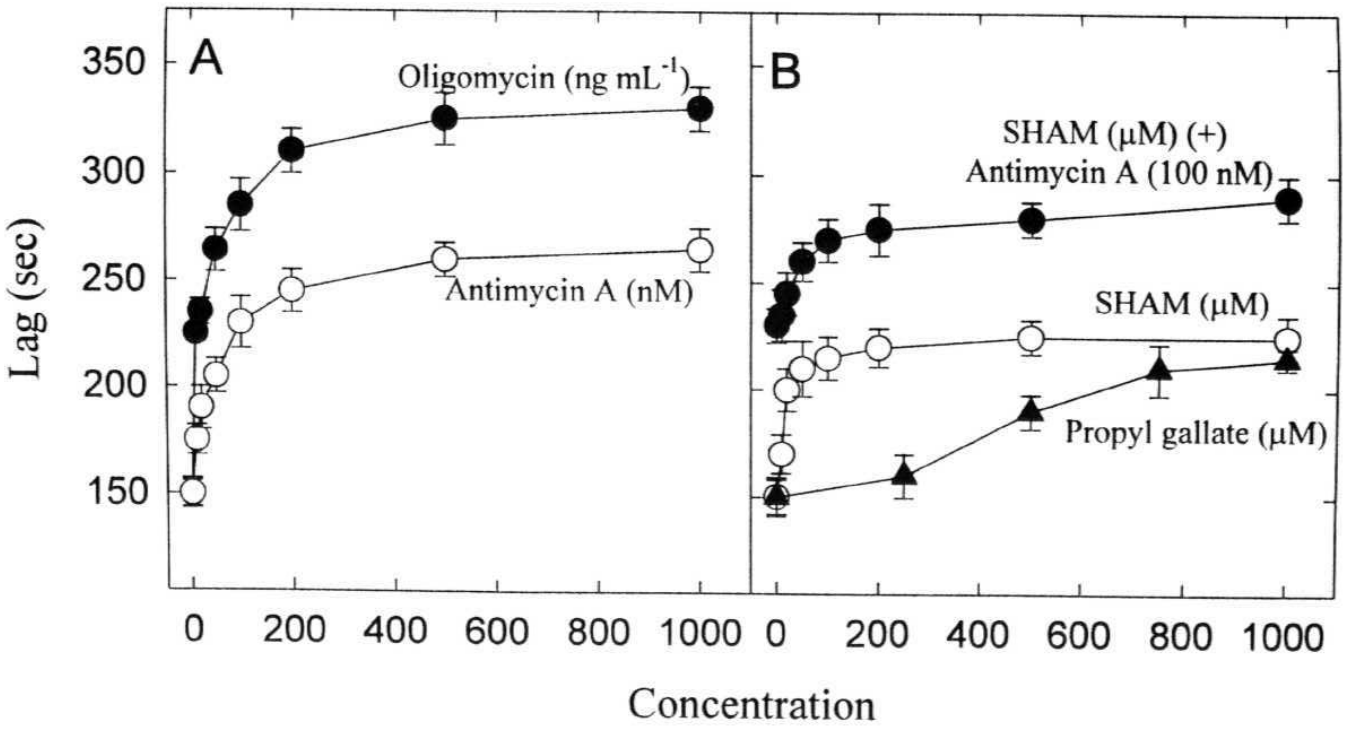

Figure 5.2. The period of photosynthetic induction at optimal CO_2 (1 mM $NaHCO_3$) as a function of concentration of mitochondrial inhibitors in the medium. The protoplasts are incubated in the dark with test inhibitors for 5 min at 25 °C before illumination. The typical patterns of lag period are illustrated in Figure 5.1. The range of the different inhibitors used were: 0 to 1 μg ml^{-1} oligomycin; 0 to 1 μM of antimycin A; 0 to 1 mM of SHAM and propyl gallate.

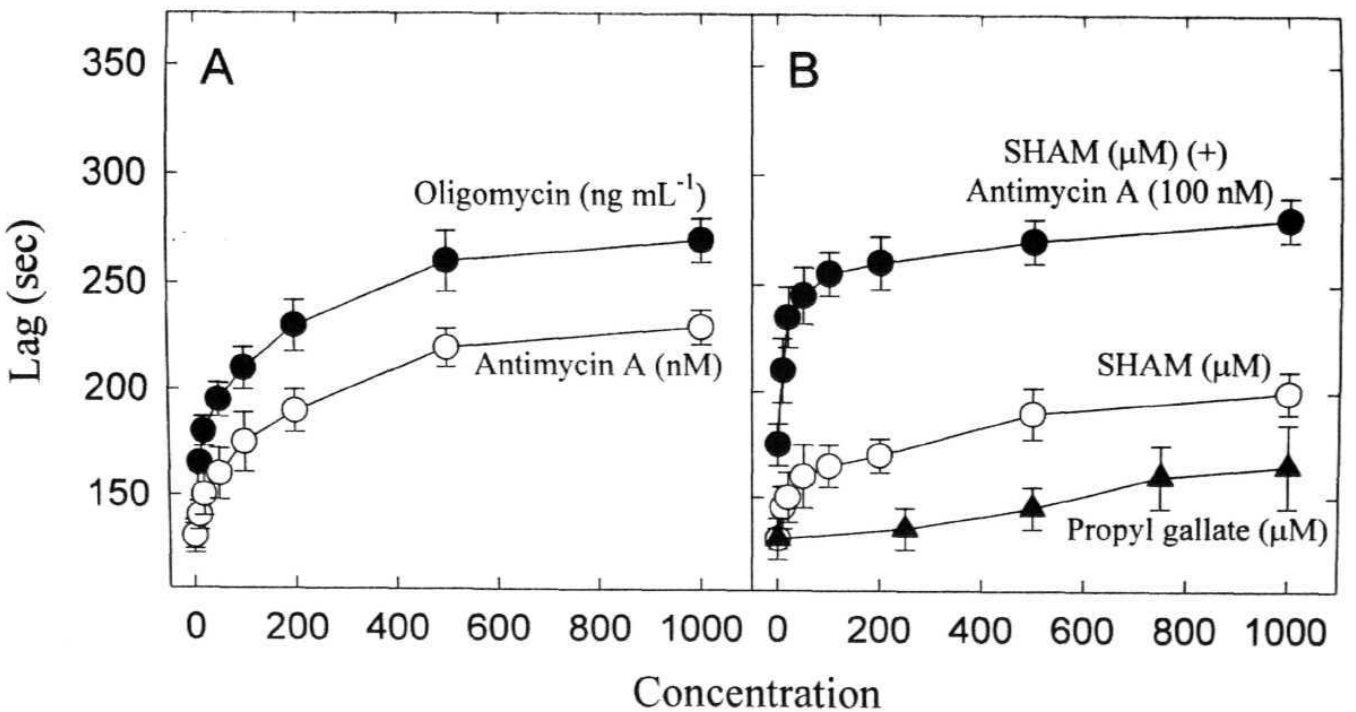

Figure 5.3. The period of photosynthetic induction at limiting CO_2 (0.1 mM $NaHCO_3$) as a function of concentration of mitochondrial inhibitors in the medium. Other details were as in Figure 5.2.

inhibitor) samples, the lag period (or induction) at optimal CO_2 (150 sec) was slightly higher than that at limiting CO_2 (130 sec). At optimal CO_2, the lag increased to >5 min in presence of 1 $\mu g\ mL^{-1}$ oligomycin and to >4 min in presence of 1 μM antimycin A (Fig. 5.2A). In contrast there was only a marginal increase in lag to <4 min at 1 mM SHAM or propyl gallate. The lag increased to almost 5 min with SHAM in presence of antimycin A (Fig. 5.2B). The increase in lag period is higher in presence of oligomycin or antimycin A compared to SHAM or propyl gallate even at limiting CO_2. However the presence of antimycin A resulted in a prolongation of lag period, even with SHAM (Fig. 5.3, A and B).

The rate of photosynthetic O_2 evolution was stable, after 4-5 min of illumination, at both optimal and limiting CO_2 (Fig. 5.4A; Fig. 5.5A). Even though the rate of photosynthetic O_2 evolution was decreased. the linearity was maintained when protoplasts were treated with: oligomycin (100 ng mL^{-1}), antimycin A (100 nM) and propyl gallate (1 mM) (Fig. 5.4, B. C and E; Fig. 5.5, B. C and E). However the stability was significantly affected and photosynthetic rate declined in presence of 500 fiM SHAM (± 100 nM antimycin A) at both optimal and limiting CO_2 (Fig. 5.4, D and F; Fig. 5.5, D and F).

In control samples the levels of RuBP increased significantly (about 30%) on illumination at both optimal (Fig. 5.6A) and limiting CO_2 (Fig. 5.7A). On the other hand the RuBP levels decreased on illumination in presence of oligomycin or antimycin A (Fig. 5.6, B and C; Fig. 5.7, B and C). However, the decrease in RuBP was more pronounced at optimal CO_2 compared to limiting CO_2. In contrast there was an increase in RuBP levels on illumination in presence of SHAM (± antimycin A) or propyl gallate (Fig. 5.6, D and F; Fig. 5.7, D and F).

The pattern of RuBP levels in presence of various metabolic inhibitors during active photosynthesis (after 10 min of illumination) are summarized in

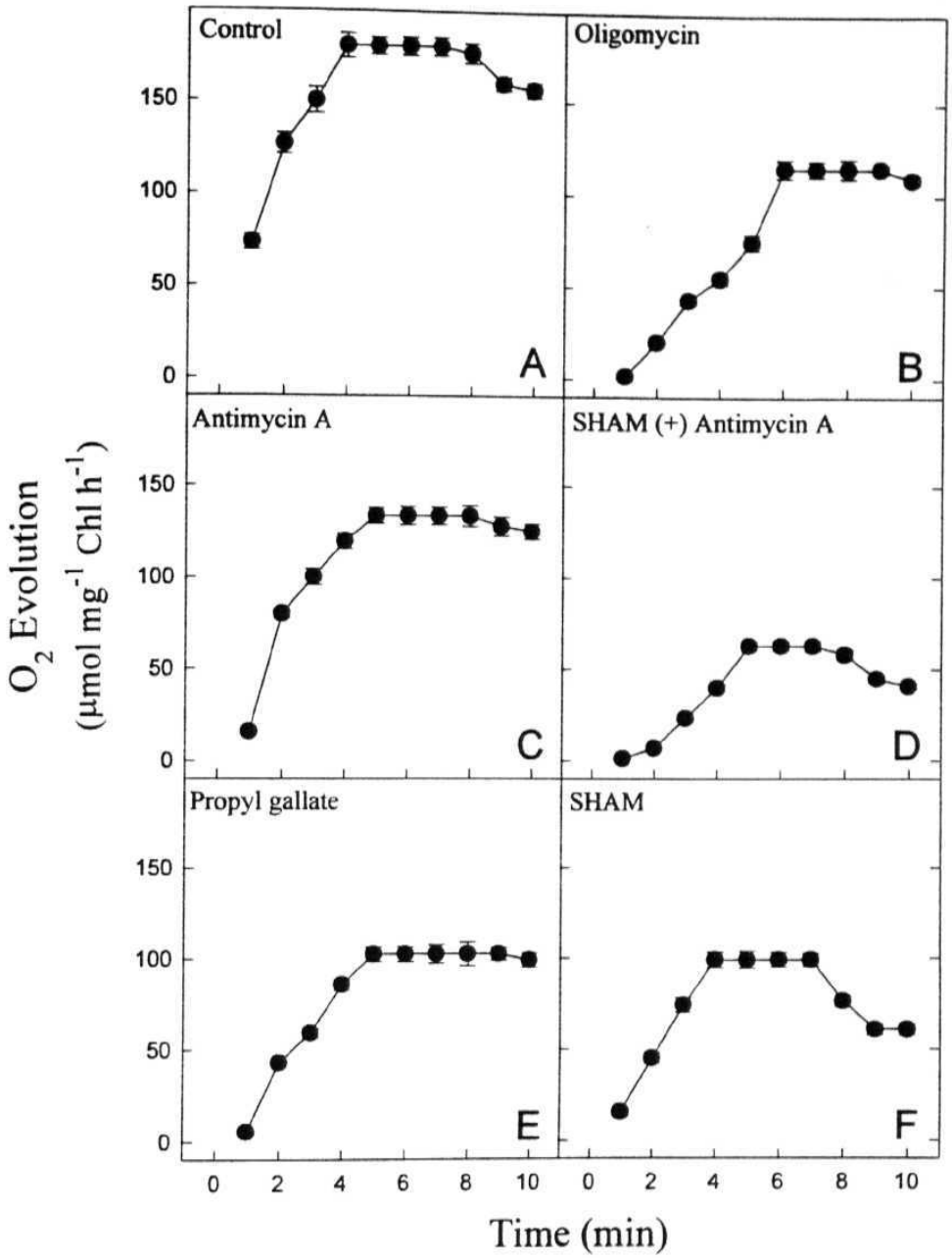

Figure 5.4. The rates of photosynthetic oxygen evolution by mesophyll protoplasts at different times after illumination at optimal CO_2 (1.0 mM $NaHCO_3$). The protoplasts were incubated in dark with test inhibitors for 5 min at 25 °C before switching on the light. A. Control (without inhibitor); B. 100 ng mL^{-1} oligomycin; C. 100 nM antimycin A; D. 500 uM SHAM (+ 100 nM antimycin A); E. 1 mM propyl gallate; F. 500 uM SHAM alone. Error bars, if not shown, are within the symbol.

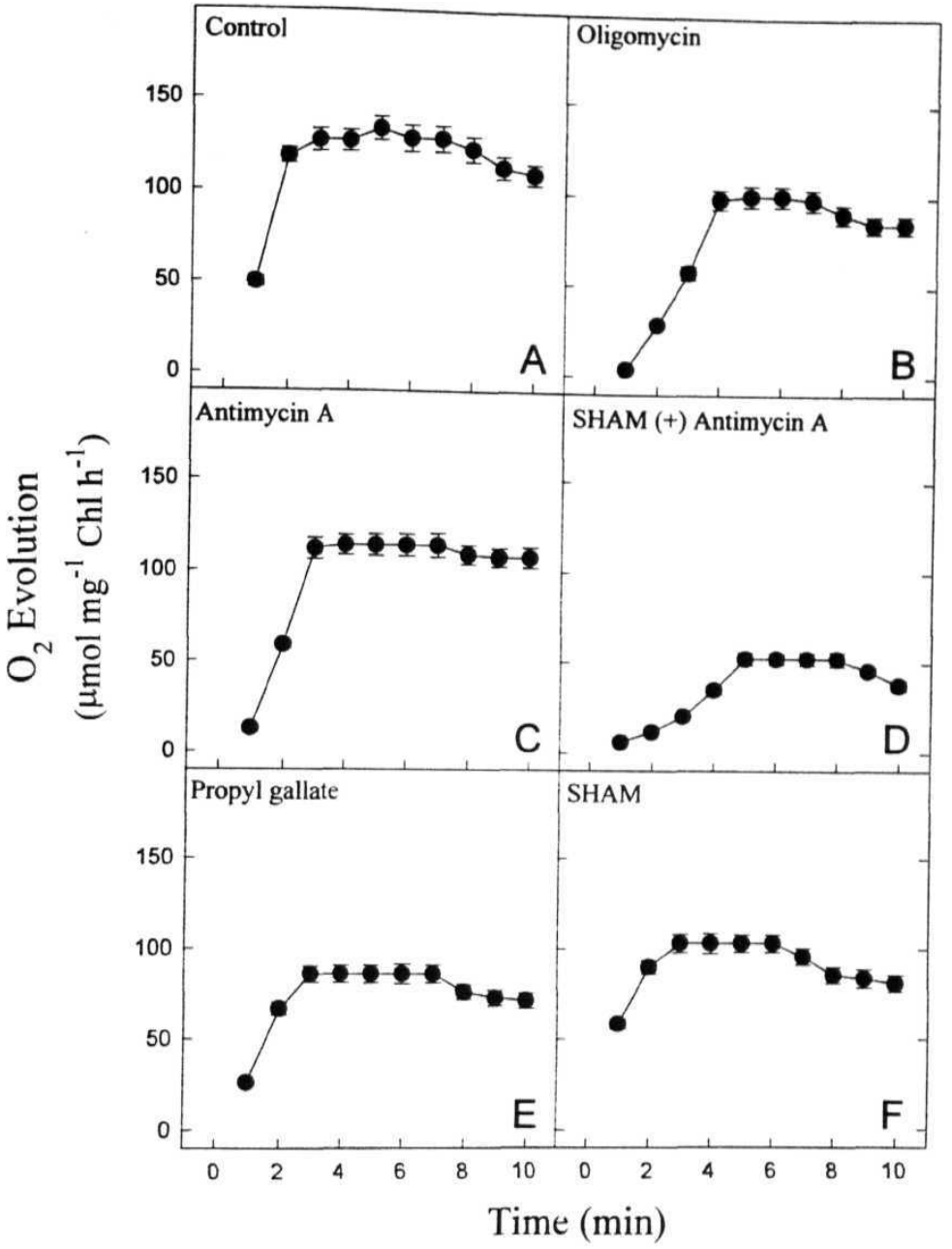

Figure 5.5. Relative rate of photosynthetic oxygen evolution by mesophyll protoplasts at limiting CO_2 (0.1 mM $NaHCO_3$) as a function of time. Other details were as in Figure 5.4.

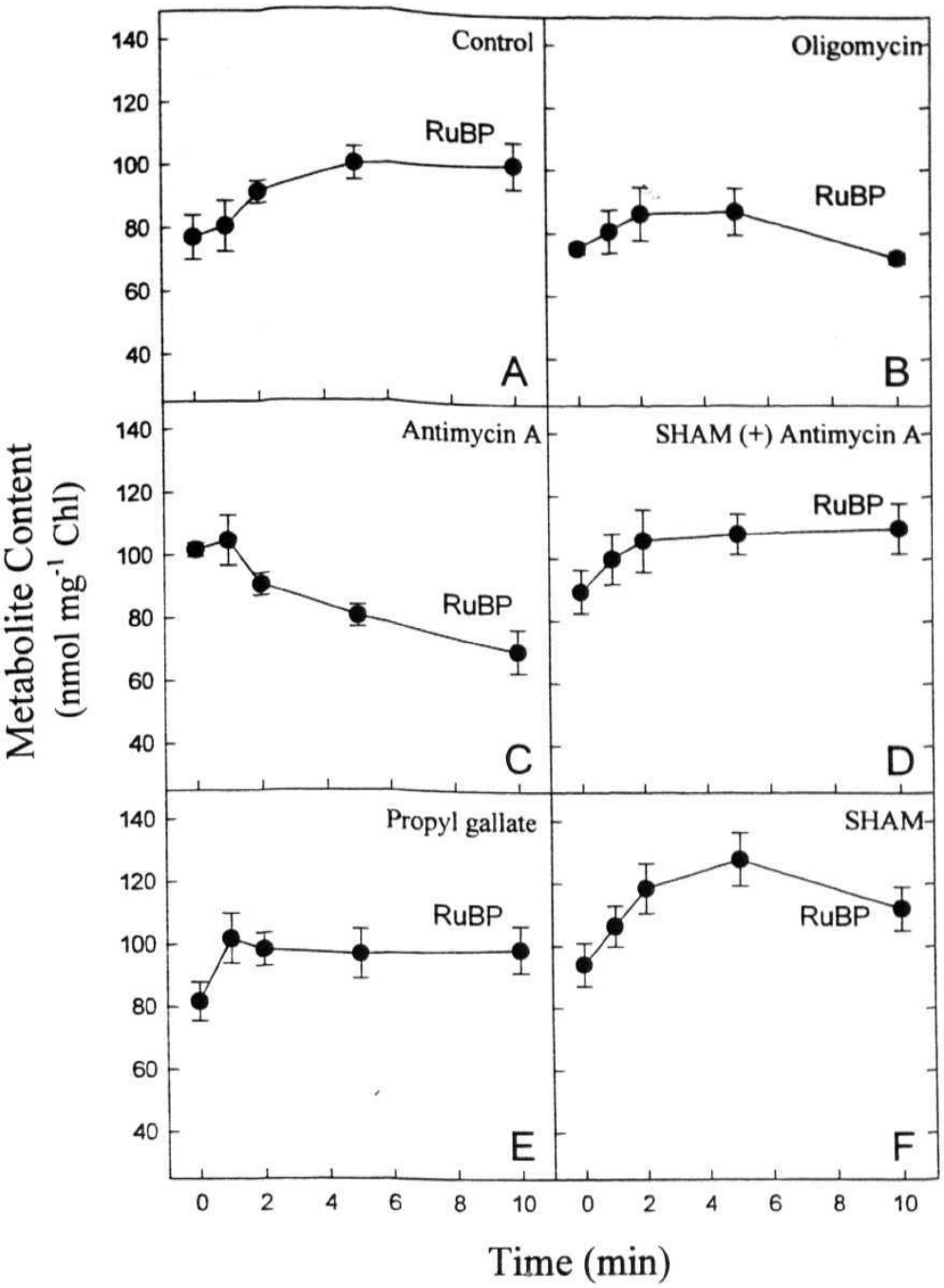

Figure 5.6. Change in the levels of RuBP in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$). The mesophyll protoplasts were incubated with test inhibitors in dark for 5 min at 25 °C before illumination. Aliquots of protoplast samples were collected at the indicated time intervals, frozen dry in liquid N_2 and were later analyzed for RuBP. Further details are described in 'Materials and Methods'. A. Control (without inhibitor); B. 100 ng mL^{-1} oligomycin; C. 100 nM antimycin A; D. 500 u,M SHAM (+ 100 nM antimycin A); E. 1 mM propyl gallate; F. 500 uM SHAM. The content of RuBP raised from 77 + 7 nmol mg^{-1} Chl at the beginning of experiment to 101 $\pm$ 7.5 nmol mg^{-1} Chl after 10 min of light in control (without inhibitor) samples.

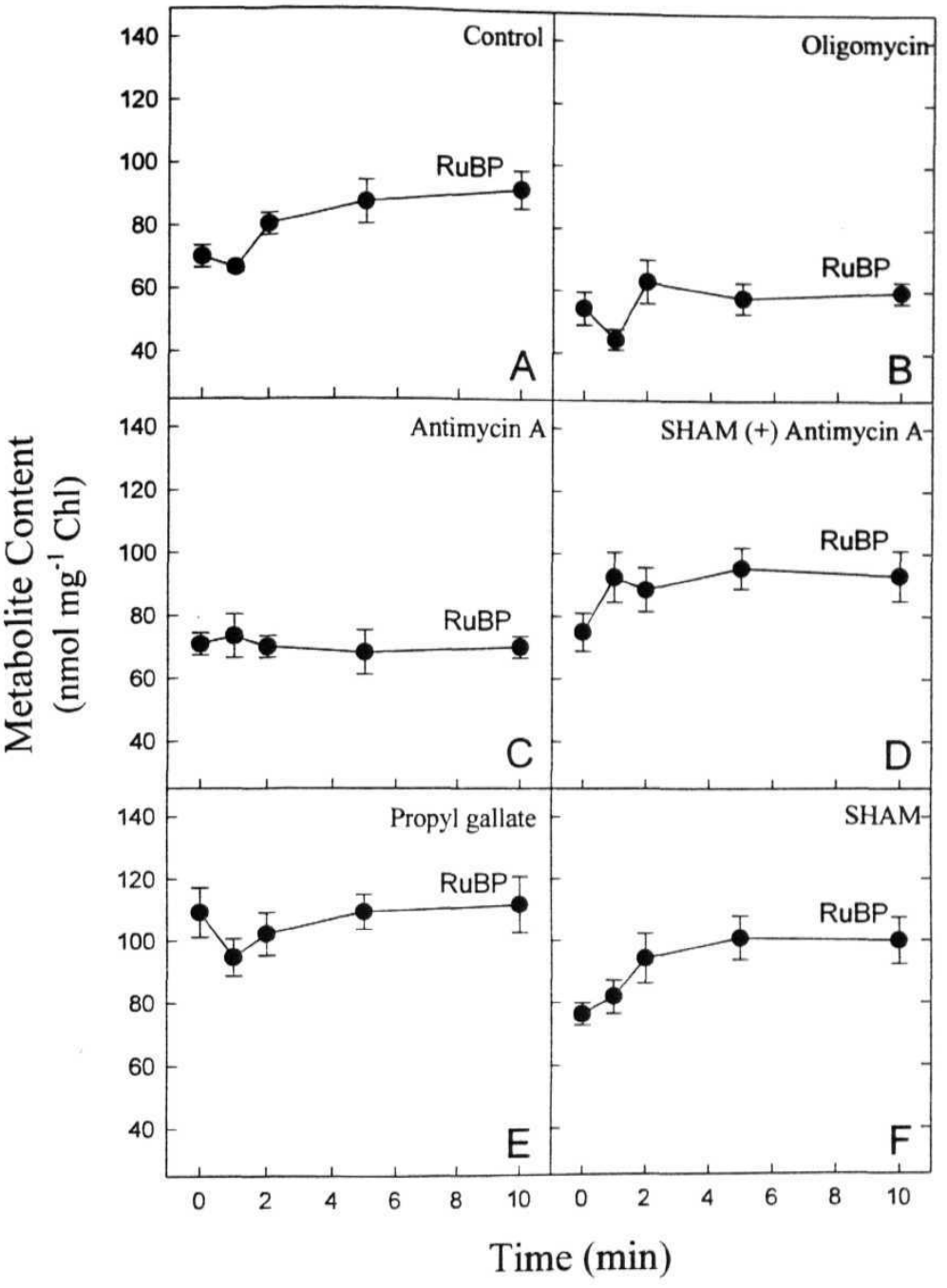

Figure 5.7. Change in the levels of RuBP in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM $NaHCO_3$). Other details were as in Figure 5.6. The content of RuBP raised from 70 + 4 nmol mg^{-1} Chl at the begining of experiment to 92 + 6 nmol mg^{-1} Chl after 10 min of light in control (without inhibitor) samples.

Table 5.1. The RuBP levels decreased significantly in presence of oligomycin or antimycin A, but kept up their increase in presence of SHAM (± antimycin A) or propyl gal late. The pattern of response to these inhibitors was similar at both optimal and limiting CO_2. However the levels of RuBP at optimal CO_2 were slightly higher than those at limiting CO_2.

Figures 5.8 and 5.9 show the time course of PRK activity during illumination at optimal or limiting CO_2. The PRK activity reached the maximum at 5 min after illumination and was steady up to 10 min. However, when incubated with mitochondrial inhibitors, the activity of PRK picked up slowly. As a result, PRK activity in presence of mitochondrial inhibitors was much less than that in control at 1 min (induction phase) but recovered during subsequent illumination (i.e., at the end of 10 min, activated phase).

The activity of PRK at limiting CO_2 was about 25% higher than that at optimal CO_2 (Fig. 5.8A; 5.9A). The decrease in the activation of PRK was more pronounced in presence of SHAM or propyl gallate (Fig. 5.8B; Fig. 5.9B) than that with oligomycin or antimycin A (Fig. 5.8A; Fig. 5.9B). Again these patterns were similar irrespective of CO_2 levels: optimal or limiting.

The effects of CCCP and DCMU on photosynthetic enzyme activation are known in the literature (See Chapter 6). They have been used as a check on the present experimental system. The restriction of light activation of PRK by DCMU, but not CCCP is expected and is logical (Fig. 5.8C: Fig. 5.9C). However, slowed down the enzyme activation, particularly under limiting CO_2.

The effects of various inhibitors on activation pattern (L/D ratios) of PRK at both optimal and limiting CO_2 are summarized in Table 5.2. All the values in the table refer to maximum rates observed during linear or activated phase of photosynthesis. The activation of PRK was affected significantly in presence of AOX pathway inhibitor SHAM ($\leq$25%) while the effect was marginal ($\leq$14%) in

Table 5.1. *Response to different metabolic inhibitors of RuBP levels in mesophyll protoplasts of pea during photosynthesis at optimal (1.0 mM NaHC03) or limiting (0.1 mM $NaHCO_3$) CO_2.*

Mesophyll protoplasts were incubated in darkness for 5 min at 25 °C with and without (control) test inhibitors. Protoplast samples were collected after 10 min illumination (corresponding to activated phase of photosynthesis), frozen, and analysed for RuBP.

Treatment	Optimal CO_2	Limiting CO_2
	nmol mg $^{-1}$ Chl	
Control	101 ± 4.5	92 ± 6.4
100 ng mL^{-1} Oligomycin	72 ± 5.3	60 ± 3.1
100 nM Antimycin A	70 ± 4.2	70 ± 3.5
500 µM SHAM	112 ± 5.9	100 ± 5.4
1 mM Propyl gallate	99 ± 4.7	112 ± 6.3
500 µM SHAM + 100 nM Antimycin A	110 ± 5.2	93 ± 6.1

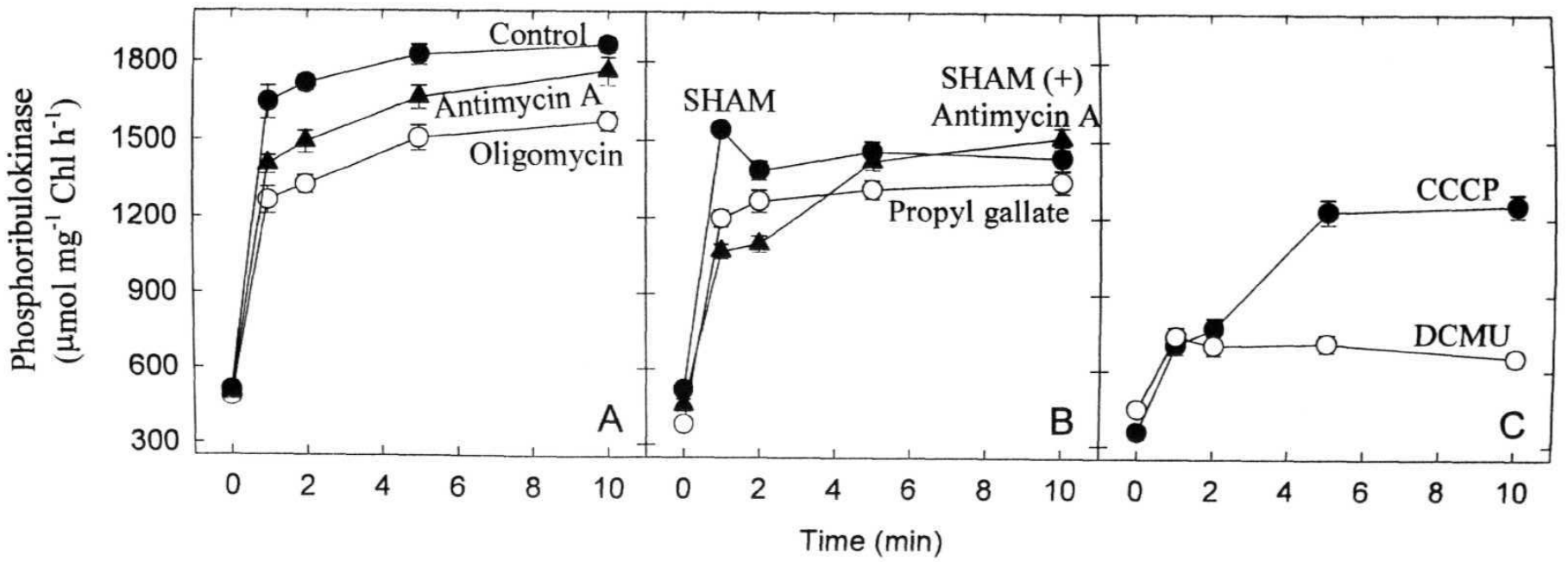

Figure 5.8. Activation of phosphoribulokinase in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$) in the absence or presence of the following inhibitors: oligomycin - 100 ng mL^{-1}; antimycin A - 100 nM; SHAM - 500 μM; propyl gallate - 1mM; CCCP - 1 μM; DCMU - 10 μM. After incubating the protoplasts in dark for 5 min at 25 °C, light was switched on at zero time. Aliquots of protoplast samples were withdrawn at the indicated time after illumination and assayed for PRK activity as described in materials and methods. The activity of PRK at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 510 ± 24 and 1866 ± 29 μmol mg^{-1} Chl h^{-1}, respectively.

