1991

Photoinhibition Of Hardened And Non-hardened Rye (secale Cereale L Cv Musketeer) Studied With Isolated Thylakoids, Isolated Mesophyll Cells, And Intact Leaves

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ISBN 0-315-64239-4

ABSTRACT

Photoinhibition of photosynthesis has been shown to be more severe when combined with other stresses. This suggests a possible correlation between the capacity to grow at low temperature and an increased resistance to photoinhibition at low temperature. Previous work has shown with chlorophyll *a* fluorescence that rye plants (*Secale cereale* L. cv Musketeer) acclimated to low temperature were more resistant to photoinhibition than non-hardened rye plants. The goal of the present study was to assess whether the morphological changes observed at the leaf level and the structural changes observed at the thylakoid level during the cold hardening process were responsible for the increased resistance of cold hardened rye to photoinhibition.

Photoinhibition was monitored in hardened and non-hardened winter rye at the leaf level, with isolated mesophyll cells, and with isolated thylakoids. They were exposed to high photon fluxes at 20 and at 5°C and recovery from photoinhibition was assessed upon return to lower photon fluxes. Room temperature chlorophyll *a* fluorescence, light limited CO_2 fixation rates, electron transport rates, photoacoustic spectroscopy and atrazine binding were measured during and following photoinhibition.

Cold-hardened isolated rye cells were more resistant to photoinhibition when monitored with chlorophyll *a* fluorescence. However, CO_2 fixation rates showed similar extent of photoinhibition in cold hardened and non-hardened isolated rye cells. Photoinhibition was similar at 5 and at 20°C but the recovery from photoinhibition was

slower when occurring at 5°C. Both groups of cells showed similar rates of recovery. In contrast, cold hardened isolated rye thylakoids were more susceptible to photoinhibition which mainly affected Photosystem II activity.

Leaf morphological differences and structural changes occurring at the thylakoid level during cold hardening of winter rye are not responsible for its increased resistance to photoinhibition. Photoinhibition as monitored with chlorophyll *a* fluorescence showed different trends and different kinetics compared to CO_2 fixation. CO_2 fixation data showed that regulatory mechanisms also play a role during photoinhibition and subsequent recovery. Therefore, CO_2 fixation should be monitored in intact leaves before the increased resistance of cold hardened winter rye is conclusively proven.

ACKNOWLEDGEMENTS

I would like to express my gratefulness to all the people who had helped me to realize that project, for their technical help, moral support and enthusiasm. I would like to thank especially the following persons:

Dr. Norm Huner, my supervisor, who provided the subject for this study and the technical support; I thank him as well for the very helpful discussions and for patiently revising this manuscript.

Mr. Douglas Campbell and Vaughan Hurry for their technical advices but especially for the moral support, the enthusiasm and friendship they expressed during all these years we spent together in the lab.

Dr. Marianna Krol, Ms. Tracey Reynolds, Dr. Steve Boese and Dr. Robert Carpentier for technical help and useful discussions.

My advisory committee, Dr. W.G. Hopkins and Dr. R.I. Greyson for their helpful suggestions and for the revision of this manuscript.

Mr. Reg Johnston and Ron Smith for their very helpful technical support and suggestions.

and finally, to my spouse Pierre, for his help with computer technicalities and very helpful discussions, but especially for his moral support and his enthusiasm all along these years in London.

Un gros merci à vous tous.

This work has been supported by a postgraduate fellowship and operational grants from NSERC.

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ABBREVIATIONS

BSA	bovine serum albumin
CAP	chloramphenicol
CH	cyclohexamide
Chl	chlorophyll
cpm	counts per minute
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-dichlorophenol-indophenol
df_1, df_2	number of degrees of freedom in F tests
DTT	dithiothreitol
EDTA	Na_2-ethylenediaminetetraacetate
F	variable F estimated in analysis of variance
F_m	maximal fluorescence
F_o	minimal fluorescence
F_v	variable fluorescence
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
K_D	rate constant for non-radiative dissipation
K_F	rate constant for fluorescence
K_P	rate constant for photochemical activity of Photosystem II
K_T	rate constant for transfer of light energy to Photosystem I
I_{50}	half-saturation energy flux

LHCII	light harvesting chlorophyll protein complexes associated with Photosystem II
M_r	relative molecular mass
MV	methyl viologen
NADP	nicotinamide adenine dinucleotide phosphate
p	probability level
P_{680}	reaction centre of Photosystem II
PAR	photosynthetically active radiation
PAS	photoacoustic spectroscopy
PPF	photosynthetic photon flux
PQ	plastoquinone
PSI	Photosystem I
PSII	Photosystem II
Q_A	primary quinone electron acceptor
Q_B	secondary quinone electron acceptor
Q_c	control photoacoustic signal obtained in absence of background saturating beam
Q_m	maximal photoacoustic signal obtained in presence of the background saturating beam
q_E	non-photochemical quenching of variable fluorescence associated with a trans-thylakoid pH gradient
q_I	non-photochemical quenching of variable fluorescence caused by photoinhibition

q_N	total non-photochemical quenching of variable fluorescence
q_o	quenching of minimal fluorescence
q_p	photochemical quenching of variable fluorescence
q_T	non-photochemical quenching of variable fluorescence associated with state transitions
RH	hardened rye grown at 5°C
RNH	non-hardened rye grown at 20°C
RuBPCase	ribulose bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tricine	*N*-tris(hydroxymethyl)-methylglycine
Tris	tris(hydroxymethyl)-aminomethane
ϕ_{app}	apparent quantum efficiency
ϕ'_r	relative energy storage yield
ϕ'_m	maximal energy storage yield

CHAPTER 1

INTRODUCTION

1.1 Studies on cold hardiness and photosynthesis in winter rye

Many research studies have been undertaken to elucidate the cold hardiness process in cold tolerant plants (Levitt, 1980). Cold hardiness is defined as the development of freezing tolerance following exposure of plants to low (below 10°C) but non-freezing temperatures. Only cold tolerant species can develop cold hardiness since exposure to low temperature induces significant damage and can even cause the death of the plant in chilling sensitive species (Levitt, 1980). A number of studies have looked for the changes occurring during cold hardening especially at the level of membrane lipids and proteins (Guy and Carter, 1984; Uemura and Yoshida, 1984; Lynch and Steponkus, 1987). However in many of these studies cold hardening is induced by a short shift to lower temperature and does not involve growth at that temperature. This protocol works for conifers where transfer of plants to cold hardening conditions stops growth and therefore cold hardening develops in mature needles (Öquist and Martin, 1986). Agricultural practices in which winter cereals are planted in the autumn to allow some growth before the winter comes suggest that these plants might require growth at low temperature in order to survive the winter.

Early work by Dexter (1933) and Tysdal (1933) established the importance of light and CO_2, possibly thus of photosynthesis, during the cold hardening period of

plants in order to reach maximal freezing tolerance. Much later, Lawrence *et al.* (1973), Andrews *et al.* (1974) and Fowler and Gusta (1977) also recognized the importance of growth at low temperature during the cold hardening process in grasses. Growth at low temperature inevitably requires the capacity to photosynthesize at low temperature after the seedling has been established (Paulsen, 1968). Comparative studies of hardened and non-hardened plants have shown a similar capacity to fix CO_2 under hardening and non-hardening conditions in many winter annuals (Regehr and Bazzaz, 1976), in *Lolium temulentum* (Pollock *et al.*, 1984), in winter rye (Huner *et al.*, 1986) and in spinach (Boese and Huner, 1990). Therefore in some cold resistant species, cold hardening does not involve changes in the capacity to photosynthesize at low temperature. Nevertheless, changes at the level of leaf morphology, pigment content and thylakoid composition and ultrastructure have been found in species such as winter rye (Huner, 1985b), spinach (Boese and Huner, 1990) and spring and winter wheat (Hurry and Huner, 1991; Huner *et al.*, 1989), that do not show cold acclimation of their photosynthetic capacity.

I will now review the major changes related to photosynthesis that have been observed following cold hardening of winter rye *Secale cereale* L. cv. Puma. All studies of winter rye by Huner and collaborators, including the present study, have grown non-hardened rye (RNH) at 20°C and hardened rye (RH) at 5°C under similar photosynthetic photon flux (PPF) and day length conditions. Rye plants grow slower at 5°C (35-40% slower rates); it was therefore essential to determine by growth kinetic study which tissues were comparable on the basis on their physiological age. Mature

leaves developed entirely at 5 or at 20°C were compared and showed a 3 to 4-fold increase in dry weight accumulation per leaf area (Table I) in RH than in RNH leaves (Krol *et al.*, 1984). RNH leaves showed a 2.7 times larger leaf area after complete expansion (12 days) than RH leaves (after 18 days). The differential response shown by dry weight and by leaf area measurements is related to higher water content and lower cytoplasmic content in RNH leaves (Krol *et al.*, 1984). Leaves fully expanded at low temperature showed a 1.6 times increase in chlorophyll (Chl) content per leaf area and concomitant increase in ß-carotene and xanthophyll content (Krol and Huner, 1985). RH chloroplasts also showed a 1.7 times increase in Chl, ß-carotene and xanthophyll compared with RNH chloroplasts (Huner *et al.*, 1984).

The photosynthetic capacity and photosynthetic efficiency of rye plants were similar when measured at 10 or at 20°C (Huner *et al.*, 1986). The photosynthetic rates were also similar between RH and RNH plants (Table I). In that respect the hardening process did not seem to modify the photosynthetic capacity nor the efficiency of rye (Huner, 1985b). In this study I will refer to photosynthetic capacity as the maximum photosynthetic rates measured under saturating PPF conditions, and to photosynthetic efficiency as a measure of the photosynthetic rates per quantum absorbed under limiting PPF conditions. The apparent quantum efficiency, ϕ_{app}, is usually estimated because it is based on the incident PPF to the leaf rather than on the absorbed PPF which would allow the estimation of the true quantum efficiency.

TABLE I: Effects of low growth temperature on leaf morphology, physiology, and photosynthetic characteristics in winter rye.

Characteristic	Changes
Dry weight per leaf area	3-4x increase
Leaf area	2.7x increase
Freezing resistance	increase from -4 to -29°C
Osmotic potential	2x increase
Chl/leaf area	1.6x increase
Photosynthetic efficiency	no change
Carboxylation efficiency	no change

Although the overall photosynthetic capacity of rye is not changed during the hardening process, isolated components of photosynthesis are modified. The first studies were done on ribulose bisphosphate carboxylase/oxygenase (RuBPCase). Growth of rye under cold-hardening temperature resulted in a *in vivo* conformational change in RUBPCase which resulted in a decreased exposure of free sulfhydryl groups (Huner and Macdowall, 1978). These structural changes were complemented by functional changes. RH RUBPCase had a higher apparent affinity for CO_2 (K_m CO_2) at low temperature while RNH had a higher K_m CO_2 at temperature higher than 10°C. Maximum reaction rates (V_{max}) were higher for RH RUBPCase at all temperatures tested (Huner and Macdowall, 1979). Nevertheless, carboxylation efficiency measured *in vivo* was similar between RH and RNH leaves (Huner 1985b). *In vitro* differences between K_m CO_2 of RH and RNH are probably not an important factor in the acclimation of photosynthesis of rye plants to low temperatures (Huner, 1985b).

More recent studies have focused on the thylakoid membrane components of the photosynthetic apparatus. Electron transport rates, chlorophyll/protein complexes and polypeptide composition of thylakoids, fluorescence characteristics and finally lipid composition of RH and RNH thylakoids have been examined in detail. Whole chain electron transport showed 1.4-fold higher rates in RH than in RNH thylakoids (Huner, 1985a). Light saturated rates through Photosystem I (PSI) were also higher in RH than in RNH thylakoids. Photosystem II (PSII) activity showed a differential response to the assay temperature, RNH having higher rates at 25°C and RH having higher rates at lower temperature. This differential response of PSII to temperature appeared to be

related to a differential sensitivity of the water splitting complex to temperature. But the quantum efficiency of PSII and PSI was similar in RH and RNH thylakoids as also shown in the present study. They also found a larger plastoquinone (PQ) pool in RH thylakoids but the pool available for reduction of PSII, as measured by room temperature fluorescence induction, was similar in both RH and RNH plants (Griffith *et al.*, 1984a). Therefore cold acclimation affects solely the electron transport capacity of rye thylakoids (Huner, 1985a).

Previous work on 77K Chl *a* fluorescence has shown the presence of a shoulder at 680 nm in RH thylakoids not present in RNH spectra (Griffith *et al.*, 1984b). This emission band has been attributed to light harvesting chlorophyll protein complexes (LHCII) that are dissociated from PSII (Homann, 1981; Briantais *et al.*, 1986). This would suggest some uncoupling of energy transfer between some LHCII and PSII units in RH thylakoids. Room temperature Chl *a* fluorescence induction study showed lower ratios of variable fluorescence/maximal fluorescence (F_v/F_m) in RH thylakoids; this suggested that RH thylakoids were less efficient at performing photochemistry than RNH thylakoids (Griffith *et al.*, 1984b). Study of the area growth over the 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) fluorescence induction curve as a function of F_v (Melis and Schreiber, 1979) suggested a better connectivity for energy transfer between the PSII reaction centres in RNH than in RH thylakoids. However, RH PSIIα units could process the energy absorbed at a faster rate than RNH PSIIα units could (Griffith *et al.*, 1984b). According to the present hypothesis, PSIIα represents the PSII population capable of linear electron transport while PSIIß do not participate in linear

electron transport and have small Chl antennae (Krause and Weis, 1991). The thylakoid fluorescence data were not consistent with similar ϕ_{app} in RH and RNH plants; they might reflect subtle changes not measurable under steady-state photosynthesis.

Polypeptide composition of RH and RNH thylakoids showed no difference (Elfman *et al.*, 1984). However, LHCII populations showed a change in the content of monomeric *vs.* oligomeric forms. The monomeric form was more abundant in RH thylakoids and the oligomeric form in RNH thylakoids. Furthermore, these changes in LHCII populations appeared to be correlated with specific lipid changes in the rye thylakoid membrane (Huner *et al.*, 1987). On a survey of different species and cultivars showing increasing levels of cold hardiness it appeared that the increase in LHCII monomeric/ LHCII oligomeric ratios was correlated with an increase in 16:0/ *trans*-16:1 content of phosphatidyldiacylglycerol in the thylakoid membranes (Huner *et al.*, 1989). So far no function has been attributed to the different populations of LHCII. Further information regarding a possible role for monomeric and oligomeric forms of LHCII is required.

In summary, the overall photosynthetic characteristics of RH and RNH leaves are very similar even though many components have been modified during the cold hardening process. These changes might be essential for long term growth and photosynthesis under low temperature.

1.2 Photoinhibition

1.2.1 Definition of photoinhibition

The definition of photoinhibition by Powles (1984) which was very similar to an earlier description by Kok (1956) is as follows:

> "reduction of photosynthetic capacity, independent of gross changes in pigment composition, induced by exposure to visible light (400-700 nm)."

Björkman (1987a) has also defined photoinhibition in similar terms:

> "In common usage the term photoinhibition included any sustained reduction in photosynthetic activity induced by excessive light, irrespective of mechanistic considerations, but does not include transient reductions that are rapidly reversible and likely to reflect short-term regulation."

However, in practice photoinhibition is usually measured as a decrease in ϕ_{app} of photosynthesis because ϕ_{app} is less sensitive to growth conditions than light saturated rates. In fact, non-stressed plants show very similar ϕ_{app} for O_2 evolution (Björkman and Demmig, 1987). Photoinhibition does not implicate necessarily damage to the photosynthetic apparatus but people often refer to photoinhibition as indicating damage. This has led to much confusion regarding photoinhibition. A new nomenclature is required to redefine actual damage caused by excessive PPF and protective mechanisms which by themselves decrease the photosynthetic activity but are aimed at protecting the photosynthetic apparatus against damage. There are also mechanisms that protect the photosynthetic apparatus without directly decreasing the photosynthetic capacity and

efficiency of plants. Following complete loss of photosynthetic activity, Chl pigments become oxidized during the so-called photooxidation process (Satoh, 1970a; Dominy and Williams, 1987). In this study I will use the term photoinhibition in the same sense as proposed by Powles and by Björkman.