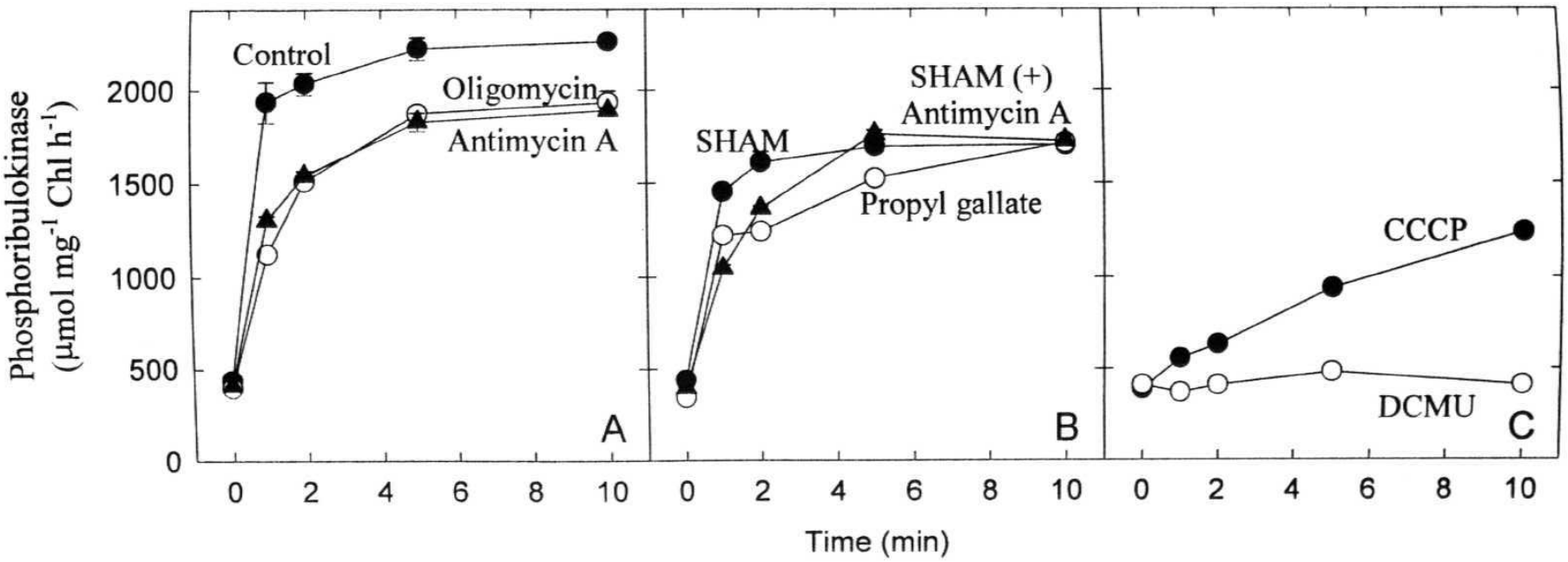

Figure 5.9. Activation of phosphoribulokinase in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM $NaHCO_3$) in absence or presence of the mitochondrial inhibitors. The other details were as in Figure 5.8. The activity of PRK at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 437 ± 9 and 2268 ± 20 μmol mg^{-1} Chl h^{-1}, respectively.

Table 5.2. *Activation pattern of phosphoribulokinase (PRK) in mesophyll protoplasts of pea in presence of different metabolic inhibitors at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

The protoplasts were incubated in darkness at 25 °C for 5 min with and without (control) test inhibitors. The protoplasts were then illuminated and the activities of PRK were determined at the begining and at the end of 10 min illumination (corresponding to dark phase and activated phase of photosynthesis). Light activation of PRK is indicated as L/D ratio.

Treatment	Optimal CO_2	Limiting CO_2
	L/D	
Control	3.7 ± 0.32	5.2 ± 0.18
100 ng mL^{-1} Oligomycin	3.2 ± 0.25	4.9 ± 0.16
100 nM Antimycin A	3.5 ± 0.17	4.6 ± 0.20
500 µM SHAM	2.8 ± 0.12	3.9 ± 0.14
1 mM Propyl gallate	3.5 ± 0.06	5.0 ± 0.09
500 µM SHAM + 100 nM Antimycin A	3.3 ± 0.28	4.4 ± 0.12
1 µM CCCP	3.5 ± 0.12	3.2 ± 0.10
10 µM DCMU	1.5 ± 0.13	1.0 ± 0.02

presence of oligomcyin or antimycin A at both optimal and limiting CO_2. In presence of CCCP, the PRK was affected more at limiting CO_2 than that at optimal CO_2. In presence of DCMU the light activation decreased to 40% and 19% of control at optimal and limiting CO_2, respectively.

Discussion

The results discussed in this chapter demonstrate that the interference with mitochondrial oxidative metabolism prolongs the induction phase of photosynthesis in mesophyll protoplasts, possibly by reducing the levels of RuBP and restricting the activation of PRK upon illumination.

The increase in induction period when protoplasts are treated with oligomycin (inhibitor of oxidative phosphorylation) or antimycin A (inhibitor of cytochrome pathway) is clearly illustrated in Figure 5.1. The increase in lag period was much more pronounced in presence of oligomycin or antimycin A than that with SHAM (± antimycin A) or propyl gallate (Fig. 5.2; Fig. 5.3), particularly at optimal CO_2. These observations suggest that mitochondrial electron transport chain through cytochrome path plays a prominent role during induction phase of photosynthesis. On the other hand, the presence of SHAM, appears to interfere with steady-state photosynthesis, as indicated by the marked decrease after 5-6 min of illumination. The exact reasons for the loss of photosynthetic stability after such a short period in presence of SHAM (Fig. 5.4F) is not clear.

The gradual increase in photosynthetic assimilation characteristic of the "induction period" is proposed to be due to autocatalytic build-up of metabolites, particularly RuBP (Walker and Crofts, 1970; Edwards and Walker, 1983; Walker, 1988) and activation of photosynthetic enzymes (Marques et al., 1987). The levels of RuBP and light activation pattern of PRK in illuminated protoplasts in

presence or absence of mitochondrial inhibitors during induction phase are therefore of great interest.

The light activation of enzymes in relation to photosynthetic induction has been earlier studied with chloroplasts and protoplasts (Leegood and Walker, 1980, 1981; Robinson and Walker, 1980a,b; Marques et al., 1987). The activity of PRK (1850 to 2250 $\mu mol\ mg^{-1}\ Chl\ h^{-1}$) observed after 10 min of illumination in mesophyll protoplasts during the present study (Fig. 5.8A; Fig. 5.9A) were similar to the activities observed in chloroplasts and leaves (Laing et al., 1981; Wirtz et al., 1982).

Paul et al. (1995) has shown that the transformed plants with PRK below 15% of wild-type concentration accumulated over two-fold higher concentrations of precursors to RuBP regeneration (Ru-5-P, ribose-5-P, ATP and fructose-6-P). In contrast, the transformed plants had two-fold lower concentrations of products of PRK reaction (RuBP, PGA and ADP). Changes in RuBP pools are therefore quite important in determining the assimilation rate, since RuBP is the substrate of Rubisco, the enzyme of primary CO_2 fixation.

The prolongation of induction by oligomycin and antimycin A, appears to be primarily due to the interference with the capacity of RuBP regeneration in protoplasts, as indicated by the marked decrease in their RuBP levels (Fig. 5.6, B and C; Fig. 5.7, B and C). On the other hand SHAM or propyl gallate did not affect RuBP levels (Fig. 5.6, E and F; Fig. 5.7, E and F). The relationship between photosynthesis and RuBP pool size has been studied, in different experimental systems: spinach cells (Collatz, 1978), soybean leaflets (Hitz and Stewart, 1980), and wheat seedlings (Perchorowicz et al., 1981). Restriction of RuBP regeneration as the main limitation on carbon assimilation has also been noticed under water stress (Sharkey and Seemann, 1989; Gimenez et al., 1992; Brestic et al., 1995). The reasons for such limited regeneration of RuBP is a

decrease in either total FBPase activity, as under drought (Sanchez-Rodriguez et al., 1997) or PRK, the enzyme involved in RuBP regeneration (Gunasekara and Berkowitz, 1993).

The pool sizes of RuBP may decrease also due to the interference with hexose monophosphate shunt. The ATP/ADP levels were lowered remarkably in presence of oligomycin or antimycin A (See Chapter 4). Krömer et al. (1992) observed that the decrease in ATP/ADP was most severe in the mitochondria and the cytosol, while being only marginal in the chloroplast. As PRK competes with PGA-kinase for ATP in the chloroplast, the increase in ADP in mesophyll protoplasts in the presence of mitochondrial inhibitors may act as a negative modulator of PGA-kinase in the regeneration of RuBP. Further, an increase in Glc-6-P levels (See Chapter 4) and triose-P levels (See Chapter 7) in mesophyll protoplasts in presence of mitochondrial inhibitors suggest that their accumulation may be at the expense of Ru-5-P needed for RuBP regeneration.

The decrease in light activation of PRK was more pronounced in presence of SHAM (± antimycin A) compared to oligomycin or antimycin A (Fig. 5.8; Fig. 5.9; Table 5.2). The exact reasons for the sensitivity of PRK to SHAM are not clear. A major mechanism of PRK activation is through the intra-subunit reduction of two cysteine residues by ferredoxin-thioredoxin system. PRK is also strongly sensitive to pH in the presence of inhibitors like PGA and FBP. Inhibition by 3-PGA *in vivo* is enhanced by the decrease in stromal pH upon darkening, which favours the protonation of glycerate 3-P^2 at the pH prevalent in the illuminated stroma (Gardemann et al., 1983; 1986). Similarly high ATP levels lead to activation of PRK due to a shift in the mid-redox potential of the enzyme from 0.58 to 0.31 V. Thus, low ATP or low redox state or both, besides the decrease in pH can reduce the activation of PRK.

These results suggest that the induction phase of photosynthesis is affected primarily through cytochrome pathway of mitochondrial oxidative electron transport and oxidative phosphorylation, while steady-state photosynthesis depends critically on AOX pathway of mitochondrial metabolism. We also propose that the decrease in photosynthetic rates in presence of mitochondrial inhibitors could be either due to limitation in regeneration of RuBP (particularly in presence of antimycin A or oligomycin) or due to restriction of light activation of PRK (in presence of SHAM or propyl gallate).

Chapter 6

Influence of Restricted Mitochondrial Metabolism on Light Activation of Photosynthetic Enzymes

Chapter 6

Influence of Restricted Mitochondrial Metabolism on Light Activation of Photosynthetic Enzymes

Introduction

Light drives photosynthesis by activating key Calvin cycle enzymes via either thioredoxin or changes of stromal pH and Mg^{2+} or both. Illumination leads to alkalization and increased free Mg^{2+} in the stroma (Portis, 1981). Enzymes of the Calvin cycle including FBPase (Gardemann et al., 1986; Leegood, 1993) and Rubisco (Andrews and Lorimer, 1987) are therefore strongly activated by elevated pH and Mg^{2+}. Typical examples of light activated enzymes of Calvin cycle, besides FBPase and Rubisco, are SBPase, PRK and GAPDH (Buchanan, 1980).

Ferredoxin:thioredoxin oxidoreductase plays a key role in activation of photosynthetic enzymes by transferring reducing equivalents from ferredoxin to thioredoxin, which in turn converts the target protein (enzyme) to an active form by modifying the cysteine groups (Buchanan, 1984). The reversible reduction of thiols in PCRC enzymes is modulated by both the supply of electrons from PS I and their utilization in metabolism (Harbinson et al.. 1990). Apart from Calvin cycle linked enzymes, thioredoxin also activates NADP-MDH (Scheibe, 1991).

Labate and Leegood (1988) showed that the activation status of the PCRC enzymes or of the sucrose synthesis can markedly affect CO_2 assimilation at low temperature. Similarly, the increases in photosynthetic capacity that occur during acclimation to lower growth temperatures could be due to the increase in the activities of enzymes such as Rubisco and stromal FBPase (Berry and Björkman, 1980; Badger et al., 1982). An increase in the activities of light activated enzymes is a strategy of increasing the photosynthetic capacity in spinach

(Holaday et al., 1992). It appears likely that the assimilation rate at low temperatures is limited, by the inability to activate key Calvin cycle enzymes in the short term or to maintain their activation state in the long term exposure to 10 °C (Holaday et al., 1992).

Thus, the interference with the activation of key photosynthetic enzymes by different stress conditions can restrict photosynthetic activation. It is possible that the restriction of mitochondrial metabolism, which affects photosynthesis in protoplasts, has its influence through the light activation of enzymes. The aim of the present study was therefore to characterize the effects of mitochondrial electron transport inhibitors on the activity of PCRC enzymes in mesophyll protoplasts of pea.

Results

The light activation pattern of three enzymes NADP-GAPDH, stromal FBPase and NADP-MDH were studied at optimal and limiting CO_2, as done in case of PRK (described in Chapter 5).

Figures 6.1 and 6.2 show the NADP-GAPDH activity at different times of illumination in mesophyll protoplasts at optimal and limiting CO_2. The GAPDH activity was maximum within 2 min after illumination and was steady up to 10 min. However when mesophyll protoplasts were incubated with mitochondrial inhibitors, the activity of NADP-GAPDH picked up slowly. The activity recovered and reached a maximum, but below the level of control-samples. The effects of the inhibitors was more at limiting CO_2 than that at optimal CO_2.

The activity of NADP-GAPDH was higher at limiting CO_2 than that at optimal CO_2 (Fig. 6.1 A; Fig. 6.2A). The activation pattern of NADP-GAPDH in presence of SHAM was unique (Fig. 6.1B; Fig. 6.2B). The GAPDH activity

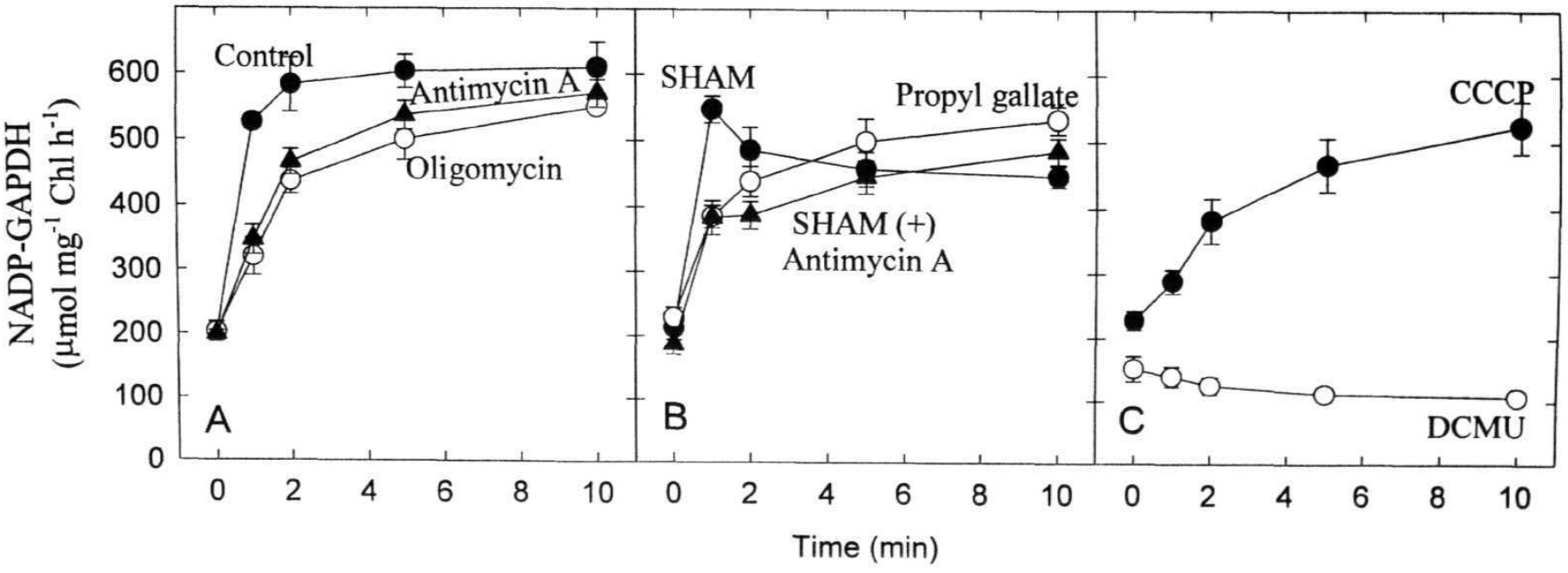

Figure 6.1. Activation of NADP-GAPDH in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$) in absence or presence of the following inhibitors: oligomycin - 100 ng mL^{-1}; antimycin A - 100 nM; SHAM - 500 μM; propyl gallate - 1 mM; CCCP - 1 μM; DCMU - 10 μM. After incubating the protoplasts in dark for 5 min at 25 ^{o}C, light was switched on at zero time. Aliquots of protoplasts were withdrawn at the indicated time after illumination and assayed for NADP-GAPDH activity as described in materials and methods. The activity of GAPDH at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 203 ± 15 and 610 ± 39 μmol mg^{-1} Chl h^{-1}, respectively.

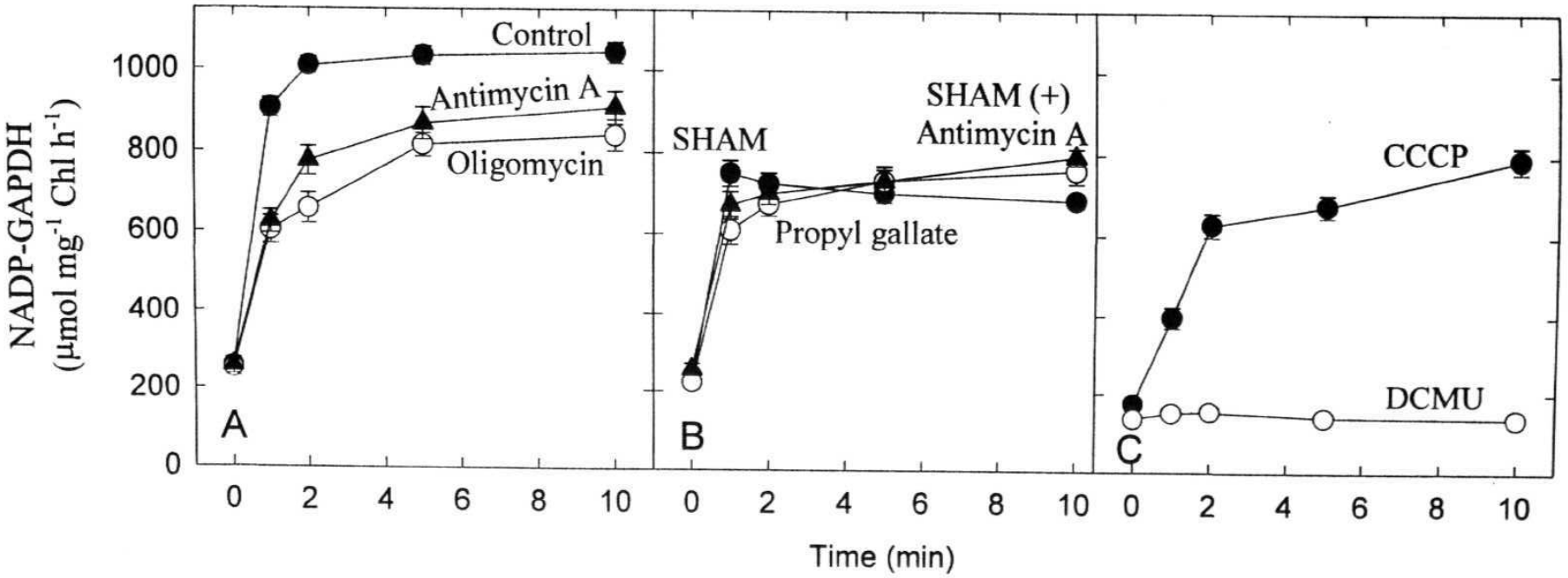

Figure 6.2. Activation of NADP-GAPDH in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM $NaHCO_3$) in absence or presence of the mitochondrial inhibitors. The other details were as in Figure 6.1. The activity of NADP-GAPDH at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 257 $\pm$ 15 and 1043 $\pm$ 25 μmol mg^{-1} Chl h^{-1}, respectively.

reached maximum during the first min of illumination and decreased during subsequent illumination at both optimal and limiting CO_2. AOX pathway inhibitors SHAM (± antimycin A) and propyl gallate decreased the activation of NADP-GAPDH more conspicuously (Fig. 6.1B; Fig. 6.2B) than oligomycin or antimycin A could (Fig. 6.1 A; Fig. 6.2A). The presence of DCMU prevented light activation of GAPDH, while the light activation was slow in presence of CCCP and picked up only after 5 min of illumination (Fig. 6.1C; Fig. 6.2C).

Table 6.1 summarizes the activation pattern of NADP-GAPDH in presence of mitochondrial inhibitors at optimal and limiting CO_2 in terms of L/D ratios. There was a significant decrease in GAPDH activation in presence of AOX pathway inhibitors (nearly 35%) compared to the limited effect of oligomycin or antimycin A (10-20%). There was a significant decrease in activation of NADP-GAPDH by about 25% in presence of CCCP while the activation decreased by >75% of control in presence of DCMU.

At optimal CO_2, FBPase was activated within 2 min of illumination (Fig. 6.3A), but at limiting CO_2, maximum activation was achieved only after 5 min of illumination (Fig. 6.4A). However the activation was steady up to 10 min at both optimal and limiting CO_2. The presence of SHAM (± antimycin A) or propyl gallate decreased the extent of FBPase activation more than that of oligomycin or antimycin A (Fig. 6.3, A and B; Fig. 6.4, A and B). The activation pattern of FBPase was not much affected by CCCP (Fig. 6.3C; Fig. 6.4C). On illumination and in presence of DCMU, the activation of FBPase either decreased (at optimal CO_2, Fig. 6.3C) or did not change (at limiting CO_2. Fig. 6.4C).

The activation pattern of FBPase at optimal and limiting CO_2 is summarized in Table 6.2. There was a significant decrease in FBPase activation in presence of SHAM at optimal and limiting CO_2 (< 40%). However the activation decreased to <25% in presence of SHAM (± antimycin A) and propyl

Table 6.1. *Pattern of light activation of NADP-GAPDH in mesophyll protoplasts of pea in presence of different metabolic inhibitors at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

The protoplasts were incubated in darkness at 25 °C for 5 min with and without (control) test inhibitors and were then illuminated. The activities of NADP-GAPDH were determined at the beginning and at the end of 10 min illumination (corresponding to dark phase and activated phase of photosynthesis). Light activation of NADP-GAPDH is indicated as L/D ratio.

Treatment	Optimal CO_2	Limiting CO_2
	L/D	
Control	3.0 ± 0.13	4.1 ± 0.10
100 ng mL^{-1} Oligomycin	2.6 ± 0.09	3.3 ± 0.15
100 nM Antimycin A	2.7 ± 0.08	3.5 ± 0.17
500 mM SHAM	2.0 ± 0.10	2.7 ± 0.09
1 mM Propyl gallate	2.2 ± 0.09	3.0 ± 0.11
500 mM SHAM + 100 nM Antimycin A	2.5 ± 0.15	3.1 ± 0.08
1 mM CCCP	2.2 ± 0.10	3.1 ± 0.14
10 mM DCMU	0.7 ± 0.04	1.0 ± 0.02

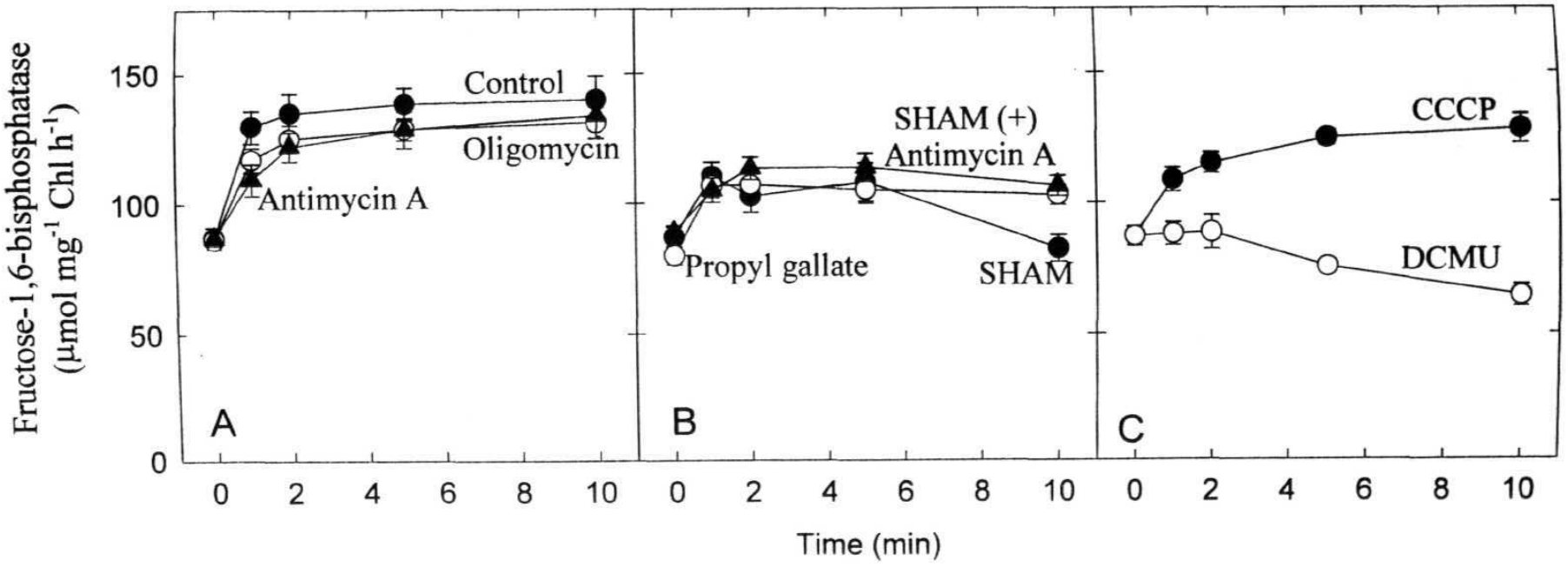

Figure 6.3. Activation of FBPase in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$) in absence or presence of the following inhibitors: oligomycin - 100 ng mL^{-1}; antimycin A - 100 nM; SHAM - 500 μM; propyl gallate - 1 mM; CCCP - 1 μM; DCMU - 10 μM. After incubating the protoplasts in dark for 5 min at 25 °C, light was switched on at zero time. Aliquots of protoplasts were withdrawn at the indicated time after illumination and assayed for FBPase activity as described in materials and methods. The activity of FBPase at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 87 ± 4 and 140 ± 9 μmol mg^{-1} Chl h^{-1}, respectively.

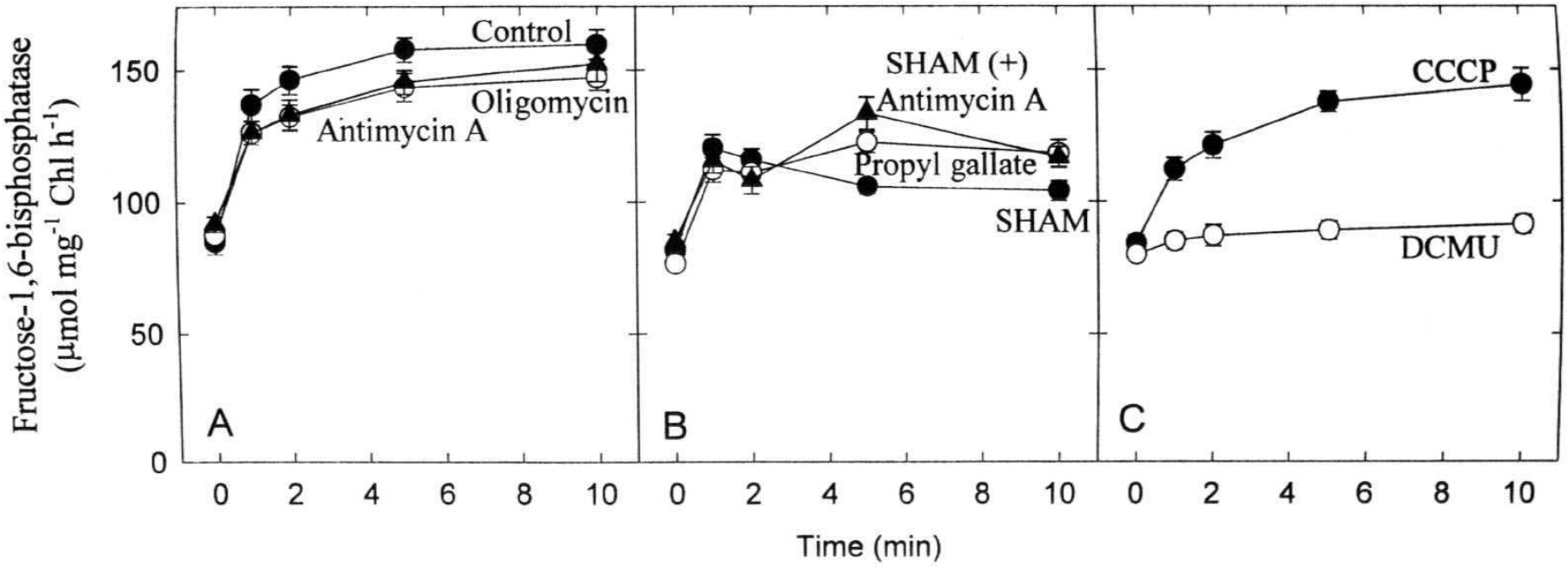

Figure 6.4. Activation of FBPase in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM $NaHCO_3$) in absence or presence of the mitochondrial inhibitors. The other details were as in Figure 5.8. The activity of FBPase at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 85 ± 4.5 and 160 ± 6.0 μmol mg^{-1} Chl h^{-1}, respectively.

Table 6.2. *Pattern of light activation of FBPase in mesophyll protoplasts of pea in presence of different metabolic inhibitors at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

The protoplasts were incubated in darkness at 25 °C for 5 min with and without (control) test inhibitors and were then illuminated. The activities of FBPase were determined at the beginning and at the end of 10 min illumination (corresponding to dark phase and activated phase of photosynthesis). Light activation of FBPase is indicated as L/D ratio.

Treatment	Optimal CO_2	Limiting CO_2
	L/D	
Control	1.6 ± 0.04	1.9 ± 0.08
100 ng mL^{-1} Oligomycin	1.5 ± 0.08	1.7 ± 0.07
100 nM Antimycin A	1.5 ± 0.07	1.7 ± 0.06
500 mM SHAM	0.9 ± 0.04	1.3 ± 0.04
1 mM Propyl gallate	1.3 ± 0.08	1.6 ± 0.06
500 mM SHAM + 100 nM Antimycin A	1.2 ± 0.06	1.4 ± 0.05
1 mM CCCP	1.5 ± 0.09	1.7 ± 0.05
10 mM DCMU	0.7 ± 0.02	1.1 ± 0.03

gallate at optimal CO_2. On the other hand, oligomycin or antimycin decreased the activation of FBPase by <10% at both optimal and limiting CO_2.

The extent of light activation of NADP-MDH was consistently higher at limiting CO_2 than that at optimal CO_2 (Fig. 6.5; Fig. 6.6). In control samples the NADP-MDH reached maximum activity within 2 min of illumination and was stable up to 5 min and thereafter declined at both optimal and limiting CO_2 (Fig. 6.5; Fig. 6.6). There was a marked stimulation in the activation of MDH in presence of oligomycin or antimycin A or CCCP (Fig. 6.5, A and C) at optimal CO_2. There was a sharp decline in NADP-MDH activation in presence of SHAM at limiting CO_2 compared to optimal CO_2 (Fig. 6.5B; Fig. 6.6B). The NADP-MDH activation was completely arrested in presence of DCMU (Fig. 6.5C; Fig. 6.6C).

Table 6.3 summarizes the activation pattern of NADP-MDH. There was a significant increase in light activation of NADP-MDH by oligomycin or antimycin A at both optimal (35%) and limiting CO_2 (<50%). This is in strong contrast to the decrease in enzyme activation by SHAM. However propyl gallate caused a marked increase (>70%) in activation at limiting CO_2. The activation was decreased in presence of SHAM at both optimal and limiting CO_2. The extent of activation was slightly reduced by SHAM alone (without antimycin A) at limiting CO_2 while there was no change at optimal CO_2. There was a significant increase in activation in presence of CCCP at limiting CO_2 but not at optimal CO_2. On the other hand, the activation was decreased to nearly 40% in presence of DCMU.

Discussion

Photosynthetic induction is dependent on light activation of Calvin cycle enzymes. The light activation of enzymes in relation to photosynthetic induction

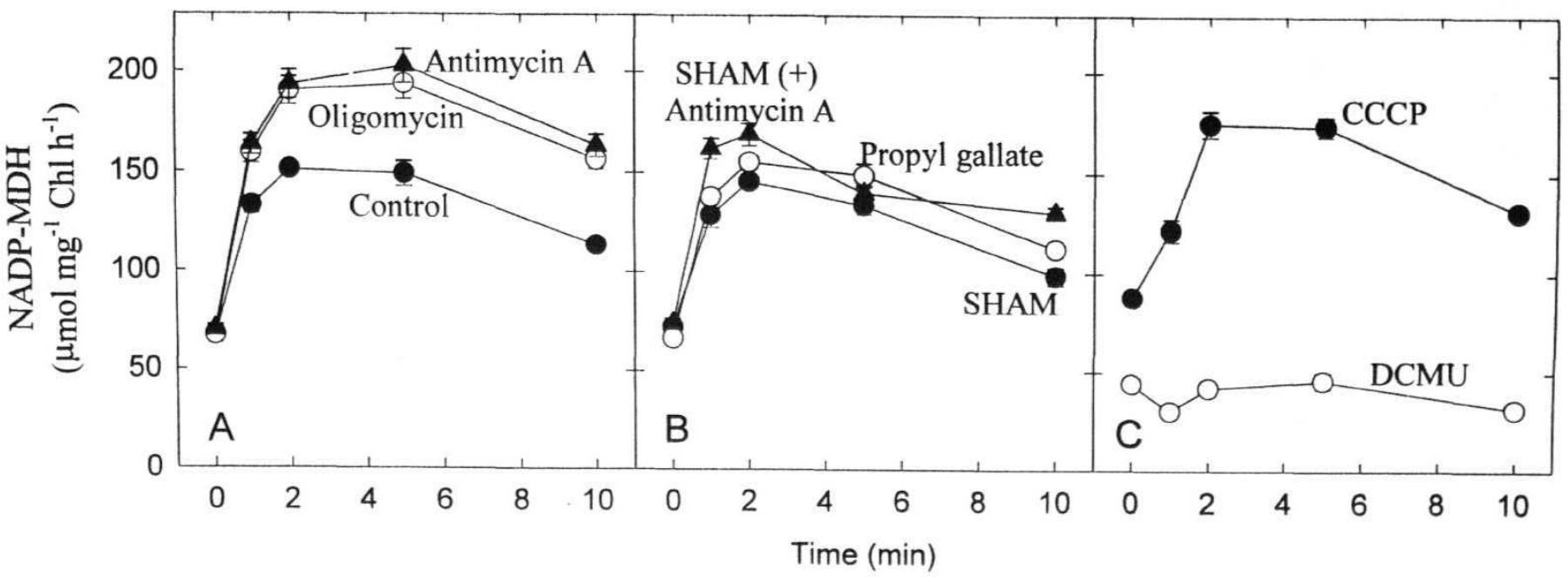

Figure 6.5. Activation of NADP-MDH in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$) in absence or presence of the following inhibitors: oligomycin - 100 ng mL^{-1}; antimycin A - 100 nM; SHAM - 500 μM; propyl gallate - 1 mM; CCCP - 1 μM; DCMU - 10 μM. After incubating the protoplasts in dark for 5 min at 25 °C, light was switched on at zero time. Aliquots of protoplasts were withdrawn at the indicated time after illumination and assayed for NADP-MDH activity as described in materials and methods. The activity of MDH at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 67 ± 4 and 113 ± 2.77 μmol mg^{-1} Chl h^{-1}, respectively.

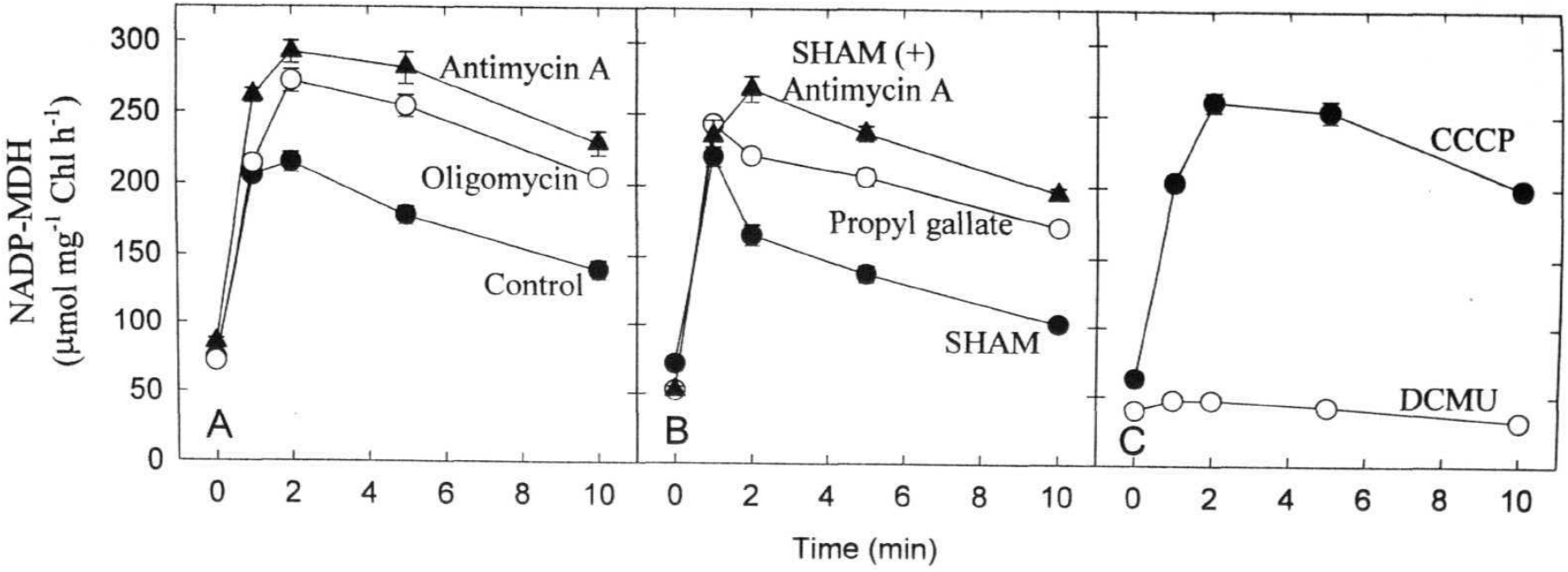

Figure 6.6. Activation of NADP-MDH in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM $NaHCO_3$) in absence or presence of the mitochondrial inhibitors. Other details were as in Figure 6.5. The activity of NADP-MDH at the beginning and at the end of 10 min of illumination in control (without inhibitor) was 74 ± 5 and 139 ± 6 μmol mg^{-1} Chl h^{-1}, respectively.

Table 6.3. *Pattern of light activation of NADP-MDH in mesophyll protoplasts of pea in presence of different metabolic inhibitors at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

The protoplasts were incubated in darkness at 25 °C for 5 min with and without (control) test inhibitors and were then illuminated. The activities of NADP-MDH were determined at the beginning and at the end of 10 min illumination (corresponding to dark phase and activated phase of photosynthesis). Light activation of NADP-MDH is indicated as L/D ratio.

Treatment	Optimal CO_2	Limiting CO_2
	L/D	
Control	1.7 ± 0.04	1.9 ± 0.08
100 ng mL^{-1} Oligomycin	2.3 ± 0.09	2.9 ± 0.15
100 nM Antimycin A	2.3± 0.10	2.7 ± 0.12
500 µM SHAM	1.4 ± 0.09	1.4 ± 0.05
1 mM Propyl gallate	1.7 ± 0.08	3.3 ± 0.10
500 µM SHAM + 100 nM Antimycin A	1.8± 0.11	3.7 ± 0.18
1 µM CCCP	1.5 ± 0.06	3.2 ± 0.19
10 µM DCMU	0.7 ± 0.03	0.8 ± 0.05

has been shown in studies with chloroplasts and protoplasts (Leegood and Walker, 1980, 1981; Robinson and Walker, 1980a,b; Marques et al., 1987).

In this chapter, we examined the influence of restriction on mitochondrial metabolism on the light activation of three photosynthetic enzymes viz. NADP-GAPDH, FBPase, and NADP-MDH. Of the three enzymes studied NADP-GAPDH and FBPase are participants of Calvin cycle, while NADP-MDH accomplishes the reduction of OAA to malate with the help of photosynthetic electron transport. All the three enzymes are regulated through reductive activation (Buchanan, 1984).

The light activation of NADP-GAPDH. FBPase, MDH has been demonstrated in leaves, mesophyll cell protoplasts, guard cell protoplasts and chloroplasts (Laing et al., 1981; Heber et al., 1982; Leegood and Walker. 1982; Wirtz et al., 1982; Gotow et al., 1985; Shimazaki et al., 1989; Scheibe et al., 1990). The activities of FBPase (140 to 160 $\mu mol\ mg^{-1}\ Chl\ h^{-1}$) observed after 10 min of illumination in the present study were comparable to the activities observed in chloroplasts and leaves (Laing et al., 1981; Wirtz et al., 1982).

The extent of light activation of enzymes depends on the limiting or optimal status of CO_2. For e.g. the activity of NADP-MDH increases at low CO_2 concentrations due to decrease in photochemical electron flow towards NADP (Harbinson et al., 1990). Similarly there is a marked increase in the extent of light activation of stromal FBPase under decreasing CO_2 concentrations (Harbinson et al., 1990). In our experiments also the extent of activation of all the three enzymes was invariably higher at limiting CO_2 (Fig. 6.2; Fig. 6.4; Fig. 6.6) than that at optimal CO_2 (Fig. 6.1; Fig. 6.3; Fig. 6.5).

Calvin cycle activity is a strong sink for reducing equivalents and when carboxylation of RuBP is high, the activation state of NADP-MDH is limited (Harbinson et al., 1990). This could be one of the reasons for the observed

decrease in the activation state of NADP-MDH during photosynthesis at optimal CO_2 (Fig. 6.5A). In physiological conditions the steady state activation of NADP-MDH is always low unless the rate of carbon assimilation is impaired as it occurs at low temperature (Scheibe and Stitt, 1988).

Reductive activation of enzymes is one mechanism through which the biochemical reactions of the PCRC are balanced and co-ordinated with the rates of electron transport and energy (ATP and NADPH) supply (Harbinson et al., 1990). Photosynthetic electron transport system can facilitate reduction of enzymes and their activation through ferredoxin-thioredoxin system. The precise affect of thioredoxin on the kinetic properties depends on the target protein. chloroplastic FBPase, thioredoxin activation leads to an increased substrate affinity (Laing et al., 1981; Leegood. 1985).

The marked restriction of light activation of photosynthetic enzymes suggests that this phenomenon could be one of the factors responsible for the observed decrease in photosynthetic rates in presence of mitochondrial inhibitors. While the light activation of two enzymes linked to Calvin cycle (NADP-GAPDH and FBPase) was restricted in presence of mitochondrial inhibitors (Fig. 6.1, A and B; Fig. 6.2, A and B; Fig. 6.3. A and B; Fig. 6.4, A and B), there was marked stimulation in the activity of NADP-MDH, which is linked to photosynthetic electron transport (Fig. 6.5, A and B; Fig. 6.6, A and B). The suppression of activation of NADP-GAPDH or FBPase by SHAM or propyl gallate, inhibitors of alternative oxidase. was more than that by oligomycin or antimycin A (Table 6.1; Table 6.2). The effect of SHAM was again unique in that the activation of NADP-MDH was not allowed. The effect of SF1AM was similar to that of DCMU (which is an inhibitor of PS II) in decreasing the NADP-MDH activity in contrast to other mitochondrial inhibitors.

The activity of chloroplast NADP-MDH is believed to be an indicator of the redox state of the stroma (Cseke and Buchanan, 1986; Scheibe and Stitt, 1988; Scheibe, 1990) with increasing activation correlating with increased reduction of the NADP pools (Scheibe, 1990; Foyer et al., 1992). Measurements of NADP-MDH activity (Scheibe and Stitt, 1988; Harbinson et al., 1990), direct measurements of the redox state of NADP in leaves and in isolated intact chloroplasts, indicate that the stroma remains relatively oxidized when CO_2 fixation is active (Takahama et al., 1981; Dietz and Heber. 1984).

NADP-MDH is regulated via mostly the redox states of the ferredoxin and pyridine nucleotide pools (Scheibe and Stitt, 1988; Crawford et al., 1989). FBPase is a hysteric enzyme, regulated also by its substrate FBP, which stabilizes the activation state (Soulie et al., 1981: Leegood et al., 1982, Leegood, 1990).

The activation of Rubisco. FBPase and NADP-MDH is dependent on photochemical electron transport, and almost complete activation of these enzymes in spinach occurs under conditions that reduce the efficiency of NADP-reduction through photochemical electron transport. A restriction in carbon assimilation, as would occur at low temperature, usually leads. through photosynthetic control, to an inactivation of redox-regulated enzymes (Leegood etaL, 1989).

The complete elimination of enzyme activation by DCMU but not by CCCP observed in present work (Fig. 6.1C; Fig. 6.2C; Fig. 6.3C: Fig. 6.4C; Fig. 6.5C; Fig. 6.6C) is in agreement with literature. Since the enzyme activation is driven by linear/non-linear electron transport, the presence of DCMU, an inhibitor of photosynthetic electron transport, prevents the enzyme activation. CCCP, an uncoupler of photophosphorylation in chloroplasts, would prevent ATP synthesis but enhances the electron flow/transport. This is the reason for

activation of enzymes to be either unaffected or marginally enhanced by CCCP (Gotow et al., 1985; Nakamoto and Edwards, 1986).

The marked light activation of NADP-MDH in guard cell protoplasts of *Vicia faba* (five-fold, in control samples) was totally inhibited by 5 μM DCMU (Gotow et al., 1985). Further, the presence of DCMU prevents malate accumulation suggesting that photosynthetic electron transport plays a role in both the activation of the enzyme and the supply of reducing power. The restriction of light activation of NADP-MDH by DCMU has also been observed in mesophyll protoplasts of maize (Nakamoto and Edwards, 1986). Further, the light activation of NADP-GAPDH and Rubisco also was completely inhibited in presence of 10 μM DCMU in guard cell protoplasts of *Vicia faba* (Shimazaki et al., 1989).

A stimulation of light activation of NADP-MDH, by CCCP has been reported in mesophyll protoplasts of maize (Nakamoto and Edwards, 1986). The stimulation of NADP-MDH in presence of CCCP may occur because of increased noncyclic electron flow due to uncoupling of photophosphorylation from electron transport. Phlorizin (which is an inhibitor of coupling factor for ATP synthesis), influences the light activation of NADP-MDH only slightly (Nakamoto and Edwards, 1986).

The interaction of antimycin A and DCMU during FBPase activation was studied in wheat chloropiasts by Leegood and Walker (1980). In the presence of DCMU at a concentration which inhibited photosynthesis by 50%, there was an increase in the state of light activation of the FBPase by addition of antimycin A. These results suggested that diversion of electrons from ferredoxin through cyclic electron flow was prevented by antimycin A and that cyclic electron flow is not required for light activation of enzymes.

The enhancement by antimycin A of NADP-MDH can be interpreted to be due to the increase in non-cyclic electron flow towards ferredoxin, since antimycin A blocks cyclic electron transport. Stimulation of light activation of NADP-MDH has earlier been observed in maize mesophyll protoplasts (Nakamoto and Edwards, 1986). However the marked stimulation by oligomycin of NADP-MDH was unexpected and surprising.

We conclude that the decrease in photosynthetic activity in presence of mitochondrial inhibitors is associated with a marked decrease in light activation of photosynthetic enzymes. These results reveal that mitochondrial metabolism through AOX pathway plays a much more significant role in modulating the photosynthetic enzymes (and thereby chloroplast metabolism) than that of cytochrome pathway. This is quite surprising and introduces a new concept of significance of AOX pathway in modulating chloroplast function in intact plant cells. The mechanism of such modulation is not clear and needs further experimentation to understand the phenomenon.

Chapter 7

Consequence of Restricted Mitochondrial Metabolism on Metabolites Related to the Phosphorylation Potential and Redox Status of the Cell

Chapter 7

Consequence of Restricted Mitochondrial Metabolism on Metabolites Related to the Phosphorylation Potential and Redox Status of the Cell

Introduction

Photosynthetic carbon assimilation in a plant cell is initiated by light, usually after an initial lag phase of photosynthetic induction. This slow transition is believed to be due to delay in light activation of stromal enzymes (Salvucci, 1989) as well as the continued build-up of metabolite levels in the chloroplast stroma (Leegood and Walker, 1981).

The levels of substrates or cofactors are among the principal factors which determine the photosynthetic rate during induction (Leegood and Walker. 1980). A short period of darkness, restores remarkably the lag period of photosynthesis in wheat protoplasts, but not in chloroplasts, while there was no difference between protoplasts and chloroplasts in relation to their light activation and dark deactivation of photosynthetic enzymes (Leegood and Walker. 1981). This difference in the response between protoplasts and chloroplasts lead to the suggestion that the control of photosynthesis during lag is associated with a decline in photosynthetic intermediates rather than any limitation on enzyme activity.

Edwards and Walker (1983) reported that the gradual accumulation of PCRC intermediates may also bring about an allosteric activation of related enzymes. For e.g. during induction triose-P is preferentially retained in the cycle, bringing about autocatalytic acceleration while during steady state triose-P is exported in exchange for external Pi. On the other hand, Marques et al. (1987) demonstrated with pea chloroplasts that light activation of enzymes preceded the build-up of metabolites and CO_2 fixation.

The metabolism of a leaf cell is distributed between various compartments, e.g. the cytosol, chloroplast, peroxisome and mitochondrion. Each of these organelle is also unique in its function. While chloroplasts tend to produce a lot of reductants through photochemical electron transport, mitochondria and peroxisomes typically oxidize the substrates. Cytosol is a physical and metabolic link between these organelles and thus an essential component.

The metabolic processes in these various compartments are coordinated through the exchange of metabolites. Redox processes occuring in the chloroplast stroma and the cytosol are interlinked by the transfer of redox equivalents from the chloroplastic stromal NADPH to the cytosolic NAD by at least two different metabolite shuttles viz. the triose-P-PGA shuttle (Heber, 1975) catalysed by the Pi triose-P 3PGA translocator (Flügge and Heldt, 1984) and the malate-OAA shuttle (Anderson and House, 1979) facilitated by specific transport of malate and OAA (Hatch et al., 1984). As both metabolite shuttles have the capacity to level the redox state of the stromal and cytosolic compartment, a regulation of these processes is required to maintain the specific redox states of the two metabolic compartments.

The effect of mitochondrial inhibitors on the light activation of enzymes has been studied in the previous Chapter: No. 6. In the present chapter, we investigated the effect of mitochondrial metabolism on plant cell photosynthesis, by analyzing changes in metabolite levels brought out by the inhibition of photosynthesis by mitochondrial inhibitors in mesophyll protoplasts of pea. The final objective is to analyse and identify which of the metabolites are crucial for mediating the beneficial effects of mitochondrial respiration to photosynthesis: metabolites related to redox state or ATP or both.

Results

The triose-P and PGA levels are determined at optimal and limiting CO_2 in mesophyll protoplasts, as shown in Figures 7.1 and 7.2. On illumination, in control samples (without inhibitors) both triose-P and PGA levels increased initially at optimal CO_2. However, after 10 min of illumination (representing steady state photosynthesis), the increase in triose-P pools was more pronounced than that in PGA levels (Fig. 7.1 A). However at limiting CO_2, only marginal change was observed in PGA levels compared to a significant increase of about 35% in triose-P (Fig. 7.2A).

On 10 min of illumination at optimal CO_2, there was a two to four fold increase in triose-P levels and a slight decrease in PGA levels in presence of oligomycin (Fig. 7.1B) or antimycin A (Fig. 7.1C). On the other hand, the PGA levels increased in presence of SHAM and propyl gallate (Fig. 7.1, E and F), but decreased in presence of SHAM with antimycin A (Fig. 7.1C).

There was a similar pattern of response in triose-P and PGA levels with oligomycin and antimycin A even at limiting CO_2. However, the increase in triose-P due to presence of oligomycin and antimycin A was only marginal at limiting CO_2. On the other hand, the decrease in PGA levels was slightly more at limiting CO_2 than that at optimal CO_2 (Fig. 7.1, B and C; Fig. 7.2, B and C). In presence of SHAM, there was a marginal decrease (Fig. 7.2E).

The ratio between triose-P and PGA indicates the relative enrichment of phosphorylation potential in protoplasts. The status of triose-P/PGA showed that the ratio increased on illumination at either optimal or limiting CO_2 indicating light induced accumulation of phosphorylation potential in protoplasts (Fig. 7.3 A; Fig. 7.4A). Further, the triose-P/PGA ratio increased in presence of all mitochondrial inhibitors at both optimal and limiting CO_2 (Fig. 7.3, B-D; Fig. 7.4, B-D). However, the ratio decreased markedly in presence of propyl

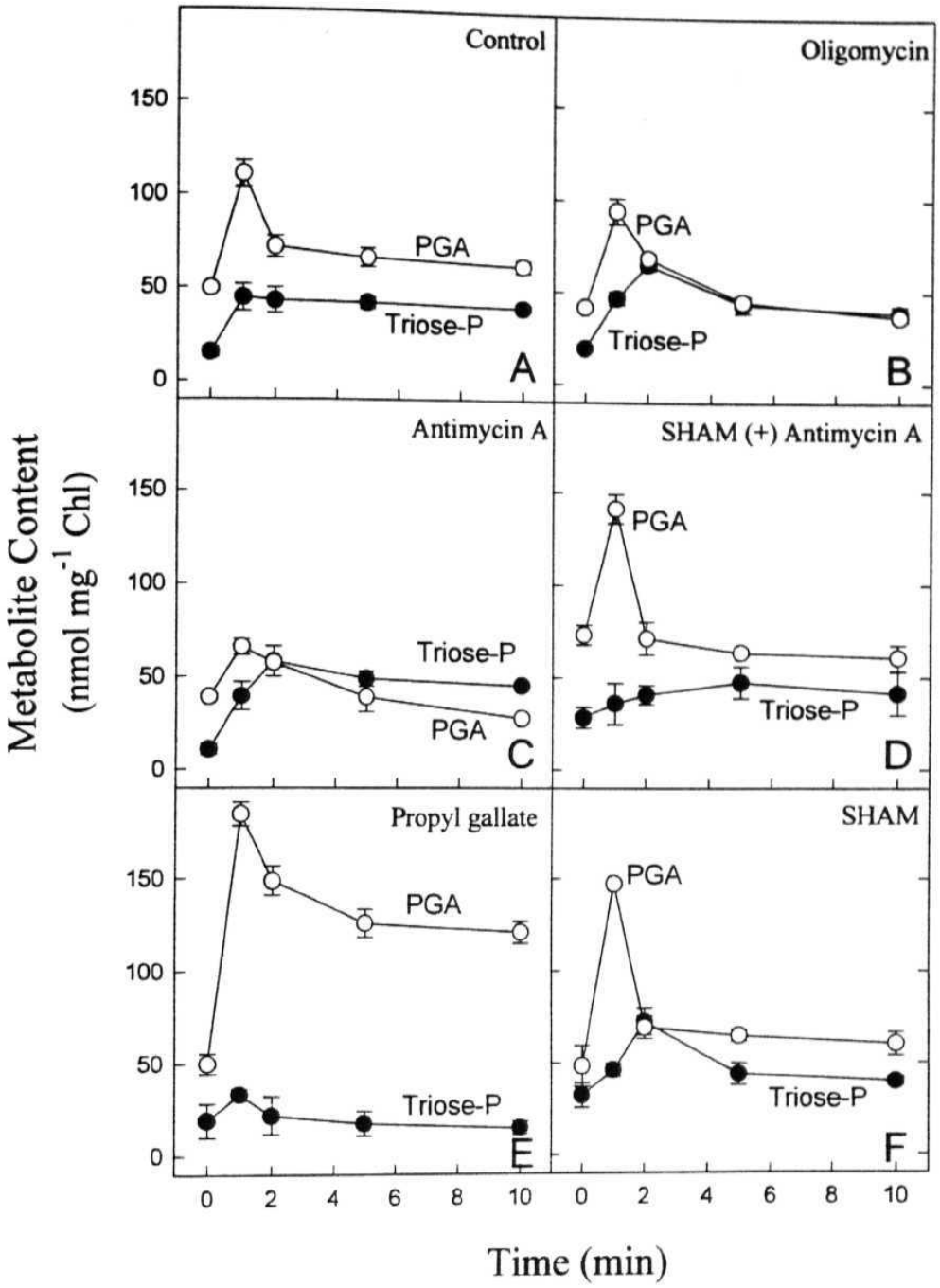

Figure 7.1. Change in the levels of triose-P and PGA in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$). A. Control (without inhibitor); B. 100 ng mL^{-1} oligomycin; C. 100 nM antimycin A; D. 500 µM SHAM (+ 100 nM antimycin A); E. 1 mM propyl gallate; F. 500 uM SHAM. The content of triose-P and PGA increased from 15 ± 1.2 and 49 ± 3 nmol mg^{-1} Chl, respectively, at the beginning of experiment to 40 + 2.5 and 63 + 3.4 nmol mg-1 Chl, respectively, after 10 min of light in control (without inhibitor) samples. Further details are as described in Figure 5.6.

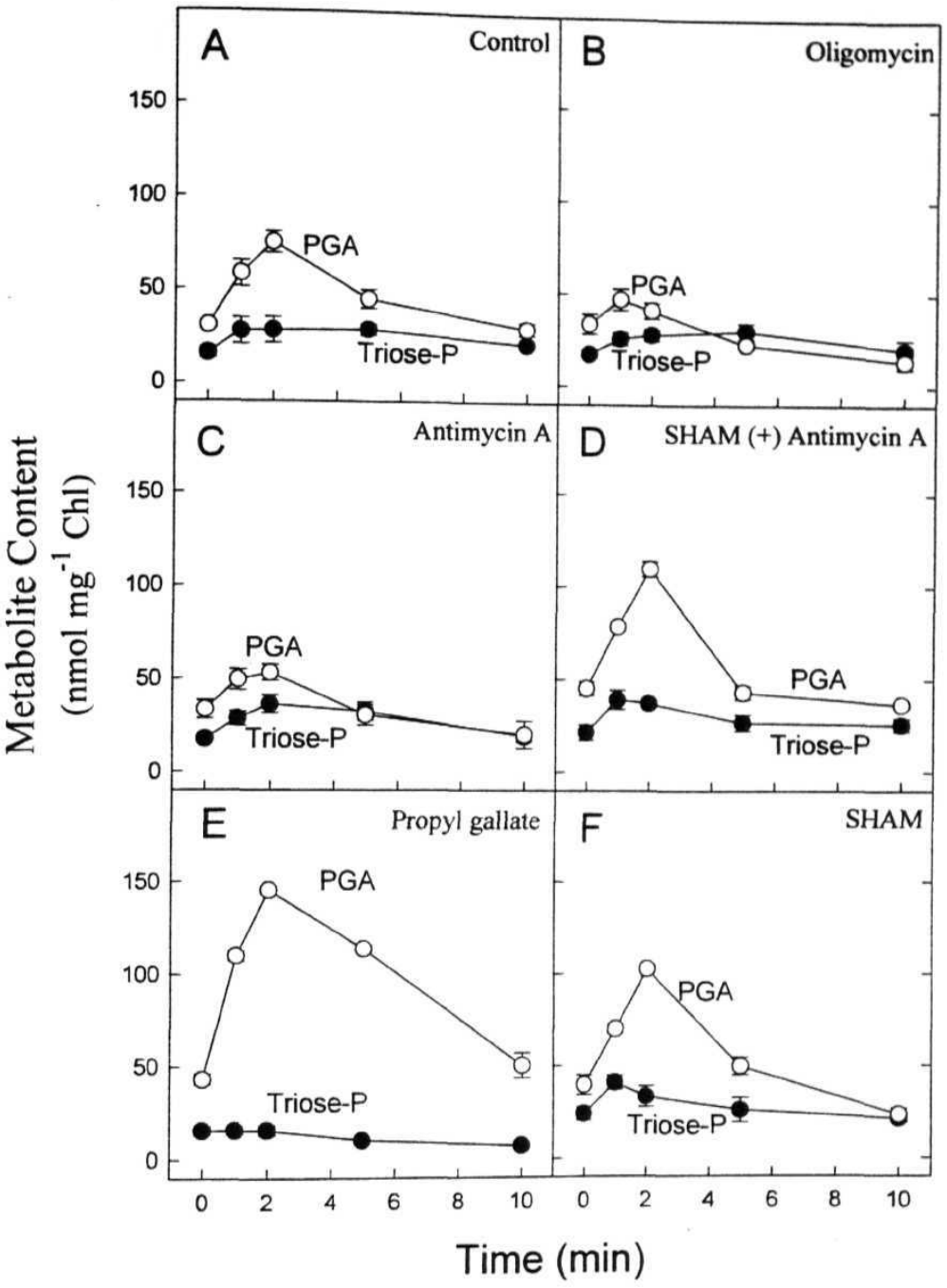

Figure 7.2. Change in the levels of triose-P and PGA in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM $NaHCO_3$). The content of triose-P and PGA were 16 + 2 and 31 ± 2 nmol mg^{-1} Chl, respectively at the beginning of experiment, while being 21 ± 1.9 and 30 ± 2.3 nmol rng-* Chl, respectively after 10 min of light in control (without inhibitor) samples. Further details are as described in Figure 7.1.