Early work by Emerson (1936), Myers and Burr (1940) and Rabinowitch (1945) already indicated the phenomenon of photoinhibition and the possible adaptation of high PPF grown plants to photoinhibition. But Kok and collaborators (1965, 1966a,b) were the first to do an extensive study on the effect of high PPF upon photosynthetic characteristics of chloroplasts. They measured the effect of very high PPF on the electron transport rates and fluorescence induction kinetics of spinach chloroplasts (Kok *et al.*, 1965). The action spectra of the incident light (400-700 nm) showed that Cnl was the sensitizing pigment during photoinhibition. They showed that the site of photoinhibition was close to PSII (Kok *et al.*, 1965) but in their study PSI was more sensitive to photoinhibition than PSII and constituted another site of damage during photoinhibition (Jones and Kok, 1966b). In these studies with spinach chloroplasts, there was no apparent effect of O_2 or temperature during photoinhibition and decreased photosynthetic activity was directly correlated with the amount of light energy received by the sample (Jones and Kok, 1966a). Malkin and Jones (1968) have shown a linear correlation between loss of F_v and of light limited PSII activity of spinach chloroplasts. Kandler and Sironval (1959) have suggested that photoinhibition was caused by the presence of O_2 free radicals during exposure to high PPF. Avron (1960) and Forti and Jagendorf (1960) have observed an important decrease in photophosphorylation when

submitting chloroplasts to high PPF under nitrogen conditions and suggested that "some of the reducing and/or oxidizing agents generated very early in the photoreaction of the chlorophyll molecule, are acting in a destructive manner on the chlorophyll molecule itself, or on some components closely associated with it" (Avron, 1960). Steemann-Nielsen (1962) have suggested that algae, and maybe higher plants as well, have the capacity to reduce the rate of photochemical reaction to protect the other photosynthetic constituents against photooxidation caused by absorption of excess light energy. Björkman and Holmgren (1963) have shown that populations of *Solidago virgaurea* from shaded areas had lower ϕ_{app} when grown at high PPF than plants from non-shaded areas and that this decrease in ϕ_{app} was ca by damage to PSII (Björkman, 1968).

In the early seventies, Satoh (1970a,b,c and 1971) published a series of papers on photoinhibition. He observed a greater increase in minimal fluorescence (F_o) under anaerobic than under aerobic conditions (Satoh, 1971) and PSI appeared to be more sensitive to the presence of O_2 than PSII (Satoh, 1970b). Similar to Jones and Kok (1966b) results, PSI was more sensitive than PSII to photoinhibition (Satoh, 1970a). However, thylakoids were less sensitive to photoinhibition at low temperature than at room temperature (Satoh, 1970a). He observed photobleaching only after all photosynthetic activity was lost in the thylakoids. And using different artificial electron donors, acceptors and inhibitors he determined that the site of inhibition was located near PSI (Satoh, 1970c).

Despite partial knowledge of electron transport intermediates and of the thylakoid structure and composition, these early *in vitro* and *in vivo* studies have shown some important characteristics of the photoinhibitory phenomenon. Chl is the pigment responsible for photoinhibition. PPF, temperature and O_2 are important factors to consider during photoinhibitory treatments and photooxidation of pigments occurs after photoinhibition. Furthermore, plants may be able to protect their photosynthetic apparatus against photoinhibition and can acclimate to resist high PPF exposure. Since then many authors have reviewed the mechanisms and possible ways to protect the photosynthetic apparatus against photoinhibition (Björkman, 1981; Osmond, 1981; Powles, 1984; Kyle and Ohad, 1986; Kyle *et al.*, 1987; Krause, 1988; Critchley, 1988; Cleland, 1988). This will be briefly summarized in this section.

Photoinhibition occurs in the natural environment. It is known to be important in aquatic ecosystems due to low PPF adaptation by most phytoplankton species. These species constantly circulate within the water column due to currents and get exposed to very different PPFs (Neale, 1987). It is also common in land plants when other stresses exacerbate photoinhibition (Powles, 1984). It has been reported under high temperature conditions in *Arbutus unedo* (Demmig-Adams *et al.*, 1989a), under saline conditions in mangrove trees (Björkman *et al.*, 1988), under water stress conditions in *Opuntia basilaris* (Adams *et al.*, 1987), and under low temperature conditions in pine (Öquist and Ögren, 1985) and in maize (Farage and Long, 1987). Photoinhibition can become an important factor when other stresses have already reduced the photosynthetic capacity of plants.

Two main targets of photoinhibition have been described, the Q_B (secondary quinone electron acceptor) binding site located on the D1 protein (Kyle *et al.*, 1984) and the P_{680} reaction centre of PSII also located on the D1 protein as shown in Fig. 1 (Cleland, 1988; Critchley, 1988). According to the Q_B site hypothesis, the turnover of D1 would not be fast enough during photoinhibition to replace the damaged D1 proteins. Therefore, ϕ_{app} of PSII would decrease since some reaction centres become nonfunctional. Normal turnover of D1 is very fast but it is uncertain if the protein is damaged or if the fast turnover is related to regulatory mechanisms (Mattoo *et al.*, 1984). Some of the possible causes of damage to the Q_B site are the prolonged presence of quinone radicals at the Q_B site due to a highly reduced PQ pool, and the formation of O_2^- free radicals in chloroplasts (Kyle and Ohad, 1986). Photoinhibition of isolated thylakoids under anaerobic conditions did not induce any loss of D1 (Arntz and Trebst, 1986; Hundal *et al.*, 1990) which supports the implication of O_2^- during damage to D1. However, Cleland (1988) has not observed a loss in the D1 protein during photoinhibition of *Oxalis oregona* leaves. Cleland (1988) and Critchley (1988) both postulated that the primary target of photoinhibition is the PSII reaction centre P_{680} and that following inactivation, it becomes a quencher of F_v. They also suggested that following inactivation of the reaction centre, the D1 apoprotein has to be replaced; therefore D1 turnover would not be the cause but the consequence of photoinhibition.

Once light energy has been absorbed by Chl it can be used for photochemistry, re-emitted as fluorescence, re-emitted as non-radiative decay or transferred to PSI by state transitions (Fig. 2). All these pathways, except fluorescence which represents only

Figure 1: Schematic representation of photosynthetic electron transport in the reaction centre of PSII. The different constituents are, D1 (32 kDa) and D2 (34 kDa) proteins; cyt b_{559}, cytochrome b_{559}; Z, the secondary electron donor; P_{680}, the reaction centre chlorophyll primary electron donor; Pheo, pheophytin; PQ, plastoquinone.

Modified from Krause (1988).