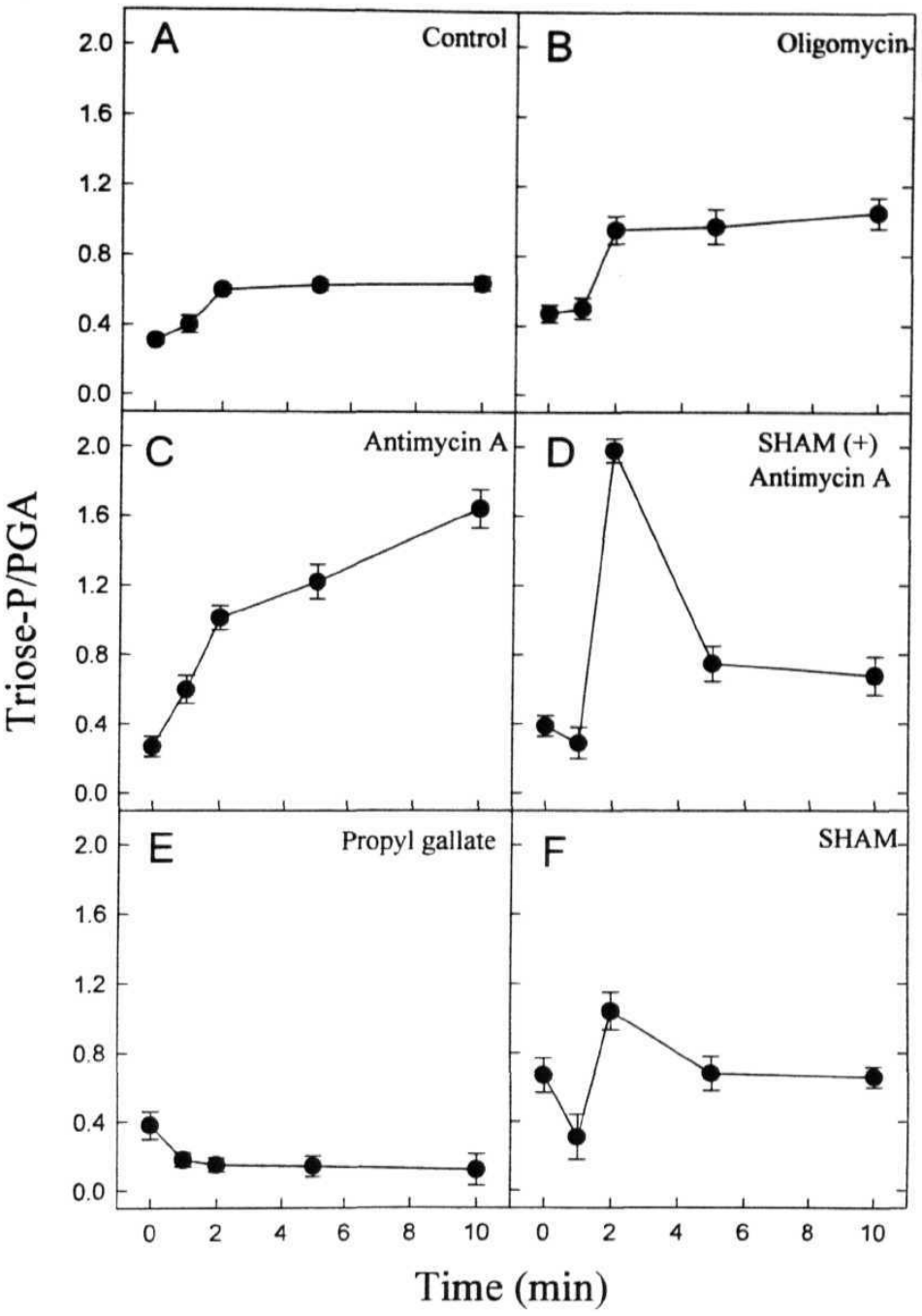

Figure 7.3. The ratio of intracellular triose-P to PGA in pea mesophyll protoplasts, at optimal (1.0 mM $NaHCO_3$) CO_2. The ratios were calculated from the data in Figure 7.1. The ratio of triose-P to PGA in control sets (in the absence of inhibitors) increased from 0.31 + 0.02 at the beginning of the experiment to 0.64 ± 0.06 after 10 min of illumination. Other details were as described in Figure 7.1.

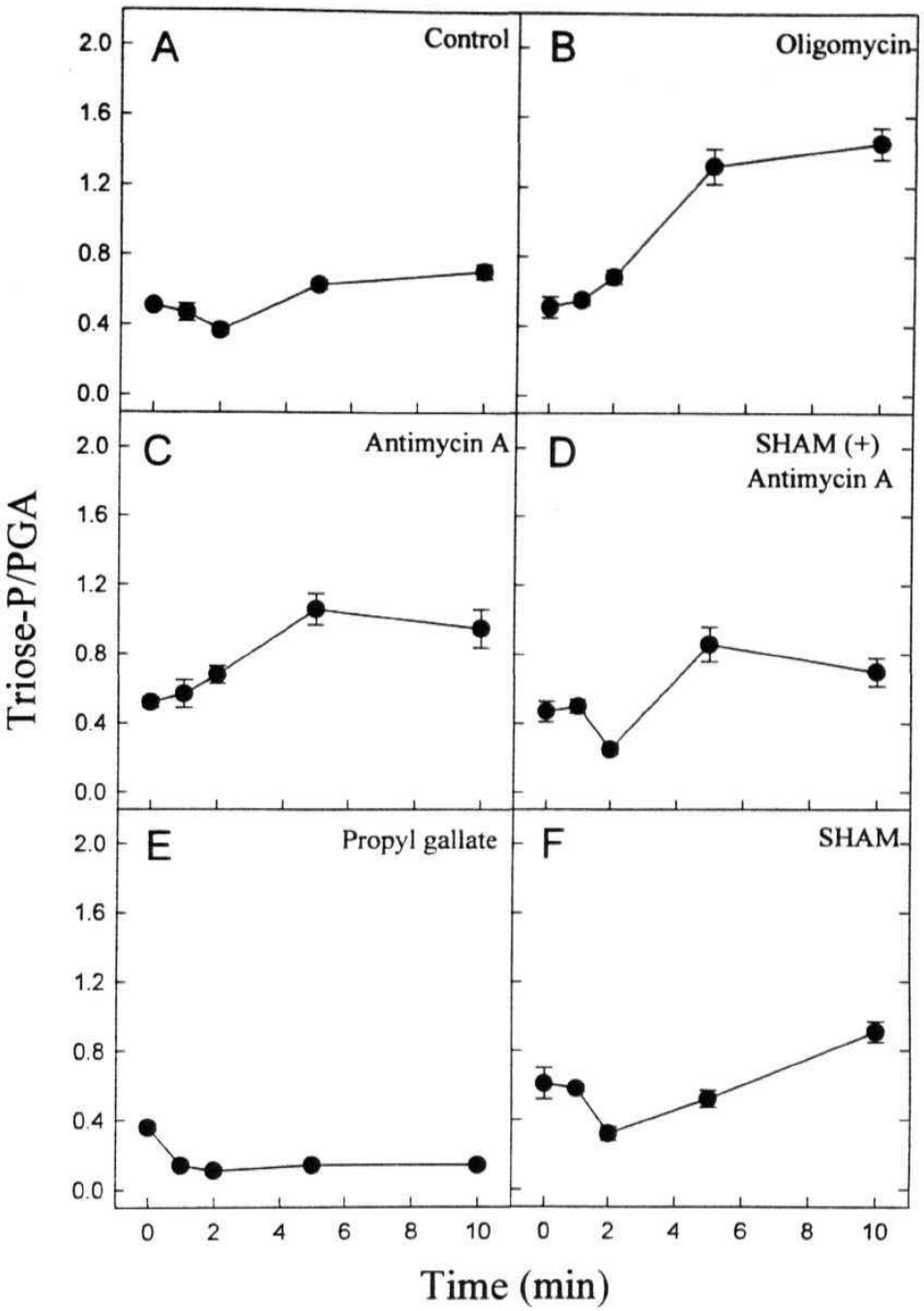

Figure 7.4. The ratio of intracellular triose-P to PGA in pea mesophyll protoplasts, at limiting (0.1 mM $NaHCO_3$) CO_2. The ratios were calculated from the data in Figure 7.2. The ratio of triose-P to PGA in control sets (in the absence of inhibitors) increased from 0.51 + 0.02 at the beginning of the experiment to 0.71 + 0.05 after 10 min of illumination. Other details were as described in Figure 7.1.

gallate (Fig. 7.3E; Fig. 7.4E), while being only marginally affected by SHAM (Fig. 7.3F; Fig. 7.4F).

A summary of the ratio of triose-P/PGA observed during steady state of photosynthesis (at the end of 10 min illumination) at either optimal or limiting CO_2 is shown in Table 7.1. There was a significant increase in triose-P/PGA ratio in presence of oligomycin or antimycin A at both optimal and limiting CO_2, while the increase was marginal in presence of or SHAM ($\pm$ antimycin A). However the ratio decreased in presence of propyl gallate at both optimal and limiting CO_2.

Figures 7.5 and 7.6 show the levels of malate and OAA in protoplasts illuminated at either optimal or limiting CO_2. The levels of malate were several fold (nearly 100 to 200-fold) higher than that of OAA. On illumination, the OAA levels increased slightly while malate decreased in the control sets, at limiting CO_2 (Fig. 7.6A). These changes were only marginal at optimal CO_2 (Fig. 7.5A). Oligomycin or antimycin A had not much effect on OAA or malate levels, compared to those of controls, both at limiting and optimal CO_2 (Fig. 7.5, B and C; Fig. 7.6, B and C). On the other hand, there was a much more significant increase in malate levels in presence of AOX pathway inhibitors (SHAM or propyl gallate) than that with oligomycin and antimycin A, at both optimal and limiting CO_2 (Fig. 7.5, D-F; Fig. 7.6, D-F).

There was a drastic decrease in malate/OAA ratio (represents the redox power) on illumination in controls samples, the decrease being more pronounced at optimal than that at limiting CO_2 (Fig. 7.7A; Fig. 7.8A). There was not much change in malate/OAA ratios in presence of oligomycin or antimycin A, both at optimal and limiting CO_2 (Fig. 7.7, B and C; Fig. 7.8, B and C). In contrast, there was a marked increase in malate/OAA ratios in presence of SHAM ($\pm$ antimycin A) at optimal CO_2 (Fig. 7.7, D and F).

Table 7.1. *Response to different metabolic inhibitors of triose-P/PGA ratio in mesophyll protoplasts of pea during photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

Mesophyll protoplasts were incubated in darkness for 5 min at 25 °C with and without (control) test inhibitors. Protoplast samples were collected after 10 min illumination (corresponding to activated phase of photosynthesis), frozen and analysed for triose-P and PGA.

Treatment	Optimal CO_2	Limiting CO_2
	Ratio	
Control	0.64 ± 0.06	0.71 ± 0.05
100 ng mL^{-1} Oligomycin	1.05 ± 0.07	1.45 ± 0.10
100 nM Antimycin A	1.64 ± 0.10	0.95 ± 0.07
500 µM SHAM + 100 nM Antimycin A	0.68 ± 0.06	0.70 ± 0.04
500 µM SHAM	0.66 ± 0 .03	0.91 ± 0.08
1 mM Propyl gallate	0.12 ± 0.02	0.14 ± 0.03

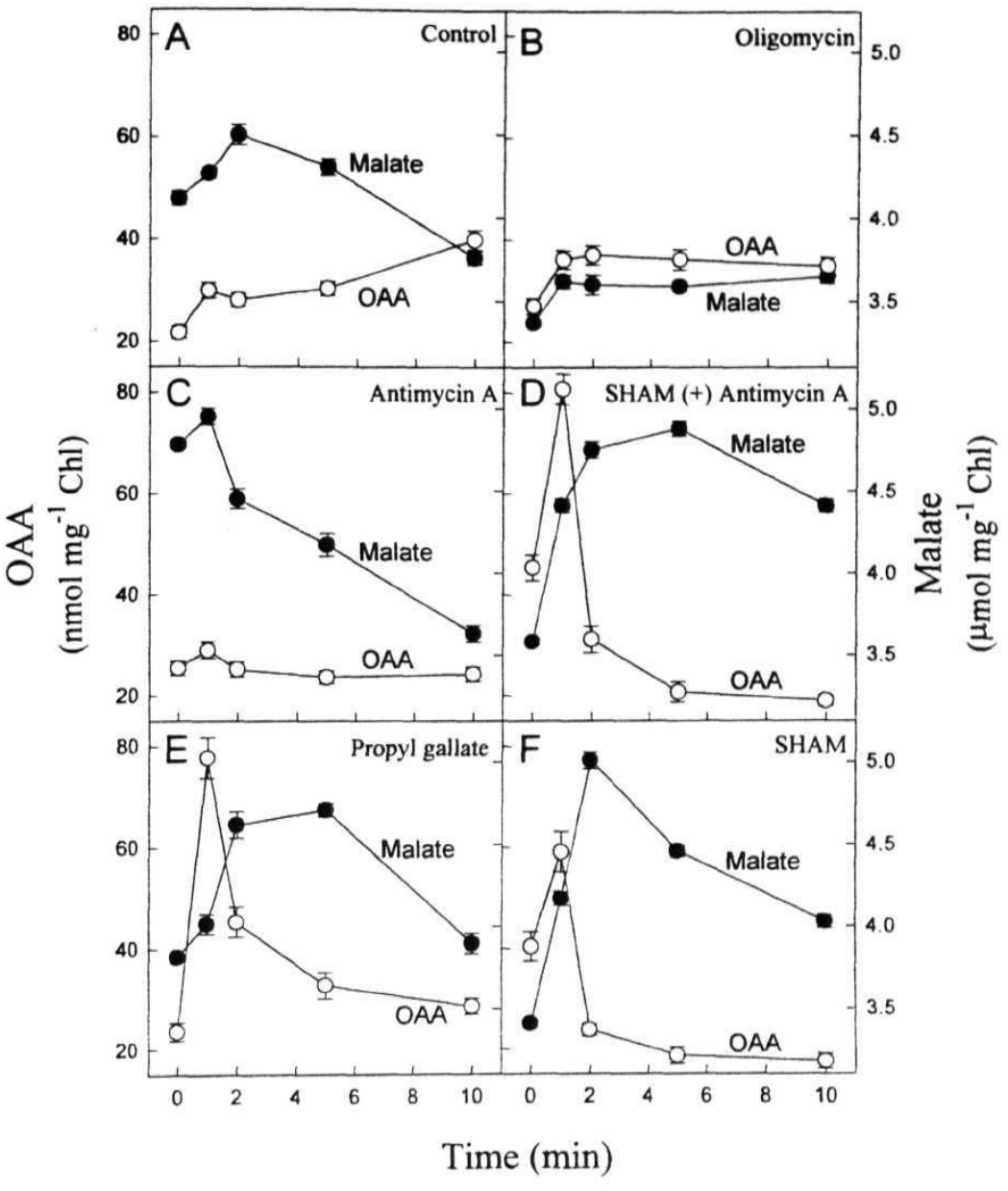

Figure 7.5. Change in the levels of malate and OAA in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM NaHCO3). The content of malate decreased from 4.1 + 0.06 μmol mg^{-1} Chl at the beginning of experiment to 3.8 ± 0.04 μmol mg^{-1} Chl after 10 min of light in control (without inhibitor) samples. The content of OAA was 22 ± 1.2 nmol mg^{-1} Chl at the beginning of experiment and 40 ± 1.9 nmol mg^{-1} Chl after 10 min of light in control samples. Further details are as described in Figure 7.1.

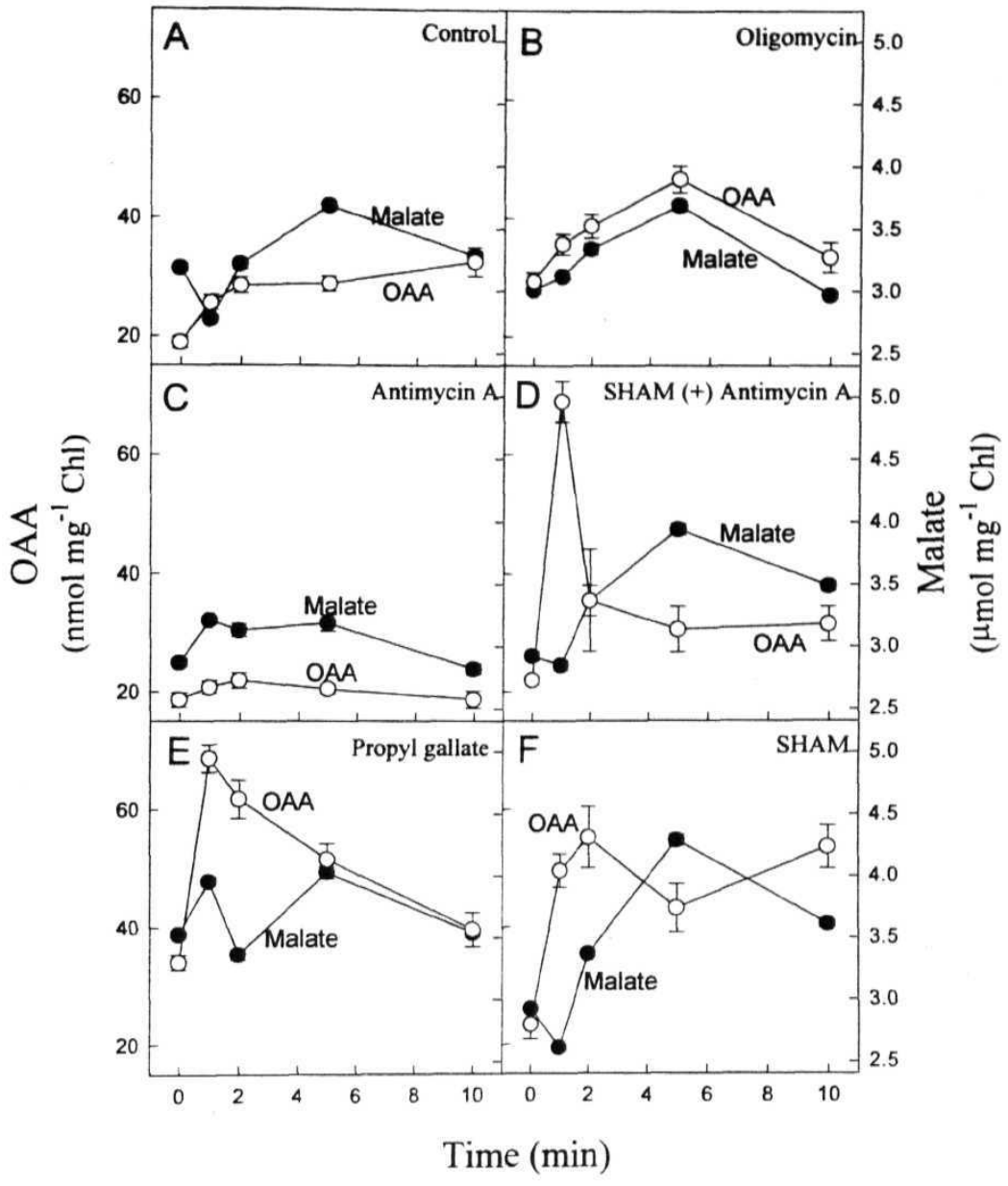

Figure 7.6. Change in the levels of malate and OAA in mesophyll protoplasts on illumination at optimal CO_2 (0.1 mM $NaHCO_3$). The content of malate was 3.2 + 0.03 μmol mg^{-1} Chl at the beginning of experiment while 3.3 ± 0.04 μmol mg^{-1} Chl after 10 min of light in control (without inhibitor) samples. The content of OAA was 19 ± 1.0 nmol mg^{-1} Chl at the beginning of experiment and 33 ± 2.5 nmol mg^{-1} Chl after 10 min of light in control samples. Further details are as described in Figure 7.1.

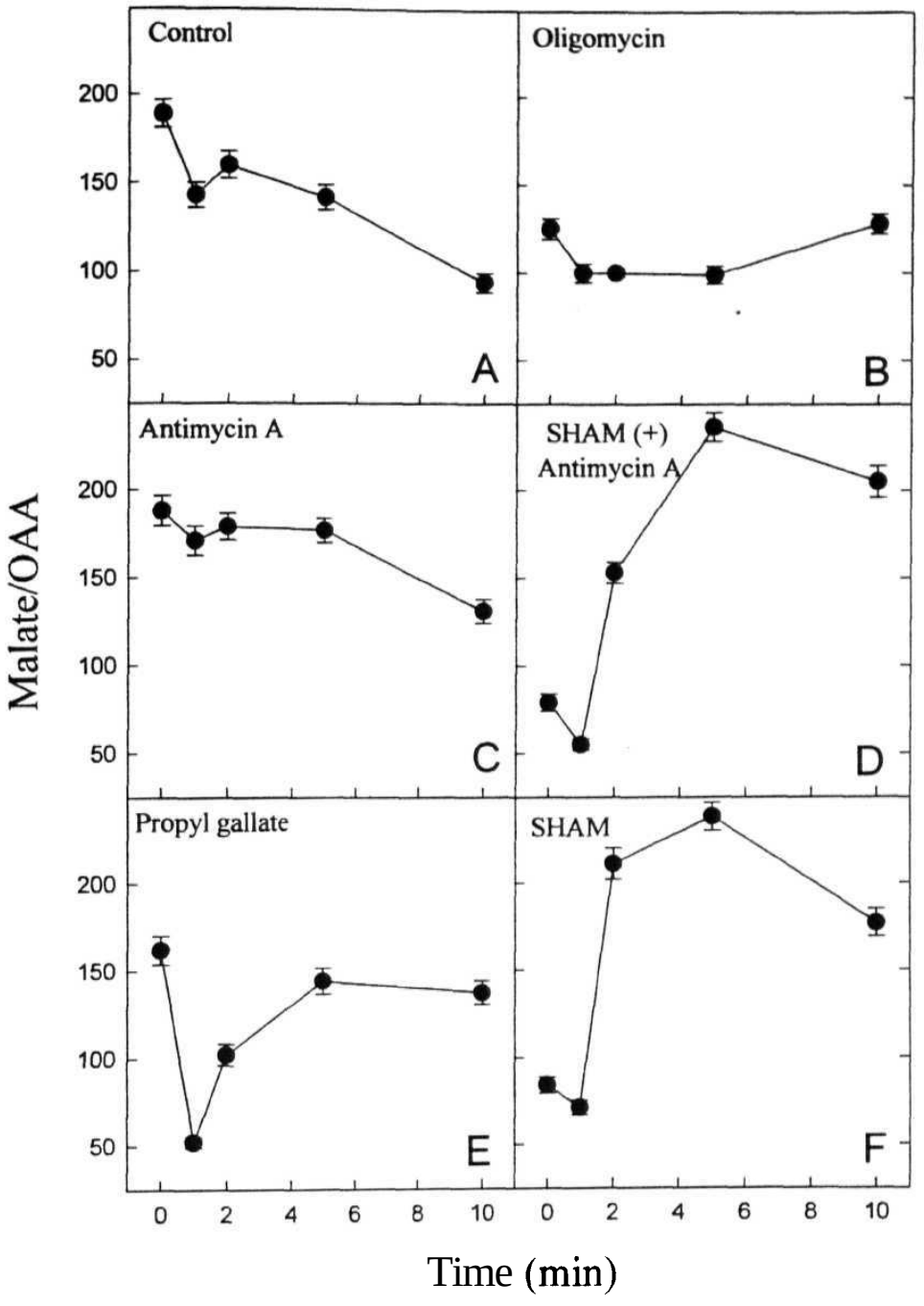

Figure 7.7. The ratio of malate to OAA in pea mesophyll protoplasts, at optimal (1.0 mM $NaHCO_3$) CO_2. The ratios were calculated from the data in Figure 7.5. The ratio of malate to OAA in control sets (in the absence of inhibitors) decreased from 189 ± 8 at the beginning of the experiment to 94 + 5.4 after 10 min of illumination. Other details were as described in Figure 7.1.

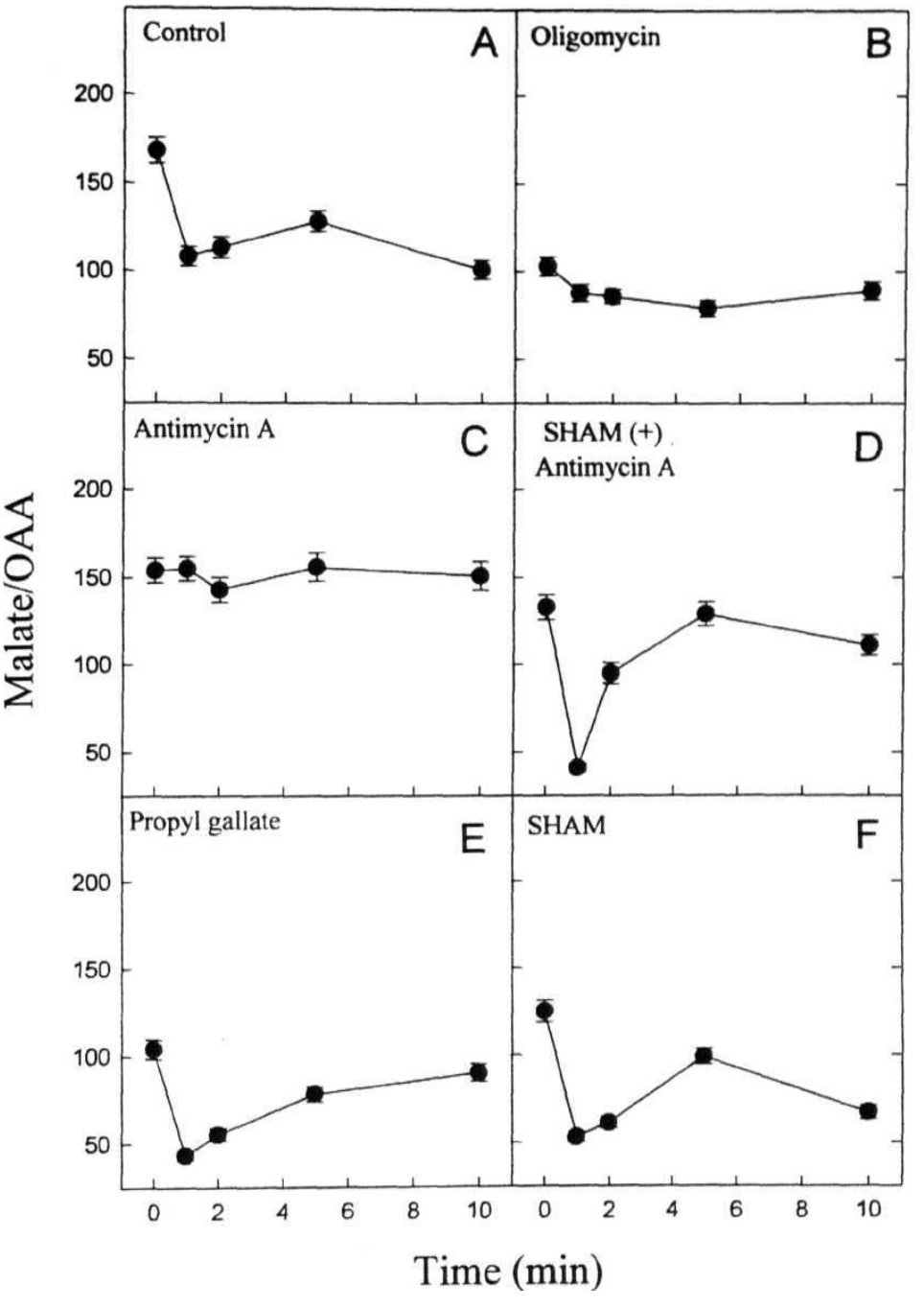

Figure 7.8. The ratio of malate to OAA in pea mesophyll protoplasts, at limiting (0.1 mM $NaHCO_3$) CO_2. The ratios were calculated from the data in Figure 7.6. The ratio of malate to OAA in control sets (in the absence of inhibitors) decreased from 168 + 7 at the beginning of the experiment to 101 ± 5 after 10 min of illumination. Other details were as described in Figure 7.1.

The summary of malate/OAA ratio at optimal and limiting CO_2 during active photosynthesis in the absence or presence of mitochondrial inhibitors is shown in Table. 7.2 At optimal CO_2, there was a two fold increase in malate/OAA ratio in presence of SHAM (± antimycin A), while there was about 40% increase in presence of oligomycin or antimycin A. In contrast, the ratio decreased in presence of all mitochondrial inhibitors at limiting CO_2, except antimycin A where there was an increase of 50% in the malate/OAA ratio.

There was a nearly 5-fold increase in FBP levels on illumination at both optimal (Fig. 7.9A) and limiting CO_2 (Fig. 7.10A) in control samples (without inhibitors). Such a marked increase in FBP was not much affected by the presence of mitochondrial inhibitors, except propyl gallate (Fig. 7.9, B-D and F; Fig. 7.10, B-D and F). A strong contrast to other inhibitors, propyl gallate decreased the FBP content, at both optimal and limiting CO_2 (Fig. 7.9E; Fig. 7.10E).

A summary of FBP levels at optimal and limiting CO_2 during active photosynthesis (at the end of 10 min illumination) is shown in Table 7.3, makes the situation clear. There was a marginal decrease in FBP levels at limiting CO_2 in presence of oligomycin or antimycin A, while there was a drastic decrease only in presence of propyl gallate.

Discussion

Marked changes occur in several metabolites of Calvin cycle on illumination of leaves or chloroplasts. Lilley et al. (1977) have shown that most spectacular rises occur in PGA and hexose monophosphates. The extent of triose-P accumulation exceeds that of PGA. A similar trend was recorded during the present observations. The concentration of PGA was about 2-3 fold higher than that of triose-P in dark-adapted protoplasts (Fig. 7.1 A; Fig. 7.2A), a pattern

Table 7.2. *Response to different metabolic inhibitors of malate/OAA ratio in mesophyll protoplasts of pea during photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

Mesophyll protoplasts were incubated in darkness for 5 min at 25 °C with and without (control) test inhibitors. Protoplast samples were collected after 10 min illumination (corresponding to activated phase of photosynthesis) frozen and analysed for malate and OAA.