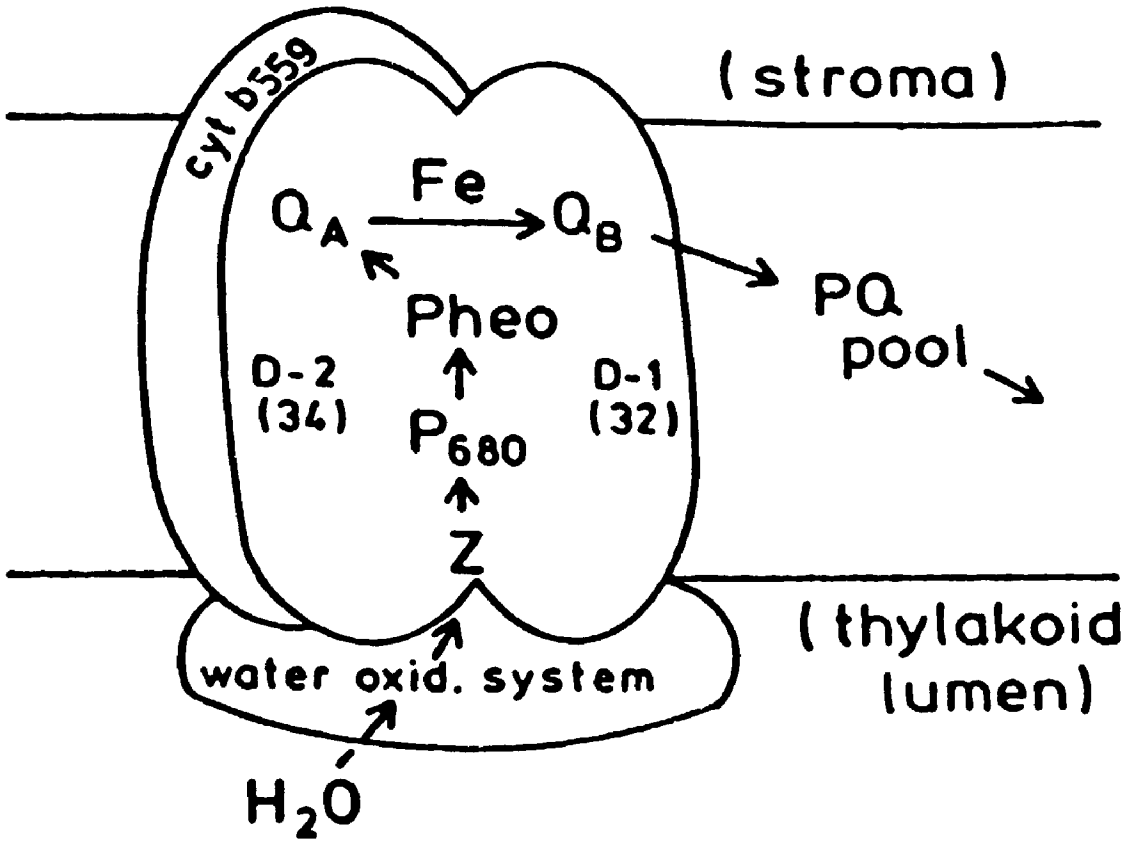
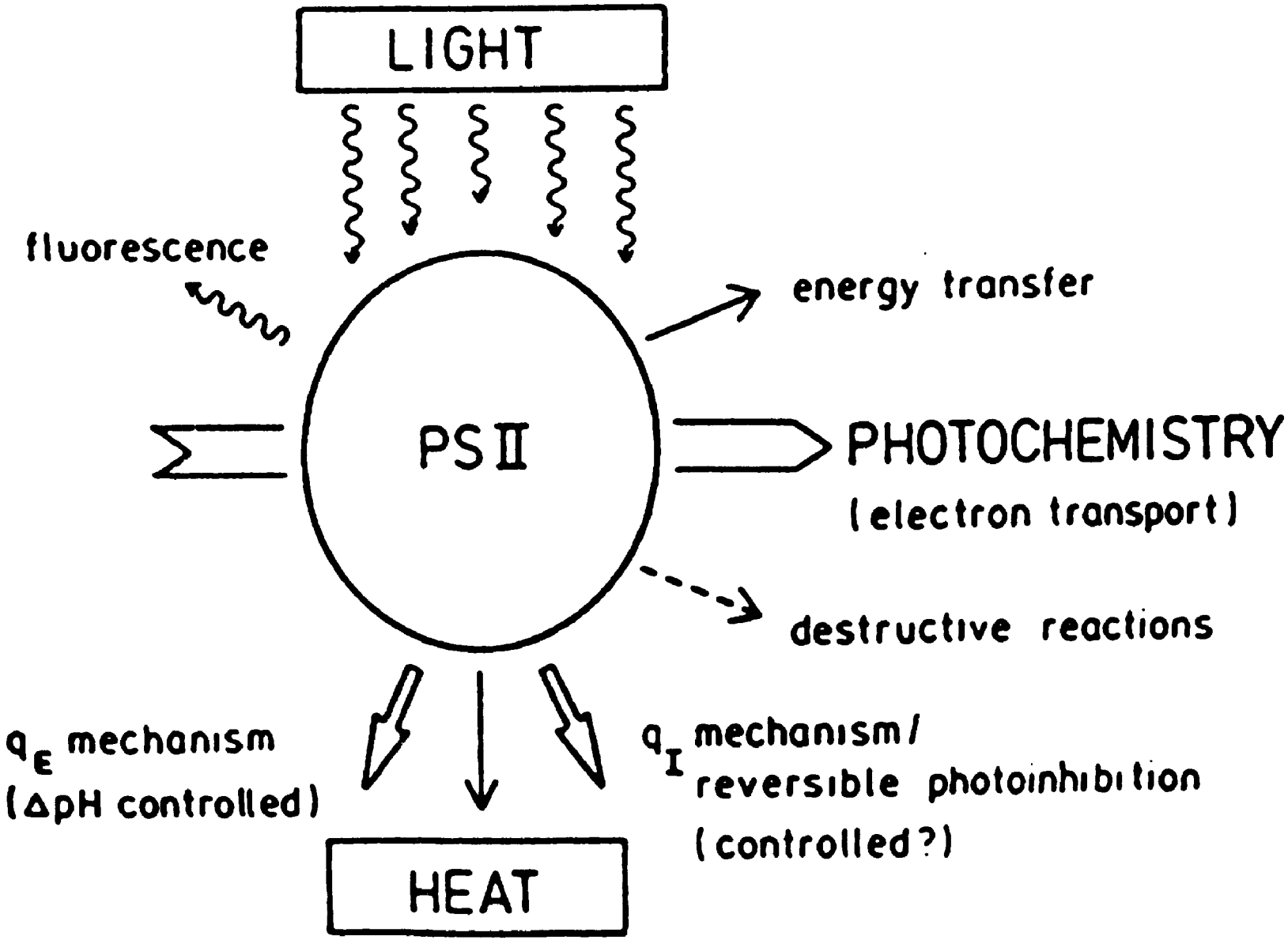

cyt b559
(stroma)
Fe
Q_A
Q_B
PQ
pool
Pheo
D-2
(34)
D-1
(32)
P_{680}
Z
(thylakoid
lumen)
water oxid. system
H_2O

Figure 2: Scheme illustrating pathways of energy conversion in PSII. The importance of each pathway depends upon the incident PPF. Modified from Krause (1988).


LIGHT
fluorescence
energy transfer
PS II
PHOTOCHEMISTRY
(electron transport)
destructive reactions
q_E mechanism
(ΔpH controlled)
q_I mechanism/
reversible photoinhibition
(controlled?)
HEAT

a small percent of the total energy released, are protective mechanisms against possible photoinhibition under high PPF conditions. High rates of photosynthesis can protect the photosynthetic apparatus by maintaining the different electron transport components in a relatively oxidized state (Powles, 1984). This has been shown by illumination of leaves in the absence of CO_2 (Powles and Osmond, 1978). Plants grown at high PPF develop less photoinhibition than plants grown under lower PPF (Powles and Critchley, 1980; Bhogal and Barber, 1987). Some sun plants can even acclimate to photoinhibition by increasing their photosynthetic capacity (Anderson and Osmond, 1987). The photosynthetic capacity of plants is therefore an important factor in their relative susceptibility to high PPF treatments (Powles, 1984). O_2 can also play a protective role by allowing photorespiration (Osmond, 1981) or by replacing NADP (nicotinamide adenine dinucleotide phosphate) as the final electron acceptor in the Mehler reaction (Egneus *et al.*, 1975; Krause, 1988). However, the effect of O_2 during photoinhibition is complex and also appears to be detrimental under certain conditions such as chilling (Lindeman, 1979; Van Hasselt and Van Berlo, 1980; Powles *et al.*, 1983).

The usual non-radiative decay mechanism is related to the build-up of a pH gradient across the thylakoid membrane (Krause *et al.*, 1983). Once the gradient is built up during photosynthetic electron transport, some of the light energy is re-emitted as heat but the mechanism of this process is unknown. Thus, the pH gradient could protect the thylakoids against photoinhibition (Krause and Behrend, 1986). Another process is triggered by the establishment of a pH gradient: the de-epoxidation of the xanthophyll violaxanthin via antheraxanthin into zeaxanthin (Yamamoto 1979).

Zeaxanthin can accept energy from Chl molecules and re-emits the energy as heat (Demmig *et al.*, 1987b). The violaxanthin/zeaxanthin cycle has been demonstrated in a number of plants and under a number of potentially photoinhibitory conditions by Demmig and collaborators (Demmig *et al.*, 1987b; Demmig *et al.*, 1989b,c,d). Zeaxanthin builds up during mild photoinhibitory conditions and by accepting energy from Chl would decrease the amount of light energy directed towards the PSII reaction centres. Under severe photoinhibitory conditions, the capacity of zeaxanthin to accept energy is overcome and damage could then possibly occur (Demmig *et al.*, 1987b). Once the stress is over, the zeaxanthin level slowly decreases and therefore ϕ_{app} of PSII is maintained at a lower level for sometime after the stress is over. The presence of zeaxanthin, as well as other mechanisms which increase the non-radiative dissipation of light energy, cause photoinhibition even though they protect the photosynthetic apparatus against damage. Other xanthophyll and carotenoids could be implicated in a similar fashion in the protection of thylakoids against photoinhibition (Siefermann-Harms, 1987) but this has not been proven yet.

The energy transfer to PSI can potentially be useful when PSII becomes overexcited. Yet many studies have shown its limited capacity to protect PSII *in vivo* (Powles and Björkman, 1982; Ögren and Öquist, 1984; Demmig and Björkman, 1987). Therefore high photosynthetic rates and increased non-radiative dissipation of light energy through the build-up of a pH gradient, via zeaxanthin and through other possible mechanisms can dissipate the excess energy and represent important protective mechanisms that have evolved to protect the thylakoid membranes against

photoinhibitory damage. Somersalo and Krause (1990) and Horton *et al.* (1989) have suggested that photoinhibition itself could become an energy dissipating mechanism once the other protective mechanisms are overtaxed.

Aside from the effect of light energy absorbed by Chl pigments, photoinhibition can be related also to the production of O_2 free radicals. The presence of O_2 free radicals has been shown in thylakoids, especially under high PPF treatments due to the high levels of O_2 released during photosynthesis (Foyer and Hall, 1980; Asada and Takahashi, 1987). These free radicals can be very damaging to proteins and to unsaturated lipids (Halliwell, 1982). Protection of isolated thylakoid membranes by O_2 radical scavengers during photoinhibition has been shown by Barényi and Krause (1985) and by Richter *et al.* (1990b). A possible way to protect thylakoid membranes would be to increase the level of O_2 radical scavengers. This has been shown during cold acclimation of spinach (Schöner and Krause, 1990). Carotenoids and xanthophylls can also protect the thylakoid membranes by reacting with O_2 free radicals (Krinsky, 1979). Vidaver *et al.* (1989) have presented another protective mechanism against photoinhibition caused by the presence of O_2 free radicals, that could occur at chilling temperature: the inactivation of the water splitting complex. This would be detected as a decrease in F_v. However, the level of damage caused by O_2 free radicals has not been clearly estimated yet.

Finally another way to avoid photoinhibition is to repair damage during high PPF treatment. One hypothesis is that net photoinhibition is the result of damage and repair

mechanisms (Kyle and Ohad, 1986). Therefore by increasing the rate of repair photosynthetic organisms can actually increase their resistance to photoinhibition (Falk *et al.*, 1990). This mechanism has been shown in algae which usually have very fast rates of recovery (Samuelsson *et al.*, 1985; Lidholm *et al.*, 1987). This has not been shown in higher plants. However, recovery is not only dependent upon protein synthesis (Greer *et al.* 1986), but also upon the decrease of non-radiative dissipation of light energy once the stress is over (Demmig and Winter, 1988b).

1.2.2 Measurement of photoinhibition

The ϕ_{app} for CO_2 fixation and O_2 evolution has been used interchangeably to assess photoinhibition but the ϕ_{app} for CO_2 fixation defines the effect of high PPF treatment on the overall photosynthetic efficiency while ϕ_{app} for O_2 evolution assesses the effect of high PPF treatment on the electron transport rates of photosynthesis. Both of these measurements require PPF response curves which are tedious and long so that some recovery from photoinhibition can occur during the measurements (Greer *et al.*, 1986).

Chl *a* fluorescence has been studied since 1931 (Kautsky and Hirsch, 1931; 1934). But Kitajima and Butler (1975) were the first to define a fluorescence parameter, F_v/F_m, which estimates the ϕ_{app} for PSII activity. Subsequently, F_v/F_m has been tested and shown to be correlated with ϕ_{app} for O_2 evolution (Demmig and Björkman, 1987). Since photoinhibition appeared to affect PSII, the use of Chl *a* fluorescence and

especially the F_v/F_m ratio, has become more and more popular. It has become one of the most popular ways to assess photoinhibition quickly, though the definition of photoinhibition implies a decrease in ϕ_{app} of photosynthesis and not solely of PSII.

Fluorescence is re-emitted from the excited Chl pigment. When the plant has been dark adapted, all reaction centres are open (oxidized state) for photochemistry. Upon turning a light source on, a fluorescence yield F_o is measured (Fig. 3a). It is caused by excitation losses during the transfer of excitation energy from the antenna to the reaction centres (Baker and Horton, 1987). Within 2 sec of illumination, the fluorescence yield rises to a maximum F_m where all reaction centres are closed (reduced state) with Q_A, the primary quinone electron acceptor, reduced in all reaction centres. Under normal incident photon flux, F_m is rarely reached since electron transport will start to oxidize Q_A before all Q_A have been reduced. According to Klimov *et al.* (1980), F_v which is F_m - F_o, rises from the charge recombination between P_{680}^+ and pheophytin$^-$ when another photon reaches a reaction centre that has its Q_A reduced. However, more recent data have supported the original hypothesis of Butler and Kitajima (1975) that attributed F_v to rapid back energy transfer of excitons from closed reaction centres to the antenna pigments (Krause and Weis, 1991).

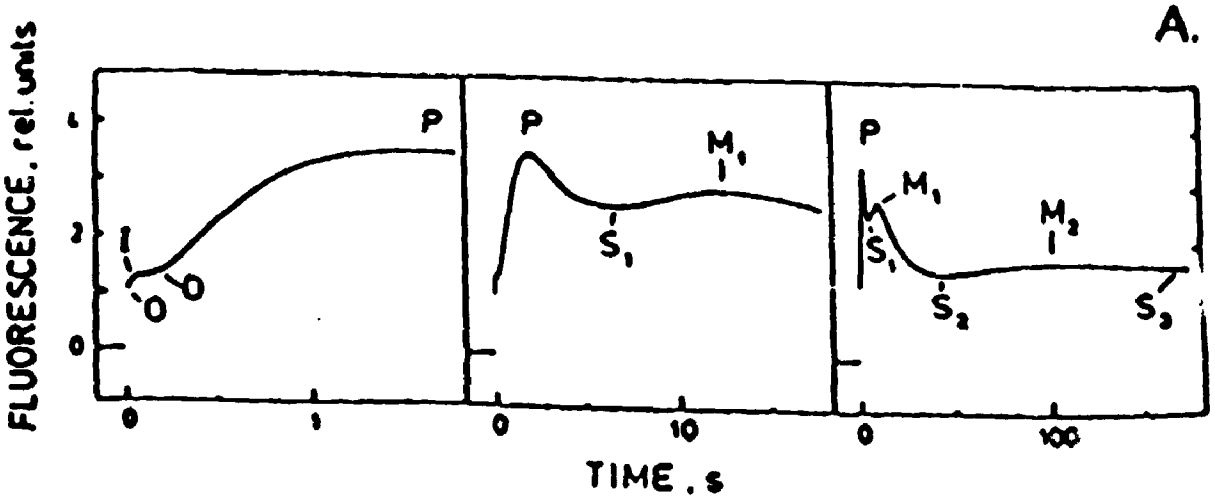
Figure 3: Room temperature Chl *a* fluorescence induction curves.

A) A typical Kautsky curve from a tomato leaf shown at three different time scales. At time zero a dark-adapted sample was illuminated with continuous blue light at 15 W m^{-2}. The characteristic fluorescence levels are: O (F_o), the initial fluorescence, P (F_m), the maximal fluorescence, S and M, oscillations occurring during induction and S_3 (or T), the steady-state level of fluorescence yield. Modified from Briantais *et al.* (1986).