Treatment	Optimal CO_2	Limiting CO_2
	Ratio	
Control	94 ± 6	101 ± 8
100 ng mL^{-1} Oligomycin	128 ± 10	89 ± 6
100 nM Antimycin A	131 ± 9	151 ± 12
1 mM Propyl gallate	137 ± 11	90 ± 8
500 μM SHAM	177 ± 13	67 ± 7
500 μM SHAM + 100 nM Antimycin A	205 ± 15	111 ± 13

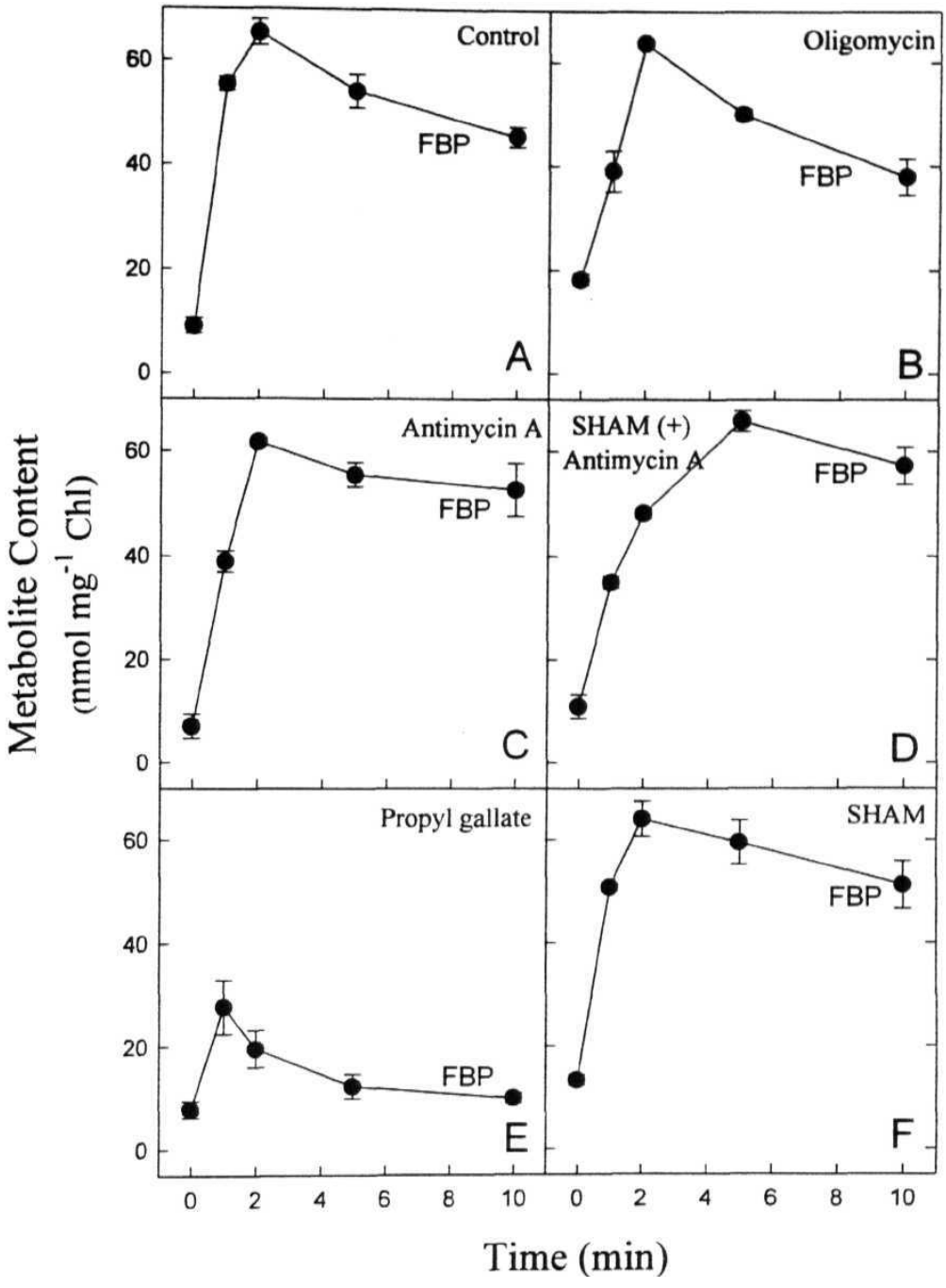

Figure 7.9. Change in the levels of FBP in mesophyll protoplasts on illumination at optimal CO_2 (1.0 mM $NaHCO_3$). The content of FBP raised from 9 ± 1.5 nmol mg^{-1} Chl at the beginning of experiment to 46 ± 2 nmol mg^{-1} Chl after 10 min of light in control (without inhibitor) samples. Other details were as in Figure 7.1.

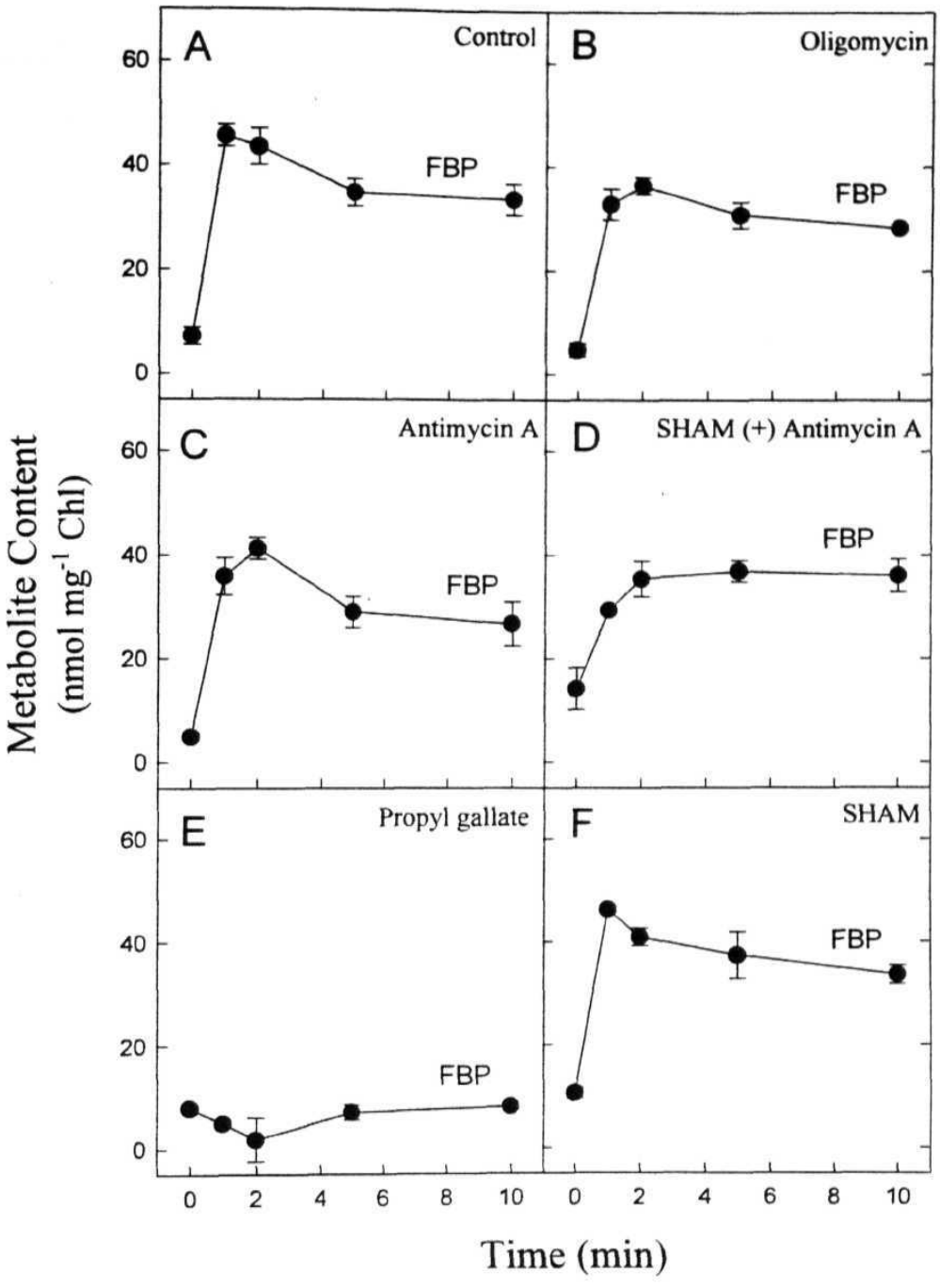

Figure 7.10. Change in the levels of FBP in mesophyll protoplasts on illumination at limiting CO_2 (0.1 mM NaHCC>3). The content of FBP was 7 ± 1.0 nmol mg^{-1} Chl at the beginning of experiment and 34 + 3 nmol mg^{-1} Chl after 10 min of light in control (without inhibitor) samples. Other details were as in Figure 7.1.

Table 7.3. *Response to different metabolic inhibitors of FBP levels in mesophyll protoplasts of pea during photosynthesis at optimal (1.0 mM $NaHCO_3$) or limiting (0.1 mM $NaHCO_3$) CO_2.*

Mesophyll protoplasts were incubated in darkness for 5 min at 25 °C with and without (control) test inhibitors. Protoplast samples were collected after 10 min illumination (corresponding to activated phase of photosynthesis), frozen and analysed for FBP.

Treatment	Optimal CO_2	Limiting CO_2
	nmol mg $^{-1}$ Chl	
Control	46 ± 2.0	34 ± 2.4
100 ng mL^{-1} Oligomycin	39 ± 2.2	29 ± 2.1
100 nM Antimycin A	53 ± 4.0	27 ± 1.5
500 µM SHAM	51 ± 3.9	34 ± 2.6
1 mM Propyl gallate	10 ± 0.5	8 ± 0.6
500 µM SHAM + 100 nM Antimycin A	57 ± 5.9	36 ± 3.1

similar to previous reports. (Stitt et al., 1985; Gardeström, 1993). In contrast, the increase in trisoe-P content during maximal photosynthesis was much higher than that in PGA, resulting in a significant increase in the ratio of triose-P/PGA (Fig. 7.3A; Fig. 7.4A).

There was a difference in the response of triose-P and PGA levels in mesophyll protoplasts to illumination depending also on the CO_2 concentration. The increase in triose-P and PGA pools was more pronounced at optimal CO_2 than that at limiting CO_2. Further, the increase in ratio of triose-P to PGA also was more significant at optimal CO_2 than that at limiting CO_2 (Fig. 7.3A; Fig. 7.4A). However, we feel that the responses of protoplasts at optimal CO_2 are more relevant than those at limiting CO_2, since there is no possibility of interference by photorespiration at optimal CO_2.

The levels of triose-P exceeded that of PGA (as indicated by the ratio of >1) only in presence of oligomycin or antimycin A (Fig. 7.3, B and C; Fig. 7.4, B and C), indicating protoplasts tended to accumulate triose-P, when oxidative metabolism through cytochrome pathway is restricted. The rise in the level of triose-P relative to that of PGA suggests an increase in the phosphorylation potential of protoplast.

The two-fold increase in triose-P/PGA ratio in the presence of oligomycin and antimycin in the present study (Table 7.1) are in agreement with the observations made with barley protoplasts in presence of oligomycin (Krömer and Heldt, 1991a). Fluctuations in 3-PGA and triose-P content in the chloroplast and extrachloroplast compartments are mediated by the 3-PGA shuttle across the envelope membrane (Gardeström, 1993). The triose-P-PGA shuttle is mainly controlled by the chloroplastic reaction of 3-PGA reduction and of the cytosolic reaction of triose-P oxidation (Heineke et al., 1991). The presence of oligomycin restricts ATP generated by oxidative phosphorylation which leads to an

accumulation of DHAP (triose-P), due to its non-utilization towards sucrose synthesis, a process which needs ATP (Krömer and Heldt, 1991a)

A critical look at the Table 7.1 and 7.3 suggests that the changes in FBP pool size were marginal in presence of mitochondrial inhibitors. However, the FBP levels represent a mirror image of triose-P/PGA ratio. The FBP levels decreased in contrast to that of an increase in triose-P/PGA ratio in presence of oligomycin and antimycin A. Similarly, the FBP levels increased, while triose-P/PGA ratio decreased in presence of SHAM and propyl gallate.

The changes in triose-P/PGA ratio and malate/OAA ratio in illuminated mesophyll protoplasts were quite in contrast. On transition from dark to light there was an increase in triose-P/PGA ratio and a fall in malate/OAA ratio during the steady state of photosynthesis (Fig. 7.3A; Fig. 7.4A; Fig. 7.7A; Fig. 7.8A).

Export of redox equivalents from the chloroplast to the cytosol can proceed via also the malate-OAA shuttle coupled to MDH activity which in its turn is regulated by the ferredoxin-thioredoxin system, which works as a 'valve' for redox regulation (Scheibe, 1987). Further. the malate-oxaloacetate shuttle itself is regulated by the chloroplastic NADP-malate dehydrogenase as well as the corresponding translocator on the envelope membrane (Heineke et al., 1991). NADPH thus transported from chloroplast to the cytosol (in the form of malate) can be oxidized by mitochondrial external dehydrogenase.

A part of the malate which enters the mitochondria is converted to pyruvate through malic enzyme. The increase in pyruvate in mitochondria can in turn stimulate the activity of the AOX (Day and Wiskich, 19950- The inhibition of AOX pathway may lead to accumulation of pyruvate and subsequent increase in malate. Thus the observed increase in malate/OAA in the presence of SHAM can also be due to the inhibition of AOX pathway.

The marked increase in the malate/OAA ratio by SHAM, particularly at optimal CO_2 (Fig. 7.7, D and F) suggests an accumulation of redox power in protoplasts when AOX pathway is restricted. AOX pathway appears therefore to be involved in the oxidation of malate in pea mesophyll protoplasts. Since malate is an important component of transfer of redox equivalents to cytosol, an accumulation of malate represents an over-reduction of chloroplasts (Backhausen et al., 1994). Thus a major function of AOX pathway in mesophyll cells of C3 and C4 plants could be to maintain the oxidation of malate, particularly under excess light.

It has already been suggested that mitochondrial electron transport in the light may be a mechanism to oxidize excess photosynthetic reducing power (Krömer, 1995) but to date the exact contribution of AOX to photosynthesis is not known. There are indications that AOX participates in the oxidation of reducing equivalents generated during either the rapid malate decarboxylation in the light that occurs in CAM species (Robinson et al., 1995) or the bundle sheath localized malate decarboxylation that occurs in C_4 species (Agostino et al., 1996). The results of this Chapter form the first report to elucidate the potential contribution of AOX to photosynthetic metabolism in C_3 plants.

The modulated 'malate valve* provided by NADP-MDH (Scheibe, 1991) also has important consequences for extra-chloroplastic metabolism, because malate is a source of NADH, and potentially, ATP in mitochondria (Heldt and Flügge, 1992). Some of the malate may enter the peroxisomes, instead of being oxidized by MDH in the cytosol. This would still allow some of the NADH formed during glycine decarboxylation to be retained in the mitochondria, rather than shuttling it to the peroxisome to support hydroxypyruvate reduction. As a result NADH can be oxidized within the mitochondria to provide additional ATP for extra-chloroplastic processes. Such ATP can be used for either sucrose

synthesis or reduction of PGA in the cytosol, in stead of being derived from the chloroplast (Krömer and Heldt, 1991a,b).

The significant increase in triose-P/PGA in presence of antimycin A and oligomycin compared to that in presence of SHAM and propyl gallate, suggest that cytochrome pathway plays a prominent role in maintaining the cytosolic triose-P/PGA ratio, particularly at optimal CO_2. On the other hand, the significant increase in malate/OAA ratio in presence of SHAM ($\pm$ antimycin A) or propyl gallate suggests that AOX pathway plays a significant role in maintaining the cytosolic redox state through, presumbly malate metabolism in mesophyll protoplasts.

Chapter 8

Effect of Mitochondrial Electron Transport Inhibitors on the Photochemical Activities of Chloroplasts

Chapter 8

Effect of Mitochondrial Electron Transport Inhibitors on the Photochemical Activities of Chloroplasts

Introduction

An inherent limitation of metabolic inhibitors is the possibility of unspecific inhibition of related activities, which makes the interpretation of the results difficult and questionable. Further, when the concentration of an inhibitor is raised, the specificity of the effect can be lost and the inhibitor may interfere with one/or more of the other metabolic pathways. It is therefore essential to ensure that the inhibitors are used at as low concentration as possible and that they do not affect any system other than that targeted for.

Since the present work is an attempt to examine the importance of mitochondrial metabolism in optimizing photosynthesis. ideal mitochondrial inhibitors should specifically affect only the respirator}' system, without any direct effect on photosynthetic reactions. Among the inhibitors, used in the present study, antimycin A has been shown to inhibit cyclic electron flow and cyclic photophosphorylation (Izawa and Good, 1972; Moss and Bendall, 1984). This point is acknowledged and discussed in Chapter 4. However we are not certain whether SHAM or propyl gallate have no direct effect on photosynthetic reactions of chloroplasts.

The occurrence of a cyanide-resistant, alternative mitochondrial respiratory pathway in mitochondria is discovered much later than the well-known conventional CN-sensitive cytochrome pathway (Schonbaum et al., 1971). Each of these pathways can be specifically inhibited by different compounds. SHAM and propyl gallate are believed to be the strong inhibitors of AOX, while KCN or

antimycin A are known to inhibit cytochrome pathway (Theologis and Laties, 1978; Møller et al., 1988; Lambers, 1990).

The AOX pathway inhibitors, *viz* SHAM, propyl gallate or disulfiram have been used frequently to assess the significance of AOX under different stress stimuli: cold and oxidative stress, pathogen attack- and by factors constricting electron flow through the cytochrome pathway of respiration (Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Day et al., 1996; Vanlerberghe and McIntosh, 1996; Popov et al., 1997).

The importance of cyanide-resistant respiration for stomatal opening under blue and red light illumination has been revealed by the usage of SHAM (Bhatia et al., 1995). The beneficial interaction between photosynthesis and respiration during short cycles of illumination and darkness in mesophyll protoplasts is elucidated by the usage of metabolic inhibitors (Vani et al., 1990; Xue et al., 1996; Igamberdiev et al., 1997). However, several authors have advised caution, while SHAM or propyl gallate are used to inhibit specifically the mitochondrial AOX pathway. Such caution applies to particularly at high concentrations of inhibitors.

Compared to the extensive literature on the use of mitochondrial inhibitors in relation to respiration only limited information is available on their possible effects on photosynthesis. A striking decrease in photosynthesis of soybean leaves of different ages was shown in presence of SHAM and propyl gallate by Diethelm et al. (1990), but the mechanism was not clear. Moreover, these authors have used rather high concentration of SHAM and employed too low light to justify the operation of active photosynthesis. Oligomycin which is known to be an inhibitor of oxidative phosphorylation has been used to elucidate the significance of dark respiration to photosynthetic metabolism in barley leaf protoplasts (Ebbighausen et al., 1987; Krömer et al., 1993). The sensitivity of

photosynthetic O_2 evolution to inhibition of mitochondrial electron transport has been shown in mutants of *chlamydomonas* by making use of antimycin A (Lemaire et al., 1988). Antimycin A and oligomycin are known to inhibit ferredoxin catalyzed cyclic photophosphorylation (Arnon, 1969). Recently Scheller (1996), reported two parallel pathways with different antimycin A sensitivity, saturation characteristics and substrate specificity.

Since different inhibitors were used to study the importance of AOX pathway or cytochrome pathways in benefiting photosynthesis, it has become necessary to establish their specificity and to clear the doubts over their non-interference with chloroplast photosynthesis. We have therefore examined in detail the effect of all the mitochondrial inhibitors, used in the present study on the photochemical activities of chloroplasts. The results were then compared with effects of two well known inhibitors, DCMU and NH_2OH, so as to identify the site of action of mitochondrial inhibitors, if any, on the chloroplast electron transport.

Results

The photochemical activities of isolated chloroplasts/thylakoids can be measured by the usage of artificial electron acceptors/electron donors and by blocking the chain at specific sites with inhibitors. Figure 8.1 depicts the flow of electrons in the photosynthetic electron transport chain from H_2O to NADP in relation to some of the known electron acceptors/donors and inhibitors. DPC is an artificial electron donor for PS II, while reduced DCIPH2 and TMPDH2 can donate electrons to PS I. *p*-BQ and MV are used as artificial electron acceptors from PS II and PS 1, respectively. NH_2OH and DCMU block the electron transport on acceptor and donor sides of PS II, respectively.

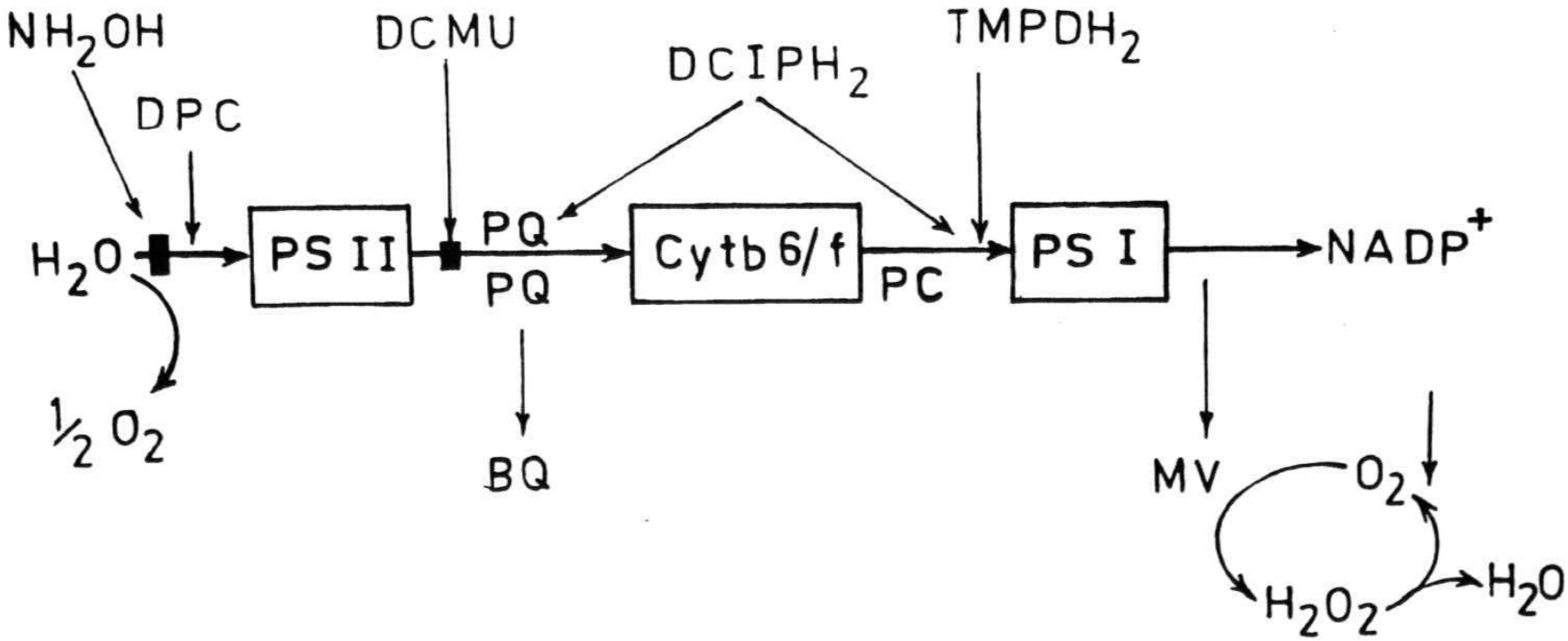

Figure 8.1. A schematic flow of electrons from H_2O to NADP through the photosynthetic electron transport chain, in relation to the measurement made in the present study. NH_2OH and DCMU are the inhibitors of electron transport chain at the acceptor and donor sites of PS II. The rate of electron transport from H_2O to MV (PS II + PS I, including oxygen evolvin complex), DPC to MV (PS II + PS I, excluding OEC) and DCPIP to MV (PS I activity) were measured as O_2 uptake while H_2O to *p*-BQ (PS II activity) was measured as O_2 evolution. DPC, DCPIP and TMPDH are artificial electron donors, while *p*-BQ and MV are artificial electron acceptors. Other details are described in 'Materials and Methods'.

Both oligomycin and antimycin A **did not** show any effect **on three different** types of reactions: (i) whole chain **(which include PS I and** PS **II through** OEC) electron transport activity measured as H_2O to MV (Fig. 8.2, A and B), or (ii) PS II activity measured as H_2O to *p*-BQ(Fig. 8.3, A and B), (iii) PS I activity measured as DCPIP to MV (Figs. 8.4, A and B).

It is possible that although oligomycin or antimycin A have no effect on chloroplast photochemical reactions, they may still exert some effects on photosystem activities in intact cells. PSII activity in intact protoplasts was therefore measured as *p*-BQ-dependent O_2 evolution in light. However there was no effect of oligomycin or antimycin A on *p*-BQ dependent oxygen evolution (Fig. 8.5, A and B) in mesophyll protoplasts. In fact, a marginal stimulation of <10% was observed at high concentrations of 1 $\mu g\ mL^{-1}$ oligomycin or 1 uM antimycin A.

In contrast to the effect of oligomycin and antimycin A (Fig. 8.5), the *p*-BQ dependent oxygen evolution in mesophyll protoplasts was marginally sensitive to SHAM and was significantly inhibited by propyl gallate (Fig. 8.6, A and B). At 1.0 mM concentration there was <10% decrease in the rate of oxygen evolution in presence of SHAM and 35% decrease in presence of propyl gallate.

Figure 8.7 shows the effect of SHAM on four different types of photochemical activities. Two PS II dependent reactions of electron transport: H_2O to MV (PS II + PS 1, including OEC) dependent O_2 uptake and H_2O to *p-BQ* (PS II) dependent O_2 evolution were only marginally affected by SHAM at the concentrations of even up to 500 uM. At 1.0 mM SHAM, there was a <15 % decrease in these two PS II dependent activities, with respect to control. However there was a 40% increase in the rate of electron transport between DPC to MV (PS II + PS I excluding OEC). while there was a marginal decrease (<5%) in the rate of transport from DCPIP to MV (PS I activity).

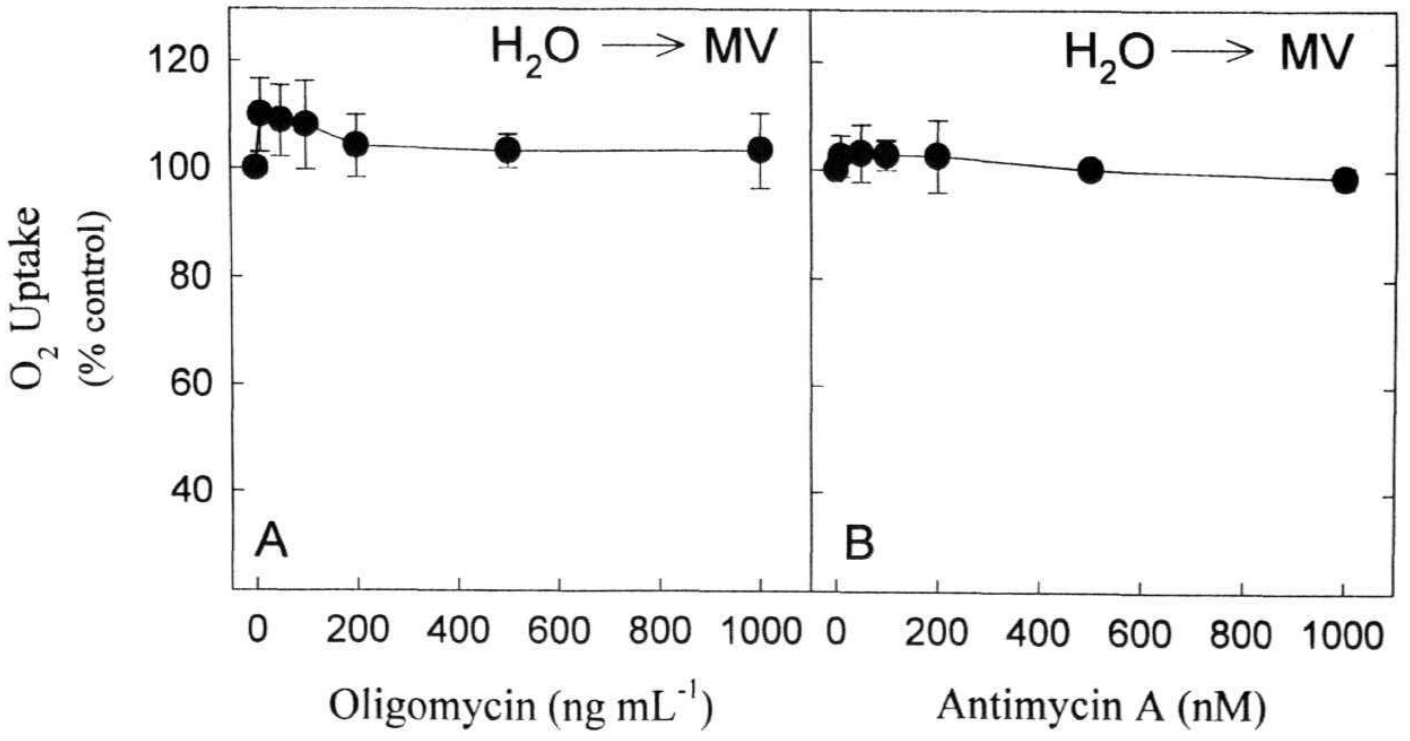

Figure 8.2. Effect of oligomycin or antimycin A on MV dependent O_2 uptake (H_2O to MV through OEC, PS II and PS I) in chloroplasts. The chloroplasts were incubated in dark at 25 °C for 5 min at the indicated concentrations of mitochondrial inhibitors before switching on the light. The average rate of MV dependent O_2 uptake in control sets (without any inhibitor) was 155 ± 12 μmol mg^{-1} Chl h^{-1}. Same scale has been used on Y-axis, of Figures 8.2 to 8.6 to make an effective comparison between them.

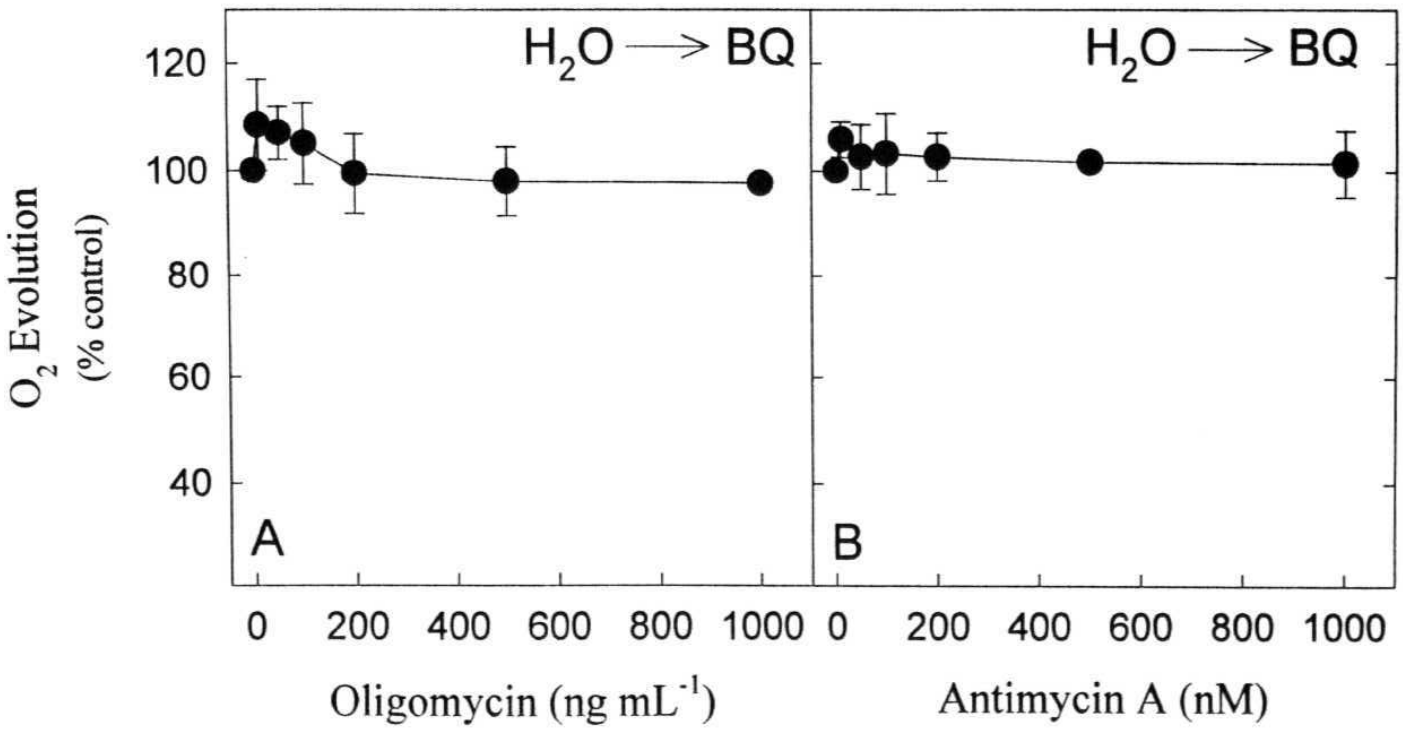

Figure 8.3. Effect of oligomycin or antimycin A on *p*-BQ dependent O_2 evolution (PS II activity) in chloroplasts. The chloroplasts were incubated in dark at 25 ^{o}C for 5 min at the indicated concentrations of mitochondrial inhibitors and *p*-BQ was added just before switching on the light. The average rate of *p*-BQ dependent O_2 evolution in control sets (without any inhibitor) was 345 $\pm$ 21 µmol mg^{-1} Chl h^{-1}.