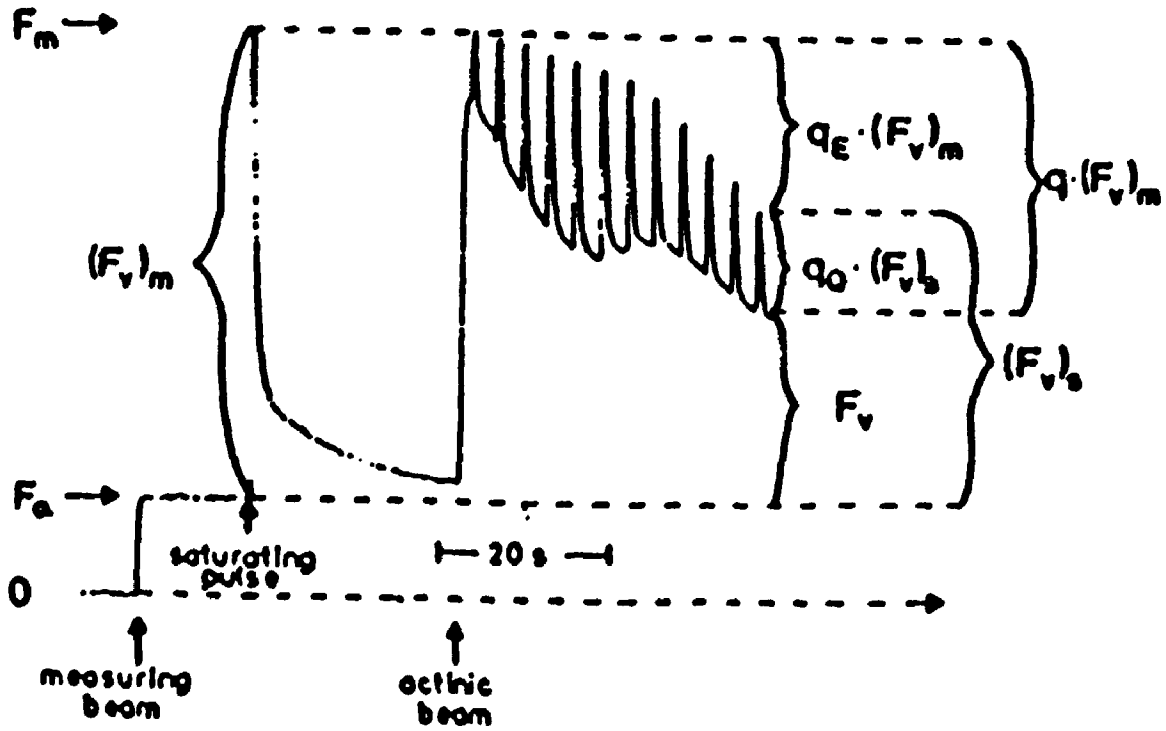
B) A curve obtained with the PAM instrument of a bean leaf showing the different quenching mechanisms. q_E (q_N), the non-photochemical component and q_Q (q_p), the photochemical component of Chl *a* fluorescence quenching.

Modified from Schreiber *et al.* (1986).

A.


FLUORESCENCE, rel. units
P
P
P
M1
M1
M2
S1
S1
S2
S3
O
O
I
0
1
0
10
0
100
TIME, s

B.


Fm
(Fv)m
qE·(Fv)m
q·(Fv)m
q0·(Fv)s
(Fv)s
Fv
Fo
0
saturating pulse
20 s
measuring beam
actinic beam

Kitajima and Butler (1975) have derived the following relationship:

$$F_o = \frac{K_F}{(K_F + K_D + K_T + K_P)} \qquad F_m = \frac{K_F}{(K_F + K_D + K_T)}$$

$$\text{and } \phi_{app} = \frac{K_P}{(K_F + K_D + K_T + K_P)} = \frac{F_v}{F_m}$$

Where K_F is the rate constant for fluorescence, K_D the rate constant for non-radiative dissipation, K_T the rate constant for transfer of light energy to PSI, and K_P the rate constant for photochemical activity of PSII.

From this model, it has been postulated that an increase in K_D would decrease F_o, F_m and ϕ_{app}, while a decrease in K_P would increase F_o, decrease ϕ_{app} and should not affect F_m (Björkman, 1987a). An increase in K_D can be related to the transformation of PSII reaction centres into fluorescent quenchers (Weis and Berry, 1987) or occurs within the antenna pigment matrices associated with PSII reaction centres (Genty *et al.*, 1990). Since an increase in F_o has been associated with a decrease in K_P, it is considered to represent damage to the reaction centre, permanent or reversible (Krause, 1988). Yet F_o could also increase if there is a partial detachment of antennae from the PSII reaction centres (Krause, 1988).

Chl *a* fluorescence measurements at 77K have been most suitable for estimation of F_o and F_m for both PSII (692 nm) and PSI (734 nm) (Björkman, 1987b). The 77K fluorescence measurements also allow an estimation of light energy transfer to PSI while room temperature Chl *a* fluorescence emanates from PSII only.

In uncoupled isolated thylakoids, fluorescence yield increases from F_o to F_m and then very slowly decreases under constant illumination. But in intact chloroplasts, cells or leaves, faster changes in fluorescence yield at room temperature are observed (Briantais *et al.*, 1986). After the onset of electron transport, the fluorescence yield is quenched by both K_D and K_P to reach a steady-state level (S_3 or T on Fig. 3a) slightly higher than F_o (Bradbury and Baker, 1981; 1984). The kinetics of quenching from F_m to T are variable and show one or more transients. One of these transients, the M peak, occurs to varying extent depending on the conditions and possibly reflects the burst of O_2 evolution and CO_2 uptake following illumination. The M peak is usually associated with changes in K_P and K_D (Bradbury and Baker, 1981; 1984). During the induction phase and after steady-state has been reached, changes in carbon assimilation can affect the rate of electron transport and therefore the fluorescence yield (Baker and Horton, 1987).

To detect the mechanisms involved in quenching fluorescence during photosynthesis, room temperature fluorescence has to be monitored. With the use of a double flash system (Bradbury and Baker, 1981; 1984) or of a light modulated system combined with a flash lamp and an actinic light source (PAM fluorometer, Schreiber *et al.*, 1986), Chl *a* fluorescence quenching has been studied during induction and steady-state photosynthesis (Fig. 3b). One parameter, the photochemical quenching (q_p), is a measure of the level of oxidation of Q_A. High q_p values are reached under low PPF, when the electron transport chain is able to oxidize Q_A as soon as it is reduced by P_{680}. Some decrease in q_p is observed at higher PPF (close to light saturation level) when the

electron transport chain does not re-oxidize Q_A as fast as it gets reduced possibly due to a high reduction level of the PQ pool (Bilger and Schreiber, 1986). However, several points must be clarified in relation to q_p. The way q_p is estimated, it reflects the level of oxidation of Q_A in the reaction centres that fluoresce. Reaction centres transformed into quenchers do not fluoresce and therefore the state of Q_A in these centres is not taken into account. So q_p is not related to the oxidation level of the total pool of Q_A but reflects how Q_A is re-oxidized in active reaction centres. Furthermore, q_p does not correlate well with ϕ_{app} of electron transport or of O_2 evolution and appears to be under other mechanisms of control than PSII efficiency alone (Horton *et al.*, 1988; Weis and Lechtenberg, 1989).

Another component often measured is q_N or the non-photochemical quenching component of fluorescence (Fig. 3b). q_N builds up quickly during the induction of photosynthesis and reaches a steady-state level that is dependent on the incident PPF. According to the present theory (see Krause and Weis, 1984), q_N would be an estimation of the rate constant, K_D, for non-radiative decay. The state transition rate constant, K_T, is also included in q_N. The higher the q_N, the higher the fraction of absorbed PPF that is re-emitted as heat. q_N has been shown to be correlated with ϕ_{app} of O_2 evolution and of electron transport (Krause and Laasch, 1987; Weis and Berry, 1987) and therefore is an important parameter to estimate when imposing stress on photosynthesis.

A strong correlation has been established between the build-up of a trans-thylakoid pH gradient and the rise in q_N (Briantais *et al.*, 1979). The fraction of q_N related to the build-up of a pH gradient is called q_E. The latter relaxes very quickly with a $t_{½}$ of less than 1 min, upon turning off the light source. However, other factors besides the pH gradient might control the establishment of q_E (Mills *et al.*, 1979; Laasch, 1987). The fraction of q_N related to state transition, q_T, can be possibly discerned as a slower component of the relaxation of q_N with a $t_{½}$ of 5 min. The fraction of q_N not relaxed after 5 min is called q_I or quenching caused by photoinhibition but no mechanism has been identified yet to cause q_I (Horton and Hague, 1988). Since q_N is calculated usually from each measure of F_v of the sample after dark adaptation rather than from F_v of the control sample, it represents the fraction of F_v present in the sample that is quenched non-photochemically. During photoinhibition, F_v can be decreased by q_I because it is maintained for a long time even after dark adaptation. However, the decrease of F_v in dark adapted samples could also be related to other quenching mechanisms or to damage at the reaction centre. More recent data have questioned the use of different relaxation times to separate the different q_N components (Lee *et al.*, 1990). There might be more than 3 quenching components measured as q_N.

A third mechanism has been defined q_o or the quenching of F_o (Bilger and Schreiber, 1986). The quenching of F_o may occur under conditions of strong energization and is possibly related to the establishment of q_N via q_T (Bilger and Schreiber, 1986) or q_E (Weis and Berry, 1987).

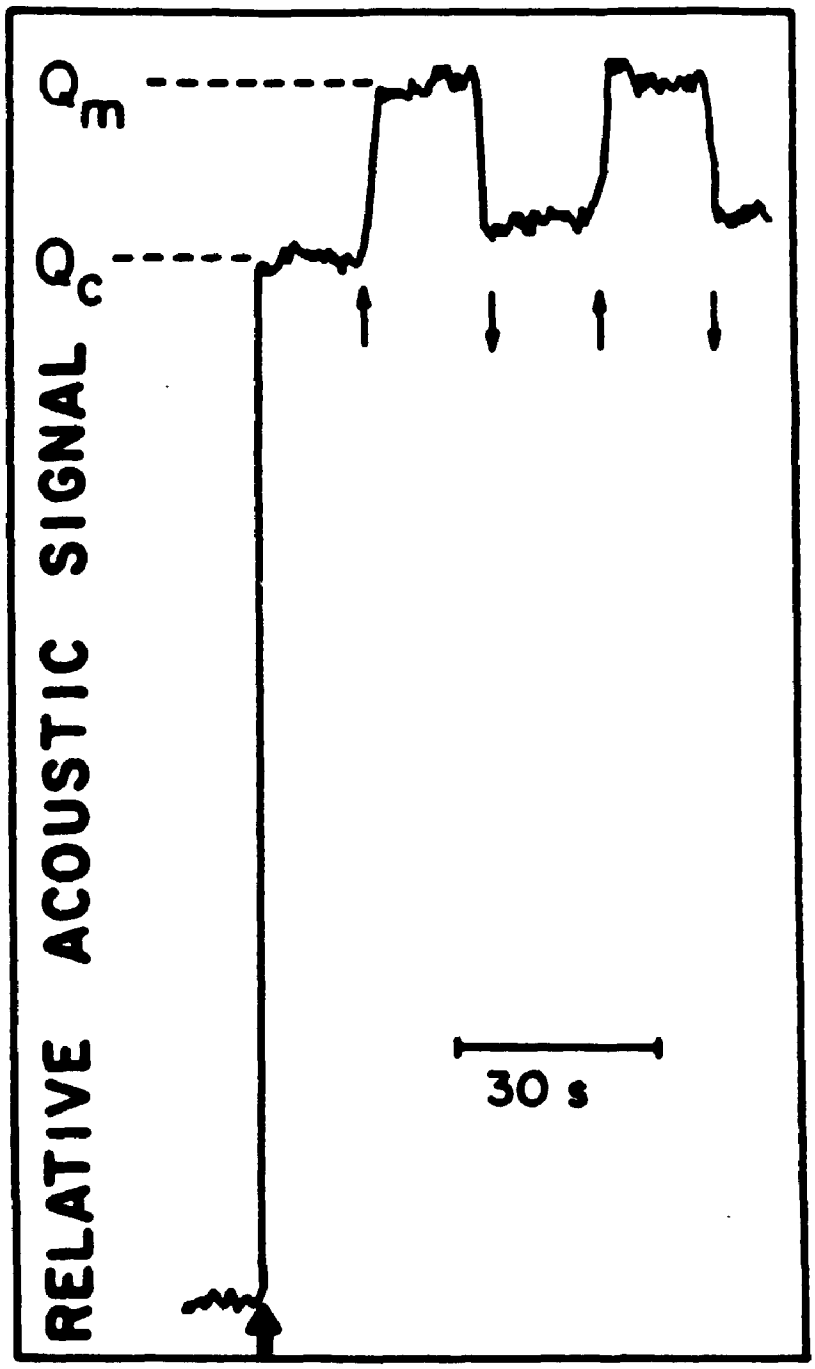
The ϕ_{app} for CO_2 fixation and O_2 evolution and Chl *a* fluorescence parameters have been used with whole leaf or leaf segments (Greer *et al.*, 1986; Long *et al.*, 1987; Correia *et al.*, 1990). Photoinhibition has also been studied with other organisms such as green algae (Lidholm *et al.*, 1987; Falk *et al.*, 1990) and cyanobacteria (Samuelsson *et al.*, 1985; Vonshak *et al.*, 1988), as well as with other systems like mesophyll cells (Krause *et al.*, 1978), protoplasts (Horton *et al.*, 1987), chloroplasts (Cornic *et al.*, 1982; Satoh and Fork, 1982; Laasch, 1987) and isolated thylakoids (Cornic and Miginiac-Maslow, 1985; Arntz and Trebst, 1986; Cleland and Melis, 1987). Isolated systems have been photoinhibited after isolation (Satoh, 1970a; Dos Santos and Hall, 1982; Bradbury and Baker, 1986) or isolated from photoinhibited leaves (Powles and Björkman, 1982; Ögren and Öquist, 1984; Le Gouallec and Cornic, 1988). The use of simpler systems such as thylakoids has allowed a better definition of the target of photoinhibition since no repair occurs in photoinhibited thylakoids and some of the protective mechanisms like O_2 free radicals and zeaxanthin cycle (Demmig-Adams *et al.*, 1990) have been lost during the isolation procedure. Protoplasts and isolated cells allow the use of complete photosynthetic units but in a homogeneous system such that each individual cell receives the same incident PPF. Furthermore, this aqueous system simplifies the use of inhibitors and radioactive markers. The use of a whole leaf or a leaf segment in photoinhibitory studies have allowed one to establish the level of photoinhibition under real conditions, taking into account the photosynthetic activity of the leaves and their optical properties.

Another technique has been used also to characterize photoinhibition: photoacoustic spectroscopy (PAS). As with Chl *a* fluorescence, PAS estimates indirectly how much energy is used for photosynthesis. This is called the photochemical loss or the relative energy storage yield (Malkin and Cahen, 1979). PAS uses a modulated light beam that induces a modulated release of heat by the sample. A frequency is chosen that allows the modulated release of heat to be detected by a microphone as a change in the air pressure in the enclosed cell caused by the variation in temperature (Rosencwaig, 1980). The modulated light beam has a low incident PPF that does not saturate photosynthesis (Fig. 4). A non-modulated saturating beam is used to saturate photosynthesis and then all of the modulated PPF absorbed is re-emitted as heat (Q_m). The difference between the amplitude of the PAS signal in presence (Q_m) and in absence (Q_c) of saturating beam in relation to the Q_m value is called the relative energy storage yield, ϕ'_r (Lasser-Ross *et al.*, 1980). To estimate the maximal level of ϕ'_r, a PPF response curve is established and extrapolated to the origin of the abscissa (Carpentier *et al.*, 1985). Using this technique the fraction of incident PPF used for photosynthesis has been estimated to be about 35-40% in pea leaves (Havaux, 1990). A large fraction of the energy is therefore re-emitted as heat even under ideal growth conditions.

At low frequency (<100 Hz), a second phenomenon is also modulated in leaves and detected by the microphone: O_2 evolution. The two phenomena, heat release and O_2 evolution, can be separated using a two-phase lock-in amplifier and analyzed with the vectorial method of Poulet *et al.* (1983). A PAS signal is also registered at higher frequency, where O_2 evolution produces a constant signal; but with increased frequency,

Figure 4: Photoacoustic signal from spinach thylakoid membranes. ↓ , onset of the measuring light beam; ↑ ↓ , non-modulated actinic beam, on and off respectively.