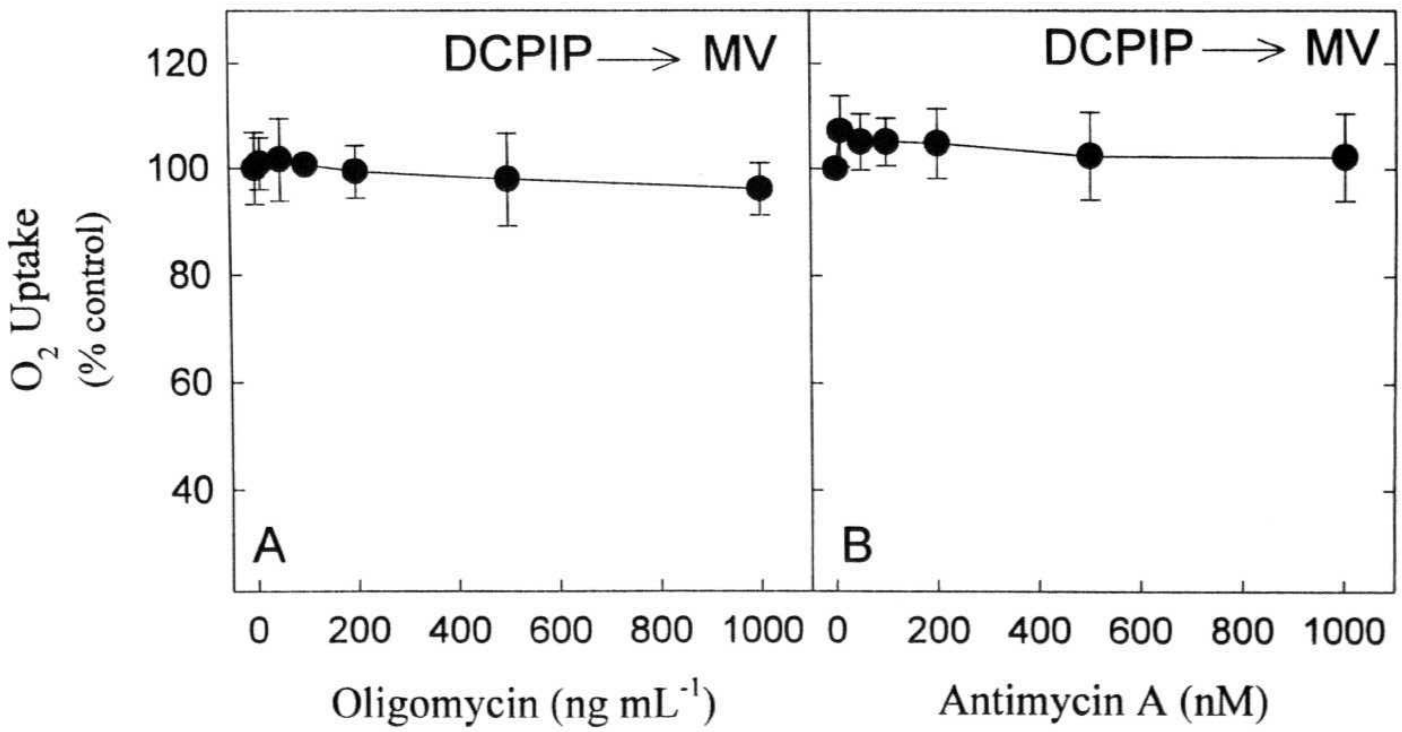

Figure 8.4. Effect of oligomycin or antimycin A on MV dependent O_2 uptake in presence of DCPIP + ascorbate (PS I activity) in chloroplasts. The average rate of MV dependent O_2 uptake in presence of DCPIP + ascorbate in control sets (without any inhibitor) was 385 $\pm$ 26 μmol mg^{-1} Chl h^{-1}. Further details were as in Figure 8.3.

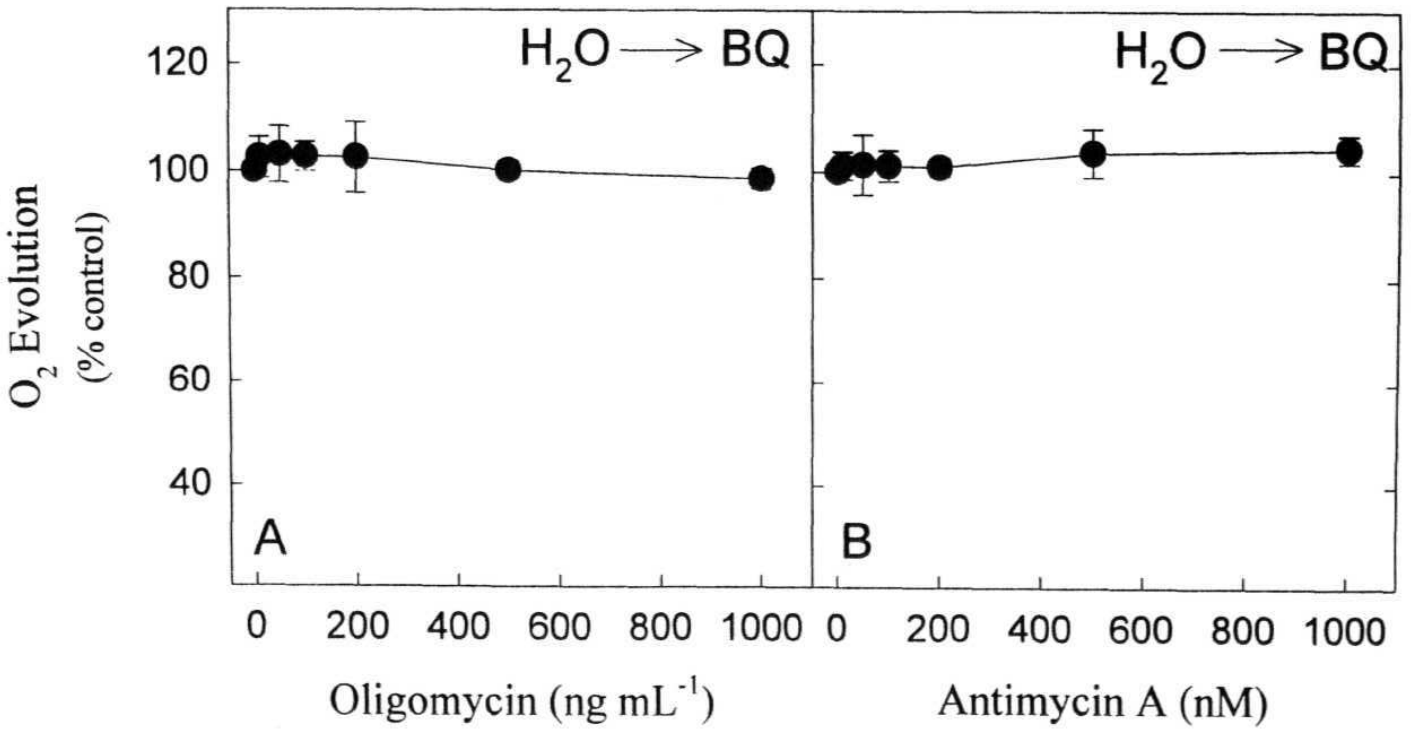

Figure 8.5. Effect of oligomycin or antimycin A on *p*-BQ dependent O_2 evolution (PS II activity) in mesophyll protoplasts. Protoplasts were kept in darkness at 25 ^{o}C for 5 min and *p*-BQ was added just before switching on the light. The reaction medium contained the test compounds at the indicated concentrations. The average rate of *p*-BQ dependent O_2 evolution in control sets (without any inhibitor) was 300 $\pm$ 20 μmol mg^{-1} Chl h^{-1}.

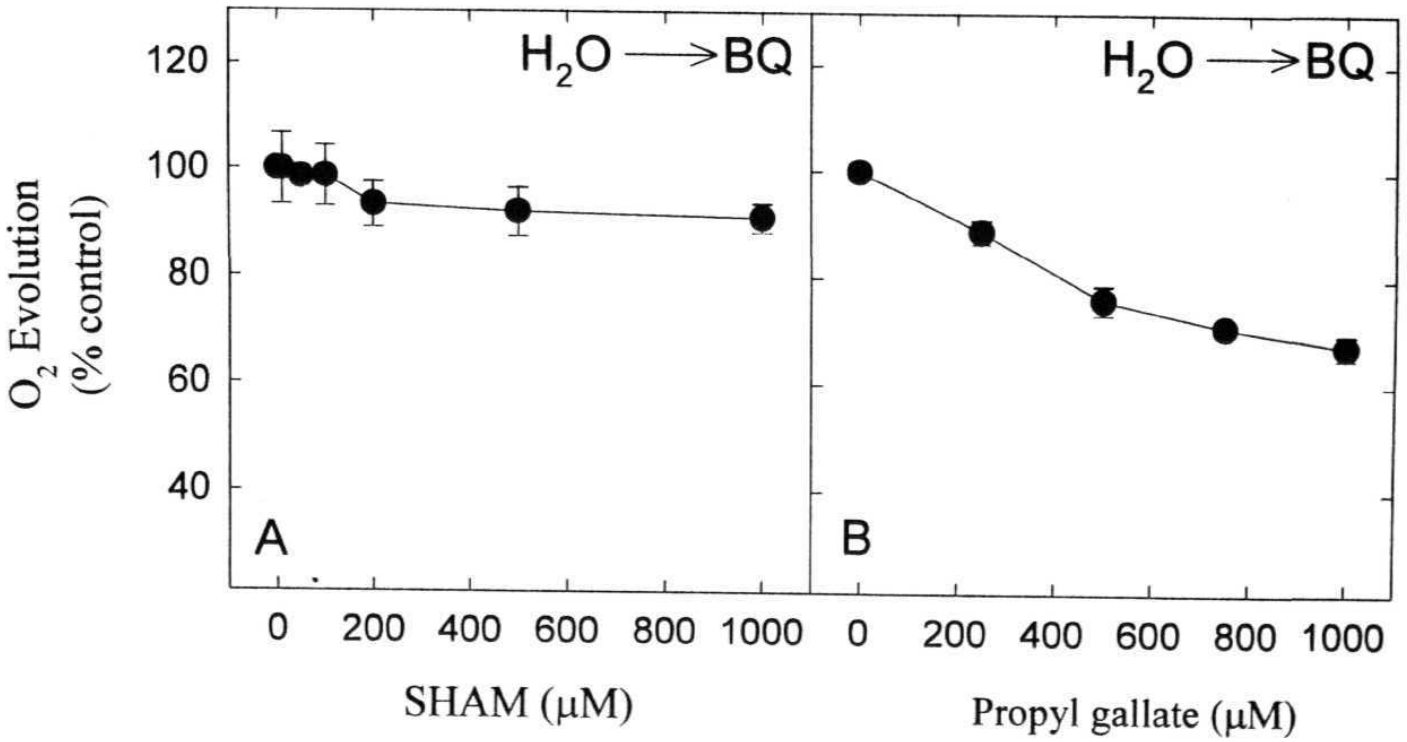

Figure 8.6. Effect of SHAM or propyl gallate on *p*-BQ dependent O_2 evolution (PS II activity) in mesophyll protoplasts of pea. Other details were as described in Figure 8.5. Same scale has been used on Y-axis of Figure 8.2 to Figure 8.6 to facilitate an easy comparison.

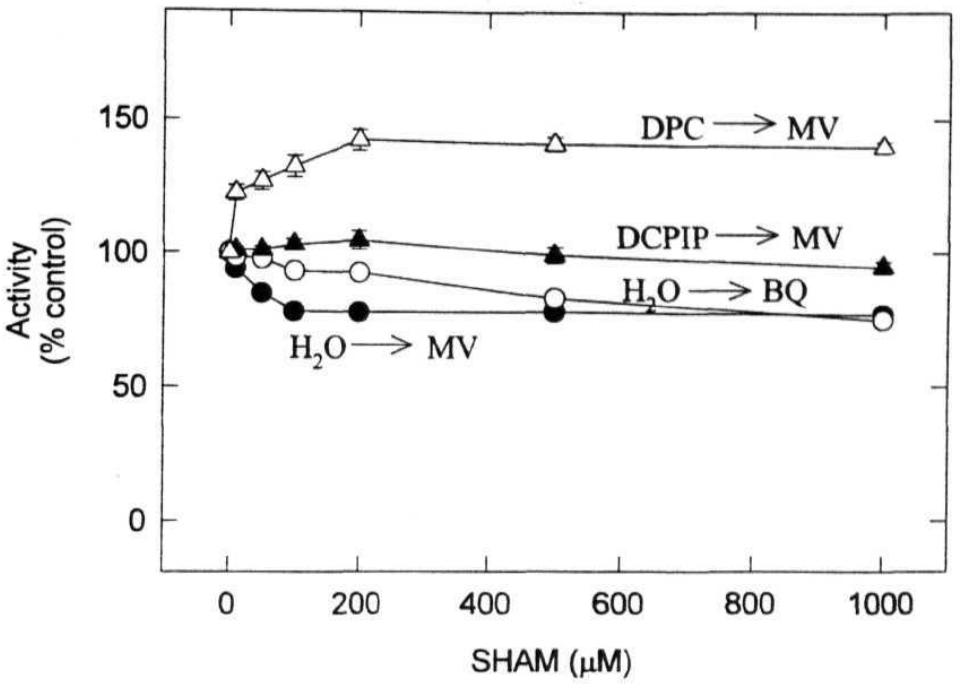

Figure 8.7. Effect of SHAM on four different photochemical reactions: i) MV dependent O_2 uptake (involving PS II + PS I, including OEC); ii) *p*-BQ dependent O_2 evolution (PS II activity); iii) MV dependent O_2 uptake in presence of DCPIP + ascorbate (PS I activity); iv) MV dependent O_2 uptake in presence of NH_2OH + DPC (through PS II + PS I, excluding OEC) in chloroplasts. The average rates of MV dependent O_2 uptake in control sets (without any inhibitor) were: 160 + 9 μmol mg^{-1} Chl h^{-1} through OEC, PS II and PS I; 360 ± 10 μmol mg-l Chl h^{-1} in presence of DCPIP + ascorbate; 273 + 14 umol mg-l Chl h-l in presence of NH_2OH + DPC, respectively while the average rate of *p*-BQ dependent O_2 evolution was 325 + 20 fimol mg^{-1} Chl h-l. Other details were as described in Figures 8.3 - 8.5.

Propyl gallate had a marked inhibitory effect on photochemical reactions, particularly of PS II (Fig. 8.8). In presence of 1 mM propyl gallate there was a 50 to 75% decrease in the rate of electron transport, from H_2O to *p*-BQ**(PS II activity) and H_2O to MV** (PS **I** + PS **II,** including OEC). **On the the other hand, there** was a **60%** stimulation in the photochemical activity of electrons moving from DPC to MV (PS II + PS I, excluding OEC) while **there** was **marginal** (<5%) **stimulation in** the rate of electron transport from DCPIP to MV (PS I activity).

The presence of DCMU resulted in a typical inhibition of the three photochemical reactions involving PS II activity (Fig. 8.9). The rates of electron transport from DPC to MV, H_2O to MV and H_2O to *p*-BQ decreased by 50 to almost 100%, compared to the rates of control. However DCMU had no effect **on** the rate of electron flow from DCPIP to MV (PS I activity).

The photochemical activities of PS II, particularly involving OEC, were sensitive to NH_2OH (Fig. 8.10). At 1.0 mM NH_2OH, the electron transport from H_2O to MV (PS II + PS I, including OEC) dependent O_2 uptake and H_2O *to p*-BQ (PS II activity) dependent O_2 evolution decreased by 70 to 100%. There was a slight stimulation in the extent of electron trasnport from DCPIP to MV (PS I activity) at lower concentrations of NH_2OH. On the other hand, there was stimulation of >50% in the rate of flow from DPC to MV (involving PS II + PS I, excluding OEC).

Discussion

The final objective of the experiments designed for this chapter is to ensure that the suppression of photosynthetic metabolism by the different mitochondrial inhibitors is not due to their effect on the photochemical activities of chloroplasts. These inhibitors had been tested for their effect on photosynthetic carbon

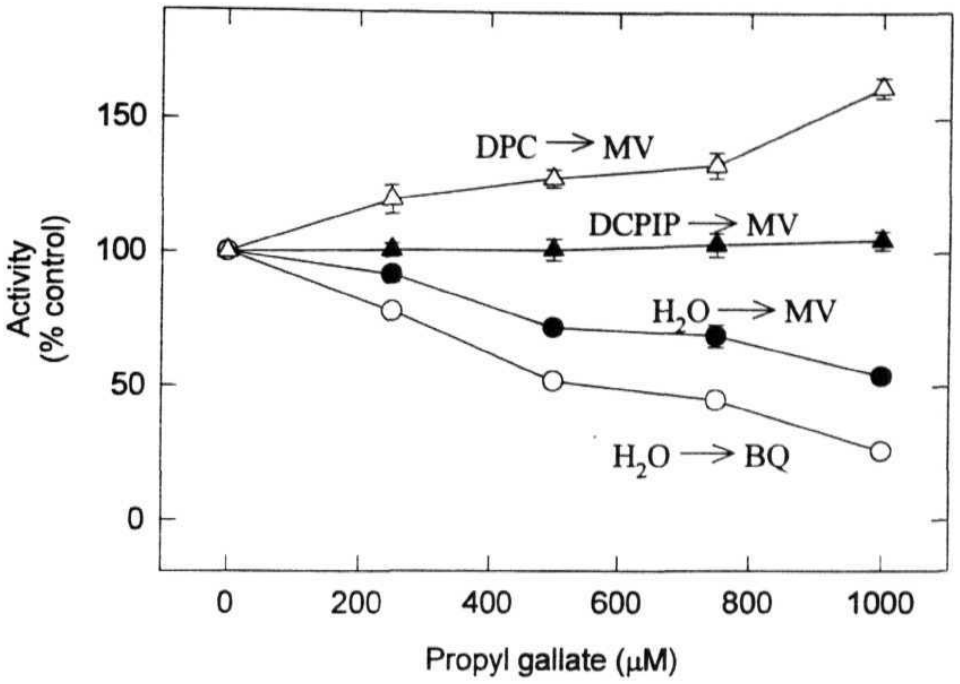

Figure 8.8. Effect of propyl gallate on four different photochemical reactions: i) MV dependent O_2 uptake (involving PS II + PS I, including OEC); ii) *p*-BQ dependent O_2 evolution (PS II activity); iii) MV dependent O_2 uptake in presence of DCPIP + ascorbate (PS I activity); d) MV dependent O_2 uptake in presence of NH_2OH + DPC (through PS II + PS 1, excluding OEC) in chloroplasts. The rates of control sets **and** other details were as in Figure 8.7.

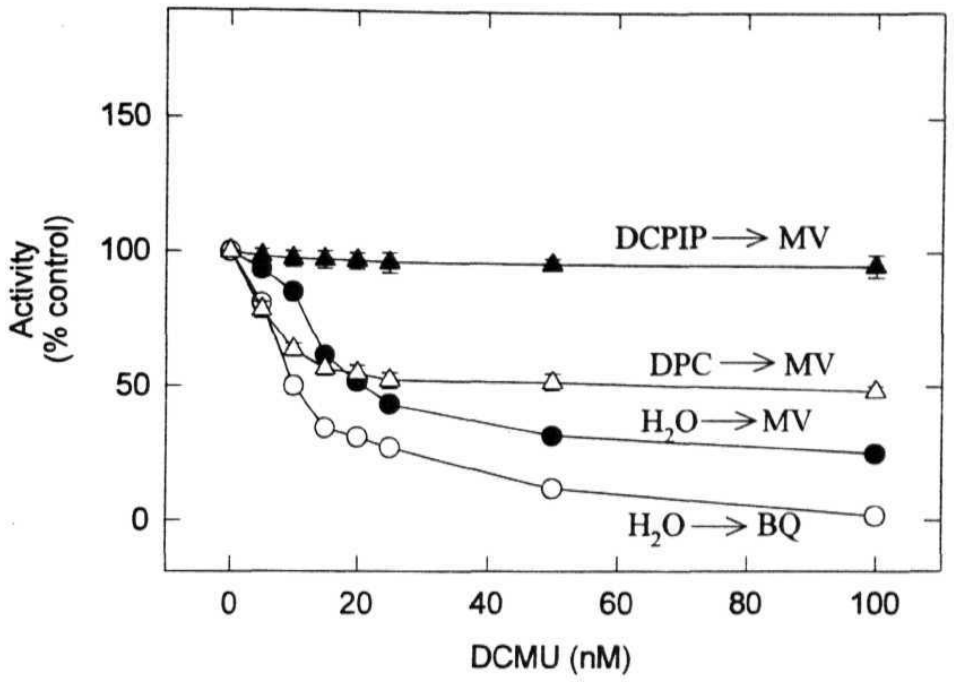

Figure 8.9. Effect of DCMU on four different photochemical reactions: i) MV dependent O_2 uptake (involving PS II + PS I, including OEC); ii) *p*-BQ dependent O_2 evolution (PS II activity); iii) MV dependent O_2 uptake in presence of DCPIP + ascorbate (PS I activity); d) MV dependent O_2 uptake in presence of NH_2OH + DPC (through PS II + PS I, excluding OEC) in chloroplasts. The rates of control sets and other details were as in Figure 8.7.

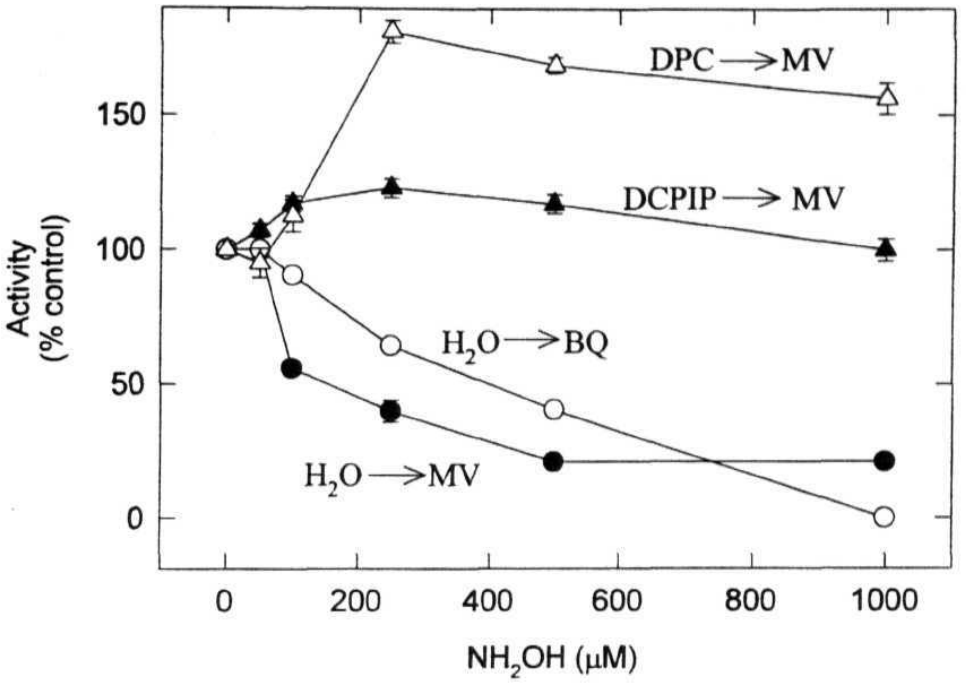

Figure 8.10. Effect of NH_2OH on four different photochemical reactions: i) MV dependent O_2 uptake (involving PS II + PS I, including OEC); ii) *p*-BQ dependent O_2 evolution (PS II activity); iii) MV dependent O_2 uptake in presence of DCPIP + ascorbate (PS I activity); d) MV dependent O_2 uptake in presence of NH_2OH + DPC (through PS II + PS I, excluding OEC) in chloroplasts. The rates of control sets and other details were as in Figure 8.7.

assimilation (indicated by CO_2 dependent O_2 evolution) in isolated chloroplasts, as already shown in Chapter 4 (Fig. 4.5; Fig. 4.6).

Oligomycin, (inhibitor of oxidative phosphorylation) or antimycin A (inhibitor of cytochrome pathway) which decreased markedly the rates of carbon assimilation (see Chapter 4) did not have any effect on *p-BQ* dependent O_2 evolution in intact protoplasts (Fig. 8.5). On the other hand, despite the decrease in bicarbonate dependent O_2 evolution, the *p-BQ* dependent O_2 evolution was sensitive to both SHAM and propyl gallate, inhibitors of AOX pathway (Fig. 8.6). The results presented in this chapter reveal that neither oligomycin nor antimycin A exerted any significant effect on the photochemical activities of chloroplasts mediated by PS 1 or PS II or both.

Two more classic inhibitors of photochemical electron flow: DCMU and NH_2OH, were also included, so as to make an easy and effective comparison, while identifying the site of action, if any, of mitochondrial inhibitors. DCMU, blocks the flow between PS II complex and PQ (Hind, 1985; Hall and Rao, 1994). The inhibition by DCMU of PS II activity (e.g. *p*-BQ dependent O_2 evolution) while not affecting that of PS I, e.g. DCPIP to MV (Fig. 8.9) is therefore quite logical. Similarly NH_2OH specifically blocks electron flow from OEC to PS II (Hall and Rao, 1994). The inhibition by NH_2OH of PS II activity involving OEC, e.g. H_2O to *p-BQ* or H_2O to MV, but not the reactions of DPC to MV (Fig. 8.10) is again expected.

Among the four mitochondrial inhibitors, the most significant effect on photochemical activities was by propyl gallate, which appeared to inactivate the oxygen evolving complex in chloroplasts (Fig. 8.8). The presence of SHAM also caused an inhibition of PS II activity, but the effect of SHAM was much less than that of propyl gallate (Fig. 8.7). The decrease in electron transport activity from H_2O to MV and H_2O to *p*-BQ but not from DCPIP to MV suggest that both SHAM and propyl gallate were exerting their effect on oxygen evolving side (OEC) of

PS II in chloroplasts. A comparison of the patterns of inhibition of photochemical activities by SHAM (Fig. 8.7) or propyl gallate (Fig. 8.8) with DCMU (which blocks the activity of PS II on the donor side) and NH_2OH (which blocks the PS II activity on acceptor side) (Fig. 8.9; Fig. 8.10), confirms that the primary effect of SHAM or propyl gallate is more like a block by NH_2OH than that by DCMU.

Unlike the inhibition of photochemical reactions of H_2O to MV or H_2O to p-BQ, SHAM or propyl gallate stimulated significantly (40 - 60%) the reactions of DPC to MV (Fig. 8.7; Fig. 8.8). However, such stimulation of DPC to MV activity was prominently seen in presence of NH_2OH (Fig. 8.10) but not with DCMU (Fig. 8.9). These observations provide further evidence that the mechanism of inhibition of photochemical reactions by propyl gallate or SHAM is similar to that of NH_2OH, i.e. on the OEC of PS II complex (Hall and Rao, 1994).

The inhibition of oxygen evolving system by NH_2OH is known to be associated with removal of Mn^{2+} ions (Yamamoto and Nishimura, 1984). Further experimentation is needed to elucidate if propyl gallate inactivates the oxygen evolving system by removing the Mn^{2+} ions in a similar way to NH_2OH.

Most of the experiments were carried out at or less than 500 μM concentration of SHAM. At this concentration, the inhibition of PS II activity was marginal (<10%). We therefore confident that the inhibition of photosynthesis by SHAM is primarily due to its interference with alternative path of mitochodrial electron transport.

The use of SHAM or propyl gallate as specific inhibitors of AOX pathway in cells, protoplasts or intact leaf tissue, is frequently questioned, as these inhibitors may affect other metabolic reactions. For e.g. SHAM stimulates peroxidase activity and leads to O_2 consumption in protoplasts (Spreen-Brouwer, 1986). Our results establish that propyl gallate has a direct inhibitory effect on OEC side (possibly by sequestering the Mn^{2+} ions), while the effect of SHAM on the

photochemical activity was only marginal, particularly at concentrations of less than 200 - 500 μM. We therefore suggest that SHAM is more suitable inhibitor than propyl gallate to study the AOX pathway of mitochondrial electron transport.

Bouthyette and Jagendorf (1982) reported that 7 mg oligomycin/mg protein inhibited photophosphorylation of thylakoids in certain pea cultivars. However this is a very high concentration and is far from the range used in the present study. It is a common observation that several of metabolic inhibitors, which affect specifically a particular site at low concentration tend to be unspecific and may interfere with other systems as well, when used at high concentrations. The inhibition of photosynthesis by SHAM at 20 mM concentration reported earlier (Diethelm et al., 1990) comes under this category and has to be therefore considered with caution.

Among the two photosystems, PS II is generally more vulnerable than PS I. A marked suppression of PS II mediated chloroplast reactions was noticed during plant responses to various environmental stresses, namely cold or heat (Sharkey and Badger. 1982). On exposure to osmotic stress *in vitro*, PS II (but not PS I) and in particluar, oxygen evolving complex was inactivated in chloroplasts (Sundari and Raghavendra, 1990; Sundari et al., 1994). Studies on the fluorescence properties (Govindjee et al., 1981) or photoacoustic spectroscopy (Havaux et al., 1986) of leaves also indicated that PS II of leaves was suppressed during water stress *in vitro.*

The present observations provide the first detailed record of possible effects of two classic mitochondrial inhibitors on chloroplast photochemical reactions. While establishing that oligomycin, antimycin A or SHAM can be used as mitochondrial inhibitors, caution must be observed in case of propyl gallate. As per the literature, SHAM and propyl gallate are frequently used as inhibitors of AOX pathway (Schonbaum et al., 1971; Siedow and Bickett, 1981). We propose that SHAM is a better choice than propyl gallate as an inhibitor of AOX pathway.

Chapter 9

General Discussion

Chapter 9

General Discussion

In Eukaryotes, the photosynthetic carbon metabolism resembles respiratory carbon metabolism in several ways. (i) the cytosol is the site of the primary sugar metabolism - glycolytic breakdown of sugar in respiration, and sucrose synthesis in photosynthesis; (ii) decarboxylation occurs through Krebs cycle in the mitochondrial matrix while carboxylation occurs through Calvin cycle in the stroma of the chloroplast; (iii) a three-carbon compound crosses from the cytosol to the organelle or *vice versa* - pyruvate enters the mitochondrion in respiration and triose-P leaves the chloroplast during photosynthesis; (iv) the reactions producing or consuming carbon dioxide occur inside the organelles during both processes; (v) while electron transport occurs on internal membrane system in both organelles. Thus, many facets of photosynthesis and respiration strike sense in light of the endosymbiotic theory that chloroplasts and mitochondria were once free-living bacteria which began a symbiotic existence and eventually became dependent on each other inside the host cell (Sharkey, 1998).

In the present study, the phenomenon of interaction between photosynthesis and respiration in mesophyll protoplasts of pea was demonstrated by specifically inhibiting the oxidative electron transport chain of mitochondria and further evaluating the consequences of their effect on four different components of photosynthesis: a) bicarbonate dependent O_2 evolution; b) adenylate levels; c) light activation of PCRC enzymes; and d) metabolite levels related to phosphorylation potential, redox status and sucrose synthesis.

Mitochondria of higher plants, possess two routes of oxidative electron transport: the cyanide sensitive cytochrome pathway and the cyanide-resistant

AOX pathway. Although the role of AOX pathway is known for the rapid oxidation of substrates and generation of heat in some ariod tissues, its role in plant metabolism is not completely understood (Seymour and Shultze-Motel, 1996). In the present study, we tried to investigate the positive implication of the AOX pathway by employing SHAM or propyl gallate, as the typical inhibitors of AOX pathway (Lambers, 1990). Further, we compared the results with those of oligomycin (inhibitor of mitochondrial oxidative phosphorylation) or antimycin A (inhibitor of cytochrome pathway). The present results demonstrate that besides the cytochrome pathway, the AOX pathway may also contribute significantly to the beneficial interaction between photosynthesis and mitochondrial respiration. Thus, this is the first report to document a significant role of AOX pathway of mitochondrial electron transport in optimizing photosyntheis.

Two possible objections against the use of SHAM are that (i) the compound may not enter the protoplasts and (ii) the inhibition may be unspecific. The marked inhibition of photosynthesis (as well as respiration) by even low concentrations (Fig. 4.2; Fig. 4.4, A and B) suggest that SHAM is presumably entering the protoplasts. Similar observations are made recently by other workers (Lynnes and Weger, 1996; lgamberdiev et al., 1997). The presence of SHAM did not affect chloroplast activity, while inhibiting protoplast photosynthesis (Fig. 4.6, A and B). We are therefore confident that at the low concentrations used in the present work, SHAM affects specifically mitochondrial electron transport via AOX.

The sensitivity of photosynthetic activity to oligomycin, antimycin A, SHAM (± antimycin A) and propyl gallate (Fig. 4.3; Fig. 4.4) indicate that AOX pathway as well as that cytochrome pathway of mitochondrial metabolism is essential for photosynthesis at both optimal and limiting CO_2. However, the photosynthetic metabolism to SHAM was more sensitive at optimal CO_2 than

that at limiting CO_2. We demonstrate for the first time possibly an exciting and a very significant role for the AOX pathway that it optimizes photosynthetic carbon metabolism in protoplasts.

Furthermore, the response of leaf protoplast photosynthesis to these respiratory inhibitors was studied at limiting or optimal CO_2 levels so as to assess the relative importance of the role of ATP during the mitochondrial interaction with chloroplasts. At optimal CO_2, the photosynthetic demand for ATP is expected to be very high, while such a need for ATP would be low at limiting CO_2. In contrast to photosynthetic activity, the cellular levels of ATP (Figs. 4.9 - 4.12) as well as ATP/ADP ratio (Fig. 4.13; Fig. 4.14) were more sensitive to mitochondrial inhibitors at limiting CO_2. The differential response of photosynthetic activity versus ATP/ADP ratio to oligomycin and antimycin A as well as the lack of any correlation between the marked decrease in photosynthesis and ATP/ADP ratio of protoplasts (Fig. 4.15; Fig. 4.16) suggests that mitochondrial respiration optimizes photosynthetic metabolism primarily through oxidative electron transport (both cytochrome and AOX pathways) while oxidative phosphorylation plays a secondary role. Our results also prove that AOX pathway does contribute to the cellular needs of ATP in a photosynthesizing cell (Fig. 4.11; Fig. 4.12; Fig. 4.14).

Induction is a common feature associated with photosynthesis (Edwards and Walker, 1983). The decrease in photosynthetic activity in response to mitochondrial inhibitors at either optimal or limiting CO_2 was always associated with a prolongation in the induction period (Fig. 5.2; Fig. 5.3). However the stability of the photosynthetic activity of mesophyll protoplasts was maintained in presence of inhibitors of mitochondrial electron transport except SHAM (Fig. 5.4; Fig. 5.5).

Our results reveal that the prolongation of induction in presence of mitochondrial inhibitors was due to the decrease in RuBP levels and restricted activation of PRK. The significant decrease in RuBP levels in presence of oligomycin or antimycin A and restricted activation of PRK in presence of SHAM or propyl gallate (in contrast to control samples on illumination) suggests that oxidative phosphorylation and cytochrome pathway contribute significantly in the regeneration of RuBP, while AOX pathway was involved in the light activation of PRK The pattern of response to mitochondrial inhibitors was similar at both optimal and limiting CO_2 (Table 5.1 and Table 5.2).

The mitochondrial metabolism exerted its effect on photosynthetic activity by influencing the light activation of enzymes in a way similar to that observed when photosynthetic cells are exposed to stress conditions (Holaday et al., 1992). The significant decrease in the activation of NADP-GAPDH, FBPase, NADP-MDH in presence of SHAM and propyl gallate compared to oligomycin or antimycin A, demonstrates that the light activation of PCRC enzymes is supported by the functioning of AOX pathway (Table 6.1; Table 6.2). The significance of the AOX pathway was more pronounced at limiting CO_2 than that at optimal CO_2.

Our results also indicate that metabolites related to cellular redox status as well as ATP are influenced by the limitation in mitochondrial activity. However the results obtained in response to mitochondrial inhibitors at optimal CO_2 would be quite more relevant, as there will be no possibility of interference by photorespiration.

Cytochrome pathway and oxidative phosphorylation appear to contribute significantly in modulating the triose-P/PGA ratio as indicated by the significant increase in the ratio in presence of oligomycin and antimycin A (Table 7.1). The cytochrome pathway and oxidative phosphorylation regulate the utilization of

triose-P for synthesis of sucrose and thereby the overall process of photosynthesis by modulating triose-P/PGA ratio and ATP.

On the other hand AOX pathway may play a major role in modulating the malate/OAA ratio as indicated by the marked increase in ratio in presence of SHAM and propyl gallate (Table 7.2). The accumulation of malate in presence of malate is an indication that malate is being metabolized with the help of AOX pathway. An inhibition of AOX pathway leads to malate accumulation and increase in malate/OAA ratio. Thus AOX pathway plays an important role in regulating malate valve by modulating malate/OAA ratios. Malate valve is an important factor to transfer excess redox equivaltents from chloroplasts to cytosol and helps to keep chloroplasts away from over-reduction state (Backhausen et al., 1994).

A summary of all the components determined in the present study in relation to photosynthetic activity in presence of mitochondrial inhibitors are represented in Table 9.1. For the first time, we illustrate the importance of oxidative electron transport through both cytochrome and AOX pathways in optimizing the photosynthetic metabolism in C3 mesophyll cell protoplasts. The importance of AOX pathway in relation to photosynthetic activity is evident by i) increase in malate/OAA ratio; ii) decrease in light activation of enzymes; and iii) a small but significant decrease in the cellular ATP levels.

The increase in triose-P/PGA ratio is associated with also a decrease in ATP and increase in G-6-P levels, particularly in presence of oligomycin and antimycin A. Thus, the cytochrome pathway contributes significantly to the synthesis of sucrose and regeneration of RuBP by modulating the following components: i) ATP status of cell; and ii) cellular triose-P/PGA ratio.

Finally, the effect of four mitochondrial inhibitors on the photochemical activities of chloroplasts was ascertained so as to ensure that these inhibitors are

Table 9.1. Summary of the patterns of photosynthetic O_2 evolution, duration of induction, activity of key photosynthetic enzymes and metabolites related to phosphorylation potential and redox status of the cell in absence or presence of inhibitors of mitochondrial metabolism at optimal CO_2 (1.0 mM $NaHCO_3$). All figures in the table are represented **as % control**.

	Photosynthesis		Enzymes				Metabolites					
Treatment	O_2 evolution	Lag	NADP-GAPDH	FBPase	Ru-5-P-Kinase	NADP-MDH	RuBP	ATP	G-6-P	FBP	Malate/ OAA	TrioseP/ PGA
	Activity	Time	Light activation				Content					
No inhibitor (Control)	100	100	100	100	100	100	100	100	100	100	100	100
100 ng mL^{-1} Oligomycin	64	190	87	94	86	135	71	76	119	85	136	164
100 nM Antimycin A	74	153	90	94	95	135	69	79	130	116	139	256
500 mM SHAM	55	150	67	62	76	82	111	95	89	113	188	103
1 mM Propyl gallate	57	147	73	81	95	100	98	97	105	22	146	20
500 mM SHAM + 100 nM antimycin A	35	187	83	75	89	106	109	81	84	126	218	106

not interfering directly with the chloroplast function. At the range of concentrations used in the present study, oligomycin (inhibitor of oxidative phosphorylation) and antimycin A (inhibitor of cytochrome pathway) did not exert any effect on photochemical activities of chloroplasts (Figs. 8.2 - 8.5). On the contrary, propyl gallate inhibited markedly PS II activity on the oxygen evolving side and thus was similar to NH_2OH in such effect (Figs. 8.6 - 8.8). However at the low concentrations used in most of the experiments, SHAM did not show any effect on PS I or PS II activities. We, therefore suggest that SHAM is a better inhibitor to modulate AOX pathway of mitochondrial electron transport than propyl gallate. This is the first report in the literature describing the effect of propyl gallate on PS II activity of chloroplast.

An attractive means of possible regulation by mitochondria of chloroplast function is the mitochondrial oxidation of photosynthetically reduced pyridine nucleotides. The difference in redox potentials of the stroma (NADPH/NADP) and cytosol (NADH/NAD) is known to be quite large, particularly in light. Illuminated chloroplasts are expected to have excess NADPH or related metabolites since their electron transport activity generally exceeds the capacity of carbon fixation. The excess reducing equivalents are transported from the chloroplasts (in the form of triose-P or DHAP and malate) to the cytosol to generate NAD(P)H. The oxidation could also be indirect through the related shuttles.

Redox equivalents can be transferred from the chloroplast stroma to the cytosol by two different metabolic shuttles: the triose-P-PGA mediated by the phosphate translocator and the malate-OAA shuttle facilitated by the dicarboxylate translocator. The operation of a malate-OAA shuttle by mitochondria facilitates further the exchange of reducing equivalents between mitochondria and the cytosol or peroxisomes (Fig. 9.1). The triose-P-PGA shuttle

is controlled by Pi availability for counter-exchange by the phosphate translocator, chloroplastic PGA reduction and cytosolic triose-P oxidation. The malate-OAA shuttle is regulated by stromal NADP-MDH and the [NADPH]/[NADP], and also by the translocating step across the inner chloroplast envelope membrane.

We propose a model to show the biochemical basis of importance of mitochondrial oxidative electron transport in optimizing photosynthesis in mesophyll protoplasts. The biochemical basis of the mutually beneficial interaction between the process of photosynthesis and dark respiration would be the rapid exchange of metabolites between chloroplasts, cytosol and mitochondria (Fig. 9.1). The transport of PGA/DHAP and OAA/malate between chloroplasts and the cytosol is a well-known phenomenon (Heineke et al., 1991). Not only a rapid transport of ATP, ADP and Pi, but also metabolite shuttles of malate and OAA occur between mitochondria and the cytosol (Douce, 1985; Douce and Neuburger, 1990). Such metabolite transport systems can achieve a favourable balance of adenine and pyridine nucleotides in these compartments.

On the basis of the metabolite movements described above, the photosynthetic and respiratory activity in chloroplasts and mitochondria, respectively, appears to be modulated by one or both of the following factors: (a) the redox state due to the relative levels of NAD(P) or NAD(P)H, and (b) inter- organelle movement of metabolites such as PGA, DHAP, malate and OAA. Adenine nucleotides (ATP. ADP, AMP) and/or cytosolic pH also could regulate indirectly mitochondrial and/or chloroplastic reactions.

We conclude that a rapid and mutually beneficial interaction between chloroplasts, mitochondria, peroxisomes and cytosol occurs in plant cells. The 'coarse' (long-term) control of the interaction between photosynthesis and respiration appears to be through the levels of soluble sugars, while the 'fine'

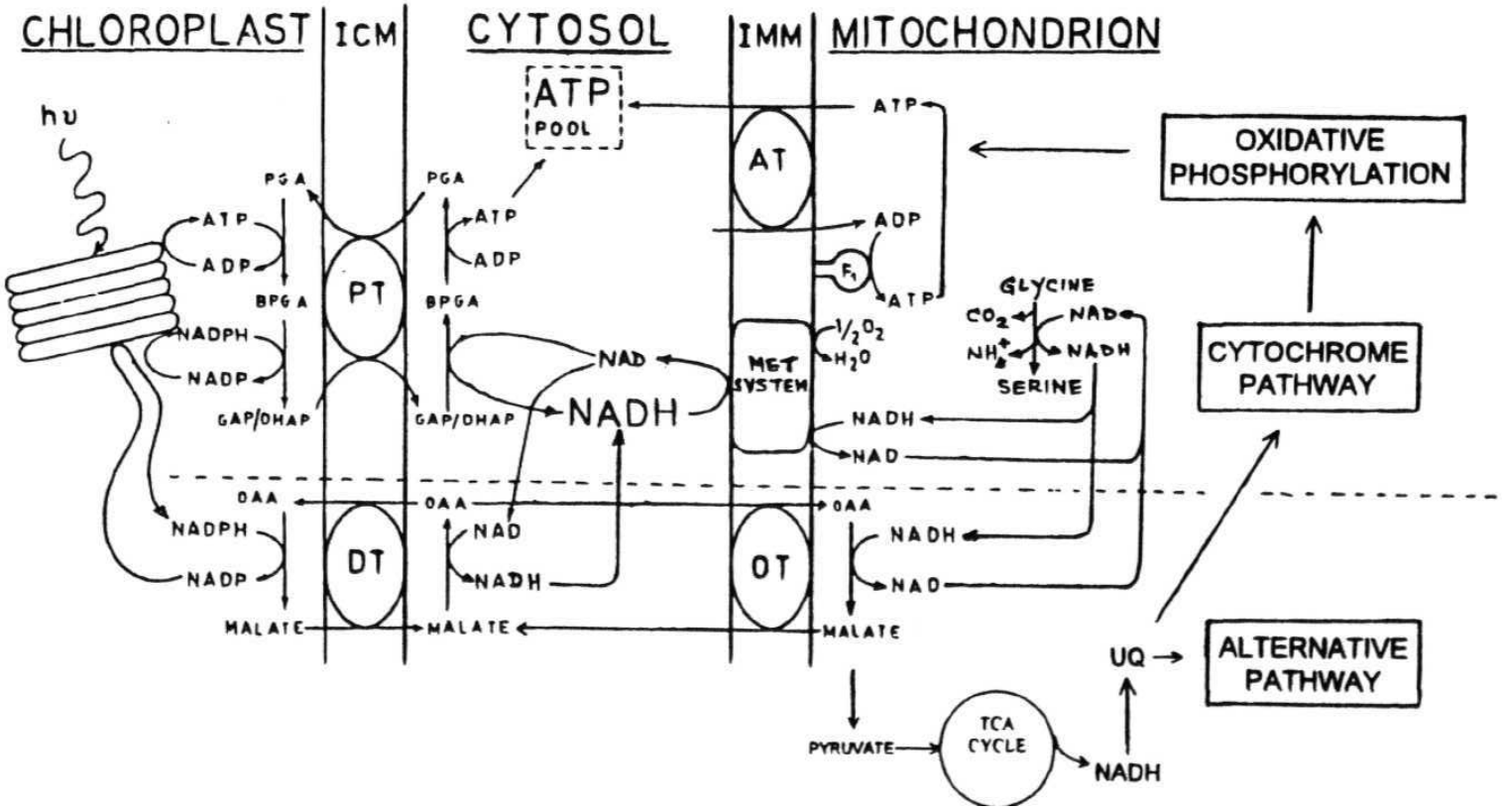

Figure. 9.1. Rapid movement of metabolites between chloroplasts and mitochondria via the cytosol forms the biochemical basis for the interaction between photosynthesis and respiration (See also Fig. 9.2). Phosphate translocator (PT) located on the inner chloroplast envelope membrane exports ATP and NADPH from chloroplasts to cytosol. Mitochondria utilize cytosolic NADH directly by the external dehydrogenase and components of the mitochondrial electron transport system (MET) and thereby synthesize ATP. The direct export of ATP by the mitochondrial adenylate translocator (AT) contributes to the intracellular ATP pool, along with the supply from the chloroplasts. Malate, exported from chloroplasts is metabolized in either cytosol or mitochondria. NADH can be oxidized by mitochondrial electron transport through either cytochrome or alternative pathway. GAP, glyceraldehyde-3-phosphate; BPGA, glycerate-1,3-bisphosphate (Adapted from Raghavendra et al., 1994).

(short-term) control is exerted by intracellular redox state or adenine/pyridine nucleotide level (Raghavendra et al., 1994).

There is a marked turnover of redox equivalents between chloroplasts, mitochondria, cytosol and peroxisomes, particularly under photorespiration/CO_2 limiting conditions (Fig. 9.2). Mitochondria and peroxisomes can prevent the over-reduction of the photosynthetic electron transport chain in illuminated chloroplasts by providing the outlet for excess reducing equivalents (Padmasree and Raghavendra, 1998). As a result, oxidative electron transport and phosphorylation in mitochondria and photorespiration in peroxisomes not only benefit photosynthesis but also protect isolated leaf protoplasts as well as PS II against photoinhibition/photoinactivation (Saradadevi and Raghavendra, 1992; Shyam et al., 1993). It should be of great interest to examine the consequence of such marked interaction between respiration, photosynthesis, photorespiration under other situations, such as CO_2 enrichment or sunflecks or shaded environment.

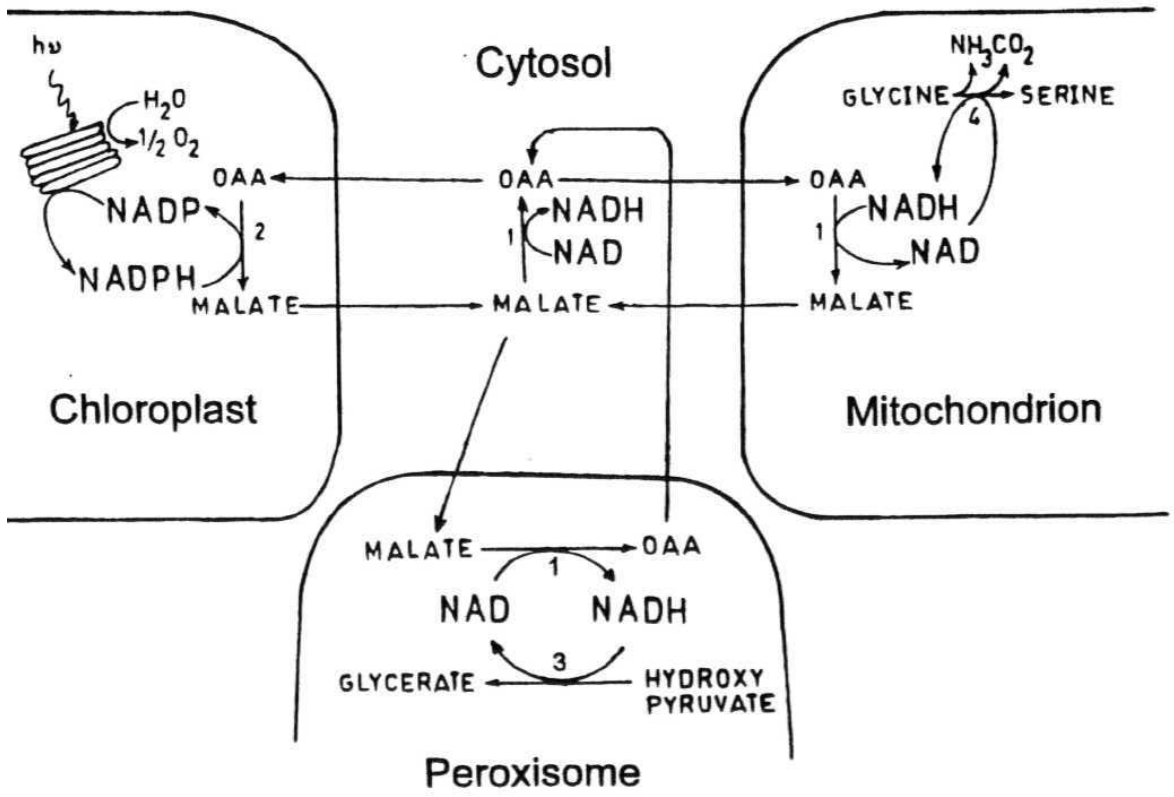

Figure. 9.2 Exchange of redox equivalents between the chloroplasts, mitochodria, peroxisomes and cytosol within a photosynthetic cell. Reduced equivalents generated from photosynthesis in chloroplasts or glycine oxidation in mitochondria are exported to peroxisomes and cytosol where they are used to reduce hydroxypyruvate. The numbers indicate the key enzymes. 1: NAD-MDH; 2: NADP-MDH; 3: hydroxypyruvate reductase; 4: GDC (Adapted from Padmasree and Raghavendra, 1998).

Chapter 10

Summary and Conclusions

Chapter 10

Summary and Conclusions

Photosynthesis and dark respiration are metabolic pathways that produce redox equivalents and ATP to meet the cell's energy demands for growth and maintenance. Although the interaction between these pathways has been debated earlier, it became evident in recent years that photosynthesis is dependent on the oxidative metabolism of mitochondria. The essential role of dark mitochondrial respiration in optimizing photosynthesis was indicated by studies with mutants of *Chlamydomonas* and tobacco (Lemaire et al., 1988; Hanson, 1992).

The essential role of mitochondrial oxidative metabolism in photosynthetic carbon assimilation is revealed by the effects of oligomycin (an inhibitor of oxidative phosphorylation in mitochondria) in leaves and protoplasts and during cycles of illumination and darkness (Vani et al., 1990; Raghavendra et al., 1994; Gardeström and Lernmark, 1995; Krömer, 1995; Padmasree and Raghavendra, 1998). The process of mitochondrial respiration is not only beneficial for photosynthesis in plant cells but also protects the illuminated cells against photoinhibition (Saradadevi and Raghavendra, 1992).

The biochemial basis of the strong interaction between chloroplasts and mitochondria, is not completely understood. Further, it is not known which of the pathways of mitochondrial electron transport influences the chloroplast metabolism in intact plant cell: the cytochrome or the alternative oxidase (AOX) pathway or both. Therefore, the present project attempts to address some of these unresolved questions on the interaction between photosynthesis and respiration using mesophyll protoplasts of pea, *Pisum sativum*.

The role of mitochondrial respiration in optimizing photosynthesis was assessed in mesophyll protoplasts of pea (*Pisum sativum)* by using low

concentrations of mitochondrial inhibitors: oliogmycin (inhibitor of oxidative phosphorylation); antimycin A (inhibitor of cytochrome pathway) and salicylhydroxamic acid (SHAM) and propyl gallate (inhibitors of alternative oxidase) (Lambers, 1990). During most of the experiments low concentrations of oligomycin (100 ng mL^{-1}), antimycin A (100 nM), SHAM (500 μM) or propyl gallate (1 mM) were used so as to minimize general perturbation of the metabolic system. Most of the studies on mitochondrial inhibitors indicate that AOX pathway is engaged only when the electron flow through cytochrome pathway is either saturated or inhibited (Møller et al., 1988). We have therefore studied the effect of SHAM, both in the absence or presence of 100 nM antimycin A.

The effect of mitochondrial inhibitors was ascertained on respiratory uptake and intracellular ATP levels in the dark. Oligomycin decreased markedly the ATP levels (by 65%) in protoplasts while inhibiting respiration marginally (<10%). On the other hand, antimycin A caused a marked decrease in both ATP levels (35%) and O_2 uptake (20%). In the concentration range of 0 to 100 uM SHAM, the respiratory O_2 uptake decreased only in the presence of antimycin A, while the decrease in ATP levels (8 to 16%) occurred both in the absence and presence of antimycin A. Contrary to the expectation that the AOX pathway is a non-phosphorylating process, the present results suggest that it may contribute to the cellular ATP to a limited, but significant extent.

The effect of these metabolic inhibitors on photosynthetic O_2 evolution was assessed at limiting (0.1 mM $NaHCO_3$) or optimal (1.0 mM $NaHCO_3$) CO_2. The rate of photosynthesis was more sensitive to oligomycin or antimycin A at optimal CO_2 than at limiting CO_2. There was a decrease of 25 to 35% in the rate of photosynthesis at optimal CO_2 while there was an inhibition of 15 to 25% at limiting CO_2. SHAM and propyl gallate also restricted photosynthetic activity, the suppression again being stronger at optimal CO_2 than that at limiting CO_2.

However, the inhibition due to SHAM in presence of antimycin A was almost similar at limiting or optimal CO_2.

The effects of these inhibitors **were evaluated at** a **wider range** of concentrations so as to ensure their direct effect on chloroplasts. **All** these compounds inhibited markedly the photosynthesis in protoplasts (up to nearly 75%), with very little effect on chloroplasts (< **8%).** On **the other hand, there was** a large depression in photosynthetic capacity compared to respiration. A general observation was that there was a large depression in photosynthetic capacity even when there was only a small decrease in the rate of respiration.

Since ATP is generated during both photosynthetic and respiratory processes, changes in adenine nucleotides could be one of the reasons for the biochemical interaction between chloroplast and mitochondria. The change in the levels of intracellular ATP and ADP during illumination and in presence of mitochondrial inhibitors at limiting CO_2 was in contrast to that of photosynthesis. The decrease in the ATP levels as well as ATP/ADP ratio by oligomycin or antimycin A was more pronounced at limiting CO_2 than at optimal CO_2. The small decrease in ATP/ADP in presence of SHAM and propyl gallate suggests that AOX pathway contributes partly to the cellular demands of ATP in a photosynthesizing cell. The Glc-6-P levels increased by 19-30% in the presence of both oligomycin and antimycin A at optimal CO_2 conditions. On the other hand, SHAM decreased markedly the Glc-6-P levels, both at optimal and limiting CO_2.

The quantitative relation between ATP/ADP ratio in protoplasts and their photosynthesis at optimal or limiting CO_2 was examined. Despite the expectation that ATP demands would be low at limiting CO_2, there was a steep positive correlation between the rates of photosynthesis and ratios of ATP to **ADP** in protoplasts in the presence of oligomycin or antimycin A. In contrast,

there was no correlation with the ratios of ATP to ADP, in spite of the marked decrease in the photosynthetic rate of protoplasts by SHAM. Nevertheless, a positive correlation could again be seen between ATP/ADP ratio and the rate of photosynthesis when antimycin A was present along with SHAM. The sensitivity of protoplast photosynthesis to mitochondria 1 inhibitors even at limiting CO_2 (when the ATP requirement for photosynthesis is low) as well as the lack of correlation between the rates of photosynthesis and the ratios of ATP/ADP in protoplasts incubated with SHAM suggest that under photorespiratory conditions mitochondrial electron transport is more crucial than oxidative phosphorylation. Similar suggestion was made by a few a workers (Gardeström, 1993; Kromer et al., 1993; Kromer, 1995; Padmasree and Raghavendra, 1998).

When leaves, protoplasts or chloroplasts are illuminated after a period of darkness, photosynthetic CO_2 fixation or CO_2 dependent O_2 evolution does not reach a maximum until after a few minutes and this initial lag is called as 'induction' (Walker, 1988). The mitochondrial inhibitors prolonged the lag period of photosynthesis at both optimal and limiting CO_2. The increase in lag period was higher in presence of oligomycin and antimcyin A compared to SHAM and propyl gallate. In presence of mitochondrial inhibitors, although the rate of photosynthetic O_2 evolution was decreased, the linearity was maintained at least up to 10 min of illumination.

The induction or lag is attributed to the delay in light activation of photosynthetic enzymes and building up of depleted Calvin cycle intermediates (Leegood and Walker, 1980, 1981). Most of the studies on photosynthetic activities under stress conditions like drought and low temperatures indicate that the decrease in photosynthetic capacity is due to two main reasons: (a) decrease in light activation of enzymes (b) limitation in RuBP regeneration (Hurry et al., 1995; Sanchez-Rodriguez et al., 1997). Therefore we examined the importance

of mitochondrial inhibitors on the light activation of photosynthetic enzymes and cellular metabolites.

First, we tested the effect of mitochondrial inhibitors on RuBP content, which is the initial acceptor of CO_2 as well as end product in the PCRC cycle and the enzyme PRK, which is involved in the regeneration of RuBP. The RuBP levels decreased significantly in presence of oligomycin or antimycin A. However, the decrease in RuBP levels was more pronounced at optimal CO_2 compared to limiting CO_2. In contrast there was an increase in RuBP levels in presence of SHAM (± antimycin A) and propyl gallate. Thus, both oligomycin and antimycin A appear to prolong photosynthetic induction, by decreasing RuBP levels.

Of the four light activated enzymes studied, NADP-GAPDH, FBPase and PRK are components of Calvin cycle, while NADP-MDH helps in generating malate which acts as a sink to the photosynthetically generated reductants from the electron transport chain. When protoplasts were incubated with mitochondrial inhibitors. the activity of photosynthetic enzymes picked up slowly compared to control samples (without inhibitors) at either optimal or limiting CO_2. As a result, the activity in presence of mitochondrial inhibitors was much less than that in control soon after the light was switched on (i.e., during induction phase), but recovered during subsequent illumination (i.e., at the end of 10 min, activated phase). The extent of activation of all the enzymes was more at limiting CO_2 than that at optimal CO_2. CCCP and DCMU, whose effects on photosynthetic enzyme activation are known in the literature (Nakamoto and Edwards, 1986) have been used as a check on the present experimental system. The restriction of light activation of enzymes by DCMU, but not CCCP was expected and was logical. However, CCCP made the activation of photosynthetic enzymes much slower than that in control.

The activation of PRK was affected markedly in presence of AOX pathway inhibitor SHAM (<25%), while the effect of oligomycin or antimycin A was marginal (≤13%). There was a significant decrease (nearly 35%) in the extent of GAPDH activation in presence of AOX pathway inhibitors compared to limited effect (10-20%) of oligomycin or antimycin A. The inhibitory effect of SHAM on FBPase activation was remarkable (40%), while the inhibition of light activation was less than 10% in presence of oligomycin and antimycin A at optimal or limiting CO_2.

On the contrary to these three enzymes, there was a significant increase in NADP-MDH activation (35-50%) in presence of oligomycin and antimycin A. In presence of SHAM, there was 18 to 27% decrease in NADP-MDH activation at optimal and limiting CO_2. Our results, suggest that AOX pathway plays a more important role than cytochrome pathway in regulating the light activation of enzymes during steady state photosynthesis. The exact molecular mechansim of such modulation is not clear.

Enzyme activation is reduced under stress conditions like drought or low temperature (Sanchez-Rodriguez et al., 1997, Holaday et al., 1992). Interference with mitochondrial respiration may also create stress on the micro-environment within the cell, for e.g. redox state (Leegood and Walker, 1980, 1981; Scheibe, 1991). Therefore, we also analysed the effect of mitochondrial inhibitors on the following metabolites: i) PGA, triose-P, malate and OAA, which are involved in maintaining the phosphorylation potential and redox status of the cell; ii) Glc-6-P and FBP, which are involved in sucrose formation. Triose-P plays a dual role in maintaining redox status of the cell as well as a precursor in the sucrose biosynthesis.

The ratio between triose-P and PGA indicates the relative enrichment of ATP and reducing power in protoplasts. On illumination at optimal CO_2, there

was a two to four fold increase in triose-P levels and decrease in PGA levels in presence of oligomycin and antimycin A. On the other hand, the PGA levels increased in presence of SHAM and propyl gallate, but decreased in presence of SHAM with antimycin A. An analysis of the triose-P/PGA ratio indicates that the ratio increased significantly in presence of oligomycin or antimycin A, while the increase was marginal in presence of SHAM (± antimycin A). However, the ratio decreased in presence of propyl gallate.

The levels of malate were several fold higher than that of OAA. There was a significant increase in malate levels in presence of SHAM and propyl gallate, unlike the situation in presence of oligomycin or antimycin A, at both optimal and limiting CO_2. Analaysis of malate/OAA ratios indicate that there was not much change in presence of oligomycin or antimycin A, while there was a marked increase in malate/OAA ratios in presence of SHAM (± antimycin A) at optimal CO_2.

In a green photosynthesizing cell, the redox processes occuring in the chloroplast stroma and the cytosol are interlinked by the transfer of redox equivalents from the chloroplast. The transfer of redox equivalents from chloroplasts to the cytosol occurs by two different metabolic shuttles, *viz* the triose-P/PGA shuttle, catalysed by the Pi-translocator and the malate-OAA shuttle, facilitated by specific transport of malate and OAA (Heineke et al., 1991). As both metabolite shuttles would have the capacity to modulate the redox state of the stromal and cytosolic compartment, a regulation of these processes is required to maintain the specific redox states of the two metabolic compartments. The present results suggest that cytochrome pathway plays a prominent role in regulating the triose-P/PGA ratio, while AOX pathways play a primary role in maintaining the malate/OAA.

A common limitation of inhibitors is their unspecified and possible interference with other metabolic systems (Diethelm et al., 1990). It is important to ascertain that the inhibitors used in the present study have no direct effect. Therefore, we analysed the effects of different mitochondrial inhibitors on photochemical activities (PS I, PS II and whole chain) of protoplasts and chloroplasts.

The effects of oligomycin or antimycin A on different photochemical activities were negligible. On the contrary, the photochemical activities of PS II and whole chain were affected in presence of propyl gallate. Therefore, we compared the effects of SHAM and propyl gallate on various photochemical activities with NH_2OH (an inhibitor of O_2 evolving system) and DCMU (an inhibitor of PS I) (Hall and Rao, 1994). The pattern of inhibition of only reactions involving OEC suggested that the effect of propyl gallate was similar to the effect of NH_2OH in interfering with OEC of PS II in chloroplasts.

There has been a constant debate on the use of inhibitors while modulating the AOX pathway, since there was always a doubt that inhibitors like SHAM or propyl gallate could be unspecific (Diethelm et al., 1990). However at the low concentrations used in the present study, the effect of SHAM on OEC was marginal compared to the marked inhibition by propyl gallate which was nearly 75% at 1 mM concentration. Therefore, we suggest that SHAM is more suitable than propyl gallate to study the AOX pathway of mitochondrial electron transport. The inhibition of PS II activity by propyl gallate is observed for the first time, in the literature.

Major conclusions from the present study are:

1. Mitochondrial metabolism is essential for optimal photosynthesis at both limiting as well as optimal CO_2.
2. Inhibitors of both cytochrome pathway (antimycin A) and AOX pathway (SHAM) or inhibitor of oxidative phosphorylation (oligomycin) suppressed evolution markedly the rate of photosynthetic O_2 in intact protoplasts, indicating the significant role of AOX pathway as well as cytochrome path in optimizing photosynthesis. These compounds had no effect on chloroplast photosynthesis.
3. Both cytochrome and AOX pathways appear to contribute to the intracellular ATP at both optimal and limiting CO_2. However, the contribution of AOX pathway to cellular ATP is not likely to exceed one-third of that by cytochrome path.
4. Oxidative phosphorylation plays a significant role in modulating photosynthetic induction, as indicated by the increase in lag period, by oligomycin or antimycin A.
5. Inhibition of AOX pathway by SHAM not only resulted in a restriction of light activation of photosynthetic enzymes but also in the marked increase in malate/OAA ratio suggesting a rise in cytosolic redox status particularly at optimal CO_2.
6. Inhibition of cytochrome pathway by antimycin A resulted in a marked decrease in sucrose formation, particularly at optimal CO_2, as indicated by the increase in triose-P/PGA ratio.
7. The co-ordination between mitochondrial respiration and chloroplast photosynthesis is mediated by both intracellular redox state and adenine nucleotides.

8. SHAM did not affect chloroplast photochemical activities. Propyl gallate appeared to interfere with PS II activity particularly at O_2 evolving system. Thus, propyl gallate appears to be similar to **hydroxylamine**.

9. We suggest SHAM as the most suitable inhibitor (compared to propyl gallate) to study the importance of AOX pathway of mitochondrial electron transport.

10. A model is proposed to illustrate the biochemical basis of the interaction between mitochondrial respiration and chloroplast photosynthesis.

Chapter 11

Literature Cited

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Literature Cited

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Appendix

List of Publications in Refereed Science Journals/Books:

1. K. Saradadevi, K. **Padmasree** and A.S. Raghavendra (1992) Interaction between respiration, photosynthesis and photoinhibition in mesophyll protoplasts of pea (*Pisum sativum*). *Research in Photosynthesis, Vol.* 4. (Ed. N. Murata), Kluwer Academic Publishers, Dordrecht. pp. 725-728.

2. A.S. Raghavendra, K. Saradadevi and **K. Padmasree** (1992) Short-term interaction between photosynthesis and respiration in leaves and protoplasts. *Malaysian Soc. Plant Physiol. Transac. (Special Edition)* Vol.3: 12-18.

3. A.M. Rao, **K.** Padmasree, P.B. Kavi Kishor and G.M. Reddy (1992) Varietal differences on plant regeneration in grain and sweet sorghum. *Plant Tissue Cult.* 2: 109-113.

4. K. Saradadevi, K. Padmasree and A.S. Raghavendra (1993) Photoinhibition of photosynthesis in mesophyll protoplasts of pea (*Pisum sativum):* Protection by dark respiration and aggravation by osmotic stress and chilling. *DAE Symposium on Photosynthesis and Plant Molecular Biology.* Department of Atomic Energy, Bombay. pp. 130-135.

5. A.S. Raghavendra, K. Padmasree and K. Saradadevi (1994) Inter-dependence of photosynthesis and respiration in plant cells: Interactions between chloroplasts and mitochondria. *Plant Sci.* 97: **1-14.**

6. K. Padmasree and A.S. Raghavendra (1995) Essentiality of both cytochrome and alternative pathways of respiration for photosynthesis in mesophyll protoplasts of pea *(Pisum sativum* L.). *Photosynthesis: From Light to Biosphere, Vol.* 2. (Ed. P. Mathis), Kluwer Academic Publishers, Netherlands. pp. 899-902.

7. A.M. Rao, K. Padmasree and P.B. Kavi Kishor (1995) Enhanced plant regeneration in grain and sweet sorghum by asparagine, proline and cefotaxime. *Plant Cell Rep A5:* 72-75

8. K. Saradadevi, **K.** Padmasree and A.S. Raghavendra (1996) Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea *(Pisum sativum). Physiol. Plant.* **96:** 395-400

Continued.

9. K. Padmasree and **A.S. Raghavendra (1998)** Interaction with respiration and nitrogen metabolism. In ***Photosynthesis: A Comprehensive Treatise,*** (ed. A.S. Raghavendra), Cambridge University Press, pp 197-211.

10.K. Padmasree and A.S. Raghavendra (1997) Mitochondrial oxidative metabolism is essential for optimizing chloroplast photosynthesis in intact cells. ***Proceeding of Symposium on Plant Physiology for National Development.*** Calcutta (in press)

11. K. Padmasree and A.S. Raghavendra (1998) Photorespiration and interaction between chloroplasts, mitochondria and peroxisomes. In ***Probing Photosynthesis: Mechanism, Regulation and Adaptation***, (eds. M. Yunus, U. Pathre, P. Mohanty), Taylor and Francis publishers. (In press).

First pages of articles at serial No. 1 - 9 are attached.

INTERACTION BETWEEN RESPIRATION, PHOTOSYNTHESIS AND PHOTOINHIBITION IN MESOPHYLL PROTOPLASTS OF PEA (*Pisum sativum*)

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1. INTRODUCTION

The interaction between photosynthesis and respiration within the leaf tissue during illumination is debated (1,2). However recent reports demonstrate a strong interaction between these two important metabolic processes. Oxidative phosphorylation is essential to sustain photosynthetic metabolism in leaves as well as in protoplasts (3,4). The respiratory rate of leaf discs is significantly stimulated after even a short period of photosynthesis, a phenomenon named as light enhanced dark respiration (5).

We have been using mesophyll protoplasts of pea to re-evaluate the interaction between photosynthesis and respiration. The system of protoplasts has certain advantages like avoiding the problem of recycling of assimilated/released CO_2 and allowing an evaluation of externally added metabolites/inhibitors. The present article summarises our observations (6-8), which demonstrate very strong and beneficial interaction between dark respiration and photosynthesis in mesophyll protoplasts. Oxidative electron transport and phosphorylation of mitochondria appear to play a much more important role than the reactions of glycolysis or TCA cycle in benefitting photosynthesis and protection against photoinhibition.

2. MATERIALS AND METHODS

Pea (*Pisum sativum* L. cv. Arkel) plants were grown outdoor (natural photoperiod of approximately 12 h, average daily temperatures of 30 °C day/20 °C night). The mesophyll protoplasts were isolated from the first and second fully expanded leaves of 8-10 day old plants as already described (9). Photosynthetic and respiratory activities of protoplasts were determined by monitoring their oxygen evolution and uptake, respectively (6,7).

The details of photoinhibitory treatment of protoplasts are described in detail elsewhere (8). The interaction between photosynthesis, respiration and photoinhibition was investigated using a range of classic metabolic inhibitors.

3. RESULTS AND DISCUSSION

A strong and rapid interaction between photosynthesis

N. Murata (ed.), Research in Photosynthesis, Vol. III, 725–728.

Transactions of Malaysian Soc. Plant Physiol. 3(1992) : 12-18

SHORT-TERM INTERACTION BETWEEN PHOTOSYNTHESIS AND MITOCHONDRIAL RESPIRATION IN LEAVES AND PROTOPLASTS[1]

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ABSTRACT

Photosynthesis and respiration are among the most important metabolic processes in plants. Respiratory rate increases after hours of illumination due to carbohydrate accumulation. Besides such long-term effect, a strong interdependence as well as a beneficial interaction exists between photosynthesis and respiration during very short periods of within a few minutes. When protoplasts are illuminated for 10-15 min, the rate of respiration immediately after darkening increases nearly three-fold. Such light enhanced dark respiration (LEDR) occurs not only in protoplasts but also in leaves. The requirement of bicarbonate by LEDR and its sensitivity to glyceraldehyde or DCMU demonstrate the important role of photosynthesis in respiration. The extreme sensitivity of photosynthesis in leaves or protoplasts to classic inhibitors of oxidative metabolism like oligomycin, sodium azide or antimycin A demonstrates the essential role of respiration in optimizing photosynthesis. Respiration not only benefits photosynthesis but also protects protoplasts against photoinhibition. Oxidative electron transport and phosphorylation play a much more important role than the reactions of glycolysis or TCA cycle in such beneficial interaction. Mitochondrial metabolism may help to prevent over-reduction of not only cytosol but also chloroplasts. The metabolite shuttles of PGA/DHAP and OAA/malate across the chloroplast membranes could form the biochemical basis for the interaction of photosynthesis and respiration.

Importance of photosynthesis and respiration

The processes of photosynthesis and respiration are among the most important metabolic processes in the plant. Photosynthesis, a process of primary carbon assimilation, leads to a net gain of biomass while respiration provides energy and helps in turnover of carbon skeletons derived from organic, amino- and fatty acids.

High photosynthetic efficiency is often essential for maximal plant growth and plant productivity (Pearson 1984; Patrick 1988). Similarly high rates of respiration are characteristic of rapidly growing tissues (Amthor 1989). Both photosynthesis and respiration therefore form an integral component of plant growth. In most of the models, two components of respiration namely growth respiration and maintenance respiration are taken into account, while describing growth. Although the topic is debated, a recent survey (Ceulemans and Saugier 1991) provided a nice demonstration of the strikingly positive correlation between photosynthetic and respiratory rates of several tree species (Fig. 1). Such positive correlation indicates a close co-operativity suggesting a strong interaction between photosynthesis and respiration in a wide range of plants.

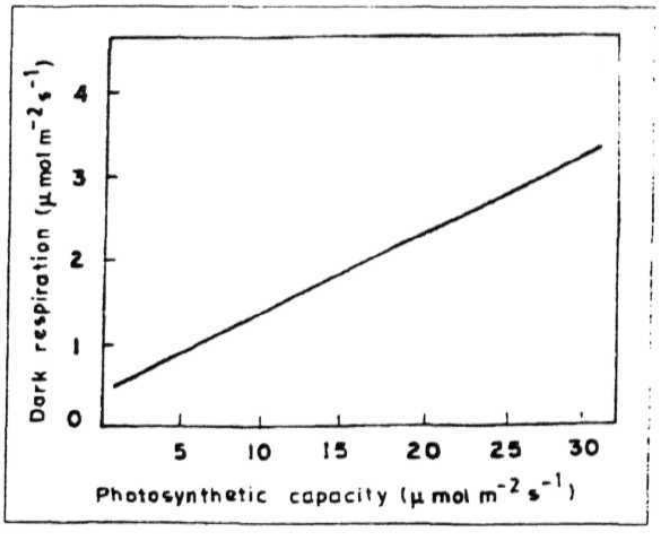

Fig. 1. Correlation between photosynthetic and dark respiratory activities. The curve is drawn from a compilation of rates from 33 tree species. The linear regression was significant at the 1% level. Modified from Ceulemans and Saugier (1991).

Long and short-term interaction

The respiratory rate of leaves increases markedly after long hours of illumination, primarily due to the accumulation of carbohydrates (e.g. Azcon-Bieto and Osmond 1983; Shishido *et al.*, 1990). Such an increase is often proportional to the hours of illumination during the preceding light period (Fig. 2). However, the high rate of respiration declines slowly to reach a steady state characteristic of dark level. Such an effect of photosynthetic activity on respiration is classified under long-term, since the interaction becomes pronounced only after hour(s) of illumination.

If one defines respiration as a process of CO_2 release or consumption, quite a few such phenomena operate in

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Varietal Differences on Plant Regeneration in Grain and Sweet Sorghum

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Key words : **Plant regeneration, Grain and Sweet sorghum**

Abstract

Kinetin and asparginc suppressed the formation of callus on MS medium from the seeds of both grain and sweet sorghum. Plant regeneration was high in grain sorghum and it ranged from 20 to 87% depending on the genotype. Lower concentrations of Kn and BAP failed to give any organogenic response, but higher concentrations of Kn and BAP were found to be good for regeneration. However, all the eight varieties tested lost their ability to regenerate within 60 to 135 days. Immature seeds gave more organogenic callus when compared to mature seeds. While mature-seed derived callus failed to differentiate, immature-seed derived callus produced plantlets with a frequency of 20-70%.

Introduction

Cell and tissue culture techniques offer a great potential for the selection of mutants and also to study genetic and physiological aspects of ontogeny. Somaclonal variants generated through callus cultures may furnish useful material for breeding programmes. Callus initiation and plant regeneration have been achieved in grain (Smith et al. 1983) as well as sweet sorghums (Mac Kinnon et al. 1986). However, this technology is limited by the inconsistent production of embryogenic tissue and the loss of the regenerating ability after a few passages in culture. The present paper deals with the identification of genotypes (both grain and sweet) capable of forming callus and regeneration from long-term cultures.

Materials and Methods

The following ten sorghum varieties were used in the present investigation, namely, IS 108, IS 305, IS 1054, IS 18417, IS 18758 (grain), IS 9901, IS 12292, IS 19273, Rio and Keller (sweet). Mature and immature seeds (12 to 14 days after fertilization) of the above varieties were surface-sterilized with a 0.1% $HgCl_2$

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PHOTOINHIBITION OF PHOTOSYNTHESIS IN MESOPHYLL PROTOPLASTS OF PEA (*Pisum sativum*): PROTECTION BY DARK RESPIRATION AND AGGRAVATION BY OSMOTIC STRESS AND CHILLING

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When mesophyll protoplasts of pea (*Pisum sativum*) were exposed to photoinhibitory light (2500 $\mu E\ m^{-2}\ s^{-1}$), their rates of photosynthesis as well as dark respiration were markedly reduced. Illumination with normal light intensity (1250 $\mu E\ m^{-2}\ s^{-1}$) enhanced the rate of dark respiration in protoplasts. The extent of photoinhibition was increased when protoplasts were incubated with even low levels of 1 μM antimycin A, 1 μM sodium azide or 1 $\mu g\ ml^{-1}$ oligomycin. Mitochondrial metabolism appears to protect the plant cell against photoinhibition by preventing the over-reduction of electron transport chain in chloroplasts. The extent of photoinhibition was aggravated on incubation in 1.0 M sorbitol and further when protoplasts were prechilled at 0 °C. The effects of chilling were reversible on exposure to 25 °C. Protoplasts offer an useful system for studying not only photosynthesis and respiration but also their interaction with light, water or temperature stress.

Photoinhibition is the phenomenon of severe reduction in photosynthetic efficiency under supra-optimal light intensity, particularly in the absence of CO_2 and 02 (1-3). Environmental stresses such as drought, high temperature and chilling/freezing enhance the extent of photoinhibition (4).

Photoinhibition was earlier demonstrated in leaves, algal cells, chloroplasts and thylakoid membranes (e.g. 2-7). We have recently reported the phenomenon of photoinhibition in isolated mesophyll protoplasts (8). Each experimental system has its own advantages and disadvantages. Leaves have a non-uniform light profile between adaxial and abaxial surfaces and exhibit variation in partial pressures of CO2/O2 levels within intercellular spaces. The interaction between different organelles can not be studied with chloroplasts. Protoplasts

For all correspondence.

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PLANT SCIENCE

Interdependence of photosynthesis and respiration in plant cells: interactions between chloroplasts and mitochondria

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Abstract

Photosynthesis and respiration in an illuminated plant cell are not only interdependent but also mutually beneficial. Respiratory rates increase after hours of illumination due to carbohydrate (substrate) accumulation. Besides such long-term effects, photosynthesis and respiration interact even during short illumination periods of a feu minutes. The rate of respiration in isolated leaf protoplasts increases severalfold after 10–15 min of illumination Such light-enhanced dark respiration (LEDR) has been demonstrated in protoplasts as well as in leaves The stimulation of LEDR by bicarbonate and its sensitivity to inhibitors of photosynthesis (DCMU) or the Calvin cycle (D,L-glyceraldehyde) point out the importance of photosynthetic carbon metabolism for respiration. From metabolite analyses of protoplasts, the majority of LEDR is due to mitochondrial oxidation of malate produced by chloroplasts. Simultaneous measurements of photosynthesis and respiration, using mass spectrometry, demonstrate that mitochondrial TCA cycle-based CO_2 evolution is inhibited by illumination while O_2 uptake is either unaffected or stimulated. The marked sensitivity of photosynthesis in leaves or protoplasts to classic mitochondrial inhibitors such as oligomycin, sodium azide or antimycin A implies that mitochondrial metabolism is essential for photosynthesis. Respiration not only benefits photosynthesis but also protects illuminated leaf protoplasts against photoinhibition. Oxidative electron transport and phosphorylation play a much more important role than the reactions of glycolysis and the TCA cycle in this beneficial interaction. The metabolite shuttles involving PGA-DHAP and/or OAA-malate across the chloroplast and mitochondrial membranes could form the biochemical basis of the interaction between photosynthesis and respiration. Alternatively, cytosolic NAD(P)H, derived from photosynthetic products, can be directly acted upon by the mitochondrial external NAD(P)H dehydrogenase and oxidised through the mitochondria) electron transport system. Mitochondrial oxidation of NAD(P)H (even if indirect) helps to prevent the over-reduction of the cytosol and. consequently, the chloroplast in illuminated leaf cells. Besides the direct interaction with chloroplasts, mitochondria can supply reducing equivalents through malate to peroxisomes during photorespiration and provide citrate as the precursor of oxoglutarate, necessary for glutamine and glutamate formation. These two phenomena further complement the strong interdependence of photosynthesis and mitochondria] metabolism.

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Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethyl urea: DHAP. dihydroxyacetone phosphate: LEDR. light-enhanced dark respiration: OAA, oxalacetate; PDH, pyruvate dehydrogenase: PGA. 3-phosphoglyceric acid: Rubisco. ribulose 1.5-bisphosphate carboxylase/oxygenase; TCA, tricarboxylic acid.

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ESSENTIALITY OF BOTH CYTOCHROME AND ALTERNATIVE PATHWAYS OF RESPIRATION FOR PHOTOSYNTHESIS IN MESOPHYLL PROTOPLASTS OF PEA (*PISUM SATIVUM* L.)

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1. Introduction

The mutually beneficial effects of photosynthesis and respiration in plant cells are believed to be due to marked interaction between chloroplasts and mitochondria, mediated through intracellular redox state and/or adenine/pyridine nucleotide levels (1,2). Mitochondrial oxidative metabolism is essential for photosynthetic carbon assimilation as revealed by the effects of oligomycin in leaves and protoplasts of barley (3-5), and studies with mutants of *Chlamydomonas* (6) and tobacco (7). Mitochondrial respiration not only benefits photosynthesis but also protects against photoinhibition, as demonstrated with mesophyll protoplasts of pea (8) and the cyanobacterial system of *Anacystis nidulans* (9).

Oxidative electron transport in plants occurs through both cytochrome pathway and an alternative cyanide resistant pathway (10). In the present work, the relative importance of these two pathways of oxidative electron transport (cytochrome and alternative pathways) in sustaining high rates of photosynthetic activity was examined by the use of antimycin A (inhibitor of cytochrome pathway), salicylhydroxamic acid (inhibitor of alternative pathway). The results were compared with the effects of oligomycin, which suppresses oxidative phosphorylation but has no direct effect on photosynthesis (3). Our results suggest that oxidative electron transport, through both cytochrome and alternative pathways, is important during the beneficial role of mitochondrial respiration in optimizing photosynthesis.

2. Materials and Methods

Pea (*Pisum sativum* L. cv. Arkel) plants were grown outdoor (natural photoperiod of approximately 12 h, average daily temperatures of 30 °C day/20 °C night). The

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Enhanced plant regeneration in grain and sweet sorghum by asparagine, proline and cefotaxime

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Summary. Cefotaxime (50 and 100 mg/l), a cephalosporin antibiotic and the amino acids asparagine and proline (200 mg/l) enhanced the production of embryogenic callus, increased the frequency of plant regeneration, and delayed the loss of regeneration potential in immature embryo-derived callus cultures of Sorghum bicolor (L.) Moench. Although these compounds did not promote callus induction or growth of callus, they influenced plant regeneration considerably in 10 low responding genotypes of grain and high anthocyanin containing sweet sorghums.

Key words: Sorghum bicolor (L.) Moench, somatic embryogenesis, plant regeneration, cefotaxime, proline, asparagine.

Introduction

The development of tissue culture protocols for embryogenic callus induction and plant regeneration is imperative for successful application of tissue culture technology for crop improvement. Sorghum is an important cereal grown in semi-arid and other regions for food and animal feed. Sweet sorghums are also used for biogas and alcohol production because of the accumulation of sucrose in the stems.

Callus initiation and whole plant rgeneration from mature and immature embryo-derived callus cultures of grain and sweet sorghums have been reported (Ma et al. 1987; Mac Kinnon et al. 1986,1987; Rao and Kavi Kishor 1989). Unfortunately, regeneration from embryogenic cultures of sorghum is often restricted to few varieties (Smith and Bhaskaran 1986) and decreases with increasing number of subcultures (Rao et al. 1992). Inclusion of certain amino acids (tryptophan, serine, proline) in the culture medium has been shown to increase the number of somatic embryos in rice (Oryza sativa) (Siriwardana and Nabors 1983) and orchardgrass (Dactylis glomerata) (Trigiano and Conger 1987).

The use of Agrobacterium in tissue culture for transformation studies has led to the inclusion of the antibiotics carbenicillin and cefotaxime in the culture medium to control growth of the bacterium (Velten et al. 1984.). Cefotaxime stimulated callus growth, embryogenesis and regeneration in wheat (Triticum aestivum) (Mathias and Boyd 1986) and barley (Hordeum vulgare) (Mthias and Mukasa 1987). In the present sudy, we investigated the effect of glutamine, asparagine, proline and cefotaxime on plant regeneration from immature embryo-derived callus cultures of grain and sweet sorghum genotypes.

Materials and Methods

Immature caryopses from Sorghum bicolor (L.) Moench genotypes IS 108, IS 305, IS 1054, IS 18417, IS 18758 (grain sorghum). Rio, Keller, IS 9901, IS 12292 and IS 19273 (sweet sorghum) were collected 9 to 15 days after pollination, surface sterilized with 0.1% (w/v) mercuric chloride for 6 to 7 min, and then washed thoroughly with sterile distilled water. Genotypes IS 1054 and IS 18417 are slightly tan in colour while rest of the genotypes are brown in colour. Immature embryos were inoculated onto Murashige and Skoog (MS) medium (1962) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2% sucrose and either 50 or 100 mg/l cefotaxime, or 200 mg/l glutamine, asparagine or proline for callus induction. Also the effects of a combination of cefotaxime plus asparagine and cefotaxime plus proline on callus induction was evaluated.

For regeneration of plants, the MS medium was supplemented with 1 and 2 mg/l kinetin (KN), 6-benzylaminopurine (BAP), 6-γ-γ-dimethylallylamino) purine (2iP) or a combination of 3 mg/l BAP plus 0.1 mg/l α-naphthaleneacetic acid (NAA) both with and without different amino acids and cefotaxime (Table 2).

Fifteen ml of nutrient medium was dispensed into each 2.5 x15 cm test tubes and were plugged with cotton. The pH of the medium was adjusted to 5.7 prior to autoclav-

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Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea (*Pisum sativum*)

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Saradadevi, K., Padmasree, K. and Raghavendra, A. S. 1996. Correlation between the inhibition of photosynthesis and the decrease in area of detached leaf discs or volume/absorbance of protoplasts under osmotic stress in pea (*Pisum sativum*). – Physiol. Plant. 96: 395–400.

Exposure to osmotic stress reduces leaf area and protoplast volume while decreasing photosynthesis. But the measurement of protoplast volume is tedious, while rapid determinations of leaf area in the field are difficult. We evaluated the quantitative relationship between the extent of decrease in area of detached leaf discs or the volume of protoplast of pea (*Pisum sativum*) and reduction in their photosynthetic capacity under osmotic stress. Osmotic stress was induced by increasing sorbitol concentration in the surrounding medium of the leaf discs from zero to 1.0 *M* (–3.1 MPa), and in case of protoplasts from 0.4 *M* (–1.3 MPa, isotonicity) to 1.0 *M* (–3.1 MPa, hypertonicity). There was a high degree of positive correlation between the extent of reduction in the area of detached leaf discs or the volume of protoplasts (indicated by diameter or absorption at 440 nm) and the decrease in photosynthesis. The correlation coefficients between inhibition of photosynthesis and the decrease in leaf disc area or protoplast volume were 0.96 and 0.99, respectively. We therefore suggest that the decrease in absorbance at 440 nm (corrected for turbidity at 750 nm) can be used as a simple measure to predict the inhibition due to osmotic stress of photosynthesis in mesophyll protoplasts. Similarly, the reduction in area of detached leaf discs could also be a very simple and useful criterion to assess osmotic tolerance of photosynthesis.

Key words – Drought tolerance, leaf disc area, osmotic stress, pea, photosynthesis, *Pisum sativum*, protoplasts, protoplast volume.

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Introduction

The inhibition of photosynthesis during water stress is due to both nonstomatal (direct) and stomatal (indirect) factors (Kaiser 1987, Graan and Boyer 1990). Direct inhibition of photosynthetic carbon fixation by water stress is confirmed by the use of experimental systems that have no limitation imposed by stomatal conductivity, e.g., thin leaf slices, cells, isolated protoplasts and isolated chloroplasts (Jones 1973, Plaut and Bravdo 1973, Kaiser et al. 1981a,b, Sharkey and Badger 1982, Saradadevi 3nd Raghavendra 1994). The sensitivity of photosynthesis to water stress was also demonstrated in leaves, at very high partial pressures of CO; (around the leaf to avoid stomatal limitation), employing the techniques of monitoring either gas-exchange (Graan and Boyer 1990) or chlorophyll fluorescence (Ogren 1990).

Osmotic adjustment is an important step during the acclimation of a plant cell to low Ψ_w (Berkowitz and Kroll 1988, Sen Gupta and Berkowitz 1988, Evans et al. 1992). Water stress leads to marked reduction in leaf expansion and leaf area (Burke et al. 198S). A reduction in size of the protoplast/chloroplast has frequently been observed along with the inhibition of photosynthesis in plant tissues during osmotic stress (Acevedo et al. 1979, Kaiser 1982, Santakumari and Berkowitz 1989, 1990).

We have been studying the effects of light- and osmotic stress on photosynthesis in protoplasts of pea,

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15 Interaction with respiration and nitrogen metabolism

K. PADMASREE AND A. S. RAGHAVENDRA

INTRODUCTION

Photosynthesis is traditionally considered to be an autonomous process, since carbon tan be assimilated even by a system of isolated chloroplasts. However, in recent years, it has become evident that photosynthetic carbon assimilation is strongl!) dependent on mitochondrial activity and that carbon partitioning is regulated significantly by nitrogen metabolism. High photosynthetic efficiency helps to achieve maximal plant growth and plant productivity. Similarly, high rates of respiration are characteristic of rapidly growing tissues. Both photosynthesis and respiration therefore, form essential components of plant growth (Amthor, 1989, 1994).

Photosynthesis results in O_2 evolution and the generation of ATP and NADPH, which are then used for the reduction of CO_2 (or other compounds like NO_3^- or SO_4^{2-}). On the other hand, respiration accomplishes oxidation of carbon compounds and evolution of CO_2. NADH produced in these reactions is utilized for ATP production and oxygen consumption. Thus, ATP is generated in both processes, while pyridine nucleotides art reduced during photosynthesis, but arc oxidized in respiration. The biochemical nature of photosynthetic and respiratory reactions implies that these two processes arc complementary to each other.

The requirements of ATP and reducing power (NADH or NADPH) for the cells arc met not only by photosynthetic reactions in chloroplasts but also by oxidative metabolism of mitochondria. Besides mitochondria and cytosol, the reduced equivalents are used up during photorespiration as well as nitrogen metabolism As a result, in a plant cell, the processes of photosynthesis, respiration, nitrogen metabolism and photorespiration become dependent on each other, underscoring the concept of organelle autonomy (Turpin & Weger, 1990, Azcón-Bieto, 1992; Raghavendra, Padmasree & Saradadevi, 1994, Gardestrom & Lernmark, 1995; Krumer, 1995)

LONG- AND SHORT-TtRM INTERACTIONS

The rate of dark respiration in leaves is higher after a few hours of illumination than that during the stead) -state in prolonged darkness Such an increase in dark respiration is often proportional to the period of illumination and depends on temperature. The high rates of respiration decline slowly to reach the lower level of a steady state, characteristic of the dark period. The increase in respiratory rate is believed to be primarily due to the accumulation of carbohydrates This effect of photosynthetic activity on respiration should be treated as a long-term effect since the interaction becomes pronounced only after hour(s) of illumination. A long-term (spanning hours or days) illumination increases the carbohydrate content and the respiratory capacity of the tissue. This occurs in several tissues, e.g in roots with increased carbohydrate flux from the shoots or in cells growing in culture. Thus, plant respiration responds readily to long-term changes in substrate availability (via photosynthesis)

Net O_2 evolution during the steady state of photosynthesis is a result of the combined effect of (a) photosynthetic O_2 evolution, (b) O_2 uptake by the Mehler reaction, (c) respirator) O_2 uptake, and (d) O_2 consumption by ovygenase activity of rubisco and glycolate ovidase, while net CO_2 evolution is a result of (a) CO_2 release from tricarboxylic acid cycle (TCA cycle), (b) glycine

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