Modified from Carpentier *et al.* (1987).


Q_m
Q_c
RELATIVE ACOUSTIC SIGNAL
30 s

the signal will come from the upper layer of the samples as observed with the Chl *a* fluorescence signal (Carpentier *et al.*, 1983). With isolated thylakoid preparations, low frequency can be used since no O_2 is evolved.

So far, PAS has been used only in a few photoinhibitory studies (Yakir *et al.*, 1985; Buschmann, 1987; Havaux, 1988; Canaani *et al.*, 1989; Jansen *et al.*, 1989) but it could represent a more direct way of assessing the importance of non-radiative decay in the protection of the photosynthetic apparatus against photoinhibition.

1.2.3 Photoinhibition and cold hardiness

Initial studies of chilling and photoinhibition were conducted by Taylor and Craig (1971), by Taylor and Rowley (1971) and by Garber (1977); they showed the importance of light during chilling stress and a differential capacity to resist the combined low temperature and light stress between cold sensitive and cold resistant plants. Chilling develops more rapidly under light conditions than in the dark (Van Hasselt and Van Berlo, 1980; Long *et al.*, 1987; Smillie *et al.*, 1988; Terashima *et al.*, 1989). From previous studies it had been concluded that photoinhibition is a primary cause of the photosynthetic decline observed at chilling temperature in the light (Long, 1983; Öquist, 1983). Chilling resistant plants are not affected by chilling in the dark but can show reduction of their photosynthetic activity when chilled in presence of light (Ögren *et al.*, 1984; Smillie *et al.*, 1988). The decrease in photosynthetic activity caused by exposure to light at low temperature occurs much more gradually in chilling resistant plants than in

chilling sensitive plants (Hetherington *et al.*, 1989). Does chilling resistance require the evolution of protective mechanisms against photoinhibition? According to Smillie *et al.* (1988) chilling resistant plants are more resistant to photoinhibition than chilling sensitive plants even at non chilling temperatures (21°C). Therefore chilling resistance would also bring about an increased resistance to photoinhibition.

Lower photosynthetic rates, lower repair mechanisms due to slow metabolic rates (Greer, 1988) and possibly a decreased capacity to dissipate energy through non-radiative decay (Havaux, 1989) could explain the development of photoinhibition at chilling temperature. Chilling resistant plants have probably increased one or many of these parameters in order to show less damage than chilling sensitive plants during exposure to high PPF treatments.

Chilling resistant plants can not only resist photoinhibition at low temperature but can also grow at low temperature. This low temperature growth requires that photosynthesis is maintained under potentially photoinhibitory conditions. Therefore, some of the changes observed during the cold hardening process in plants might be related to the adaptation of the photosynthetic apparatus to photoinhibition. Cold hardened spinach plants showed a greater resistance to low temperature photoinhibition than non hardened spinach (Somersalo and Krause, 1989; Boese and Huner, 1990). Even chilling sensitive plants like kiwifruit showed an increased resistance to photoinhibition when grown at lower temperature (Greer and Laing, 1989). However, cold hardened pine needles and cold hardened barley leaves did not show an increased

resistance to low temperature photoinhibition (Öquist and Huner, 1991).

1.3 Use of isolated mesophyll cells in photosynthesis research

Since 1969 (Gnanam and Kulandaivelu, 1969) many studies have been conducted on the photosynthesis of isolated mesophyll cells. Gnanam and Kulandaivelu (1969) studied the photosynthetic characteristics of cells isolated by mechanical grinding, but Jensen *et al.* (1971) were the first ones to study photosynthesis on cells isolated enzymatically. Some species such as soybean (Servaites and Ogren, 1977a; Oliver *et al.*, 1979; Behrens *et al.*, 1982; Cosio *et al.*, 1983; Rees *et al.*, 1985; Omielan and Pell, 1988), spinach (Aono *et al.*, 1974; Marsho *et al.*, 1979; Woo and Canvin, 1980; Larsen *et al.*, 1981) and *Asparagus spp.* (Colman *et al.*, 1979; Bown, 1982; Hills, 1986) have been used more than others, probably due to the relative facility with which cells are released from the leaves of these plants. More recently, cells have been finally isolated from rye by using a different mixture of pectic enzymes, called pectolyase, that gives good yield of isolated cells (Singh, 1981).

Regardless of the method and the species used to isolate mesophyll cells, published data showed a great variation in the photosynthetic capacity of these cells (see section 4.6). Some studies have been conducted to try to identify the factors that would improve significantly the yield and photosynthetic rates of isolated cells (Jensen *et al.*, 1971; Paul and Bassham, 1977; Aidid and Thain, 1980; Baumann and Günther, 1986). Other studies have presented data assessing the photosynthetic activity of isolated cells

under different incident PPF, CO_2 concentrations, pH of the buffer and temperature (Oliver *et al.*, 1979; Colman *et al.*, 1979; Hills, 1986). Nevertheless most studies have not been extensive; they showed the photosynthetic capacity of isolated cells and suggested their potential u e in photosynthesis research. Two groups, Servaites and Ogren (1977a,b) and Espie and Colman (1982, 1987) have used isolated cells to study CO_2 import into mesophyll cells of higher plants. Some studies have used isolated cells to look at the incorporation of photosynthetic carbon into sugars and amino acids (Rehfeld and Jensen, 1973; Paul and Bassham, 1977; Woo and Canvin, 1980; Larsen *et al.*, 1981). Omielan and Pell (1988) have studied the effect of ozone on the photosynthetic characteristics of cells isolated from *Glycine max*, Plaut *et al.* (1989) the salinity effect on photosynthesis of cowpea cells, Davis and Shimabukuro (1980) the herbicide toxicity and their mode of action on peanut cells, and De Filippis (1985) the effects of metal ions on the integrity of isolated cells of *Avena sativa*. Another interesting approach has been the isolation of cells from source and from sink leaves to study their particular photosynthetic characteristics (Cosio *et al.*, 1983). Furbank *et al.* (1982) have used cells isolated from *Xanthium strumarium* to look at the importance of the Mehler reaction *in vivo* in the energy balancing mechanism within the chloroplast. A few studies have used rye and wheat cells to assess the freezing tolerance following cold acclimation (Singh, 1981; Pomeroy *et al.*, 1983) or to do comparative spin-label studies of isolated plasma membranes of hardened and non-hardened rye mesophyll cells (Windle, 1988). Rumich-Bayer and Krause (1986) have assessed freezing damage and frost tolerance of the photosynthetic apparatus using mesophyll protoplasts of *Valerianella locusta*.

Some studies have used isolated cells or protoplasts to study photoinhibition. Krause *et al.* (1978) have shown photoinhibition in isolated spinach cells when incubated in absence of CO_2. Horton *et al.* (1987) have used barley protoplasts to characterize photoinhibition at low temperature and the effect of protein synthesis inhibitors during photoinhibition. They also looked at the Chl *a* fluorescence characteristics of protoplasts (Quick and Horton, 1986; Horton and Hague, 1988). Another study by Xu *et al.* (1989) has looked at the Chl *a* fluorescence characteristics of soybean photoautotrophic cells. Pomeroy and Mudd (1987) have shown that cucumber protoplasts responded similarly to seedlings during chilling and therefore represent a good system to study the mechanisms of chilling damage in plants. The isolated mesophyll cell system has been described by many others as very useful but only a few thorough studies have been undertaken over the last 20 years. Despite the absence of many results proving the usefulness of the system, I decided to undertake that study after having worked out the conditions required to isolate photosynthetically efficient cells.

1.4 Objectives of the present study

During an initial study on rye, RH and RNH leaves were exposed to photoinhibitory conditions at low temperatures and Chl *a* fluorescence data showed RH as more resistant than RNH to photoinhibition (Öquist and Huner, 1991). Some of the changes observed at the leaf level during cold hardening of rye are possibly involved in increased resistance to photoinhibition. But which ones? The present study will try to

answer that question. Two systems will be used to look at the increased resistance to photoinhibition in RH leaves: isolated mesophyll cells and isolated thylakoids.

Isolated cells represent complete photosynthetic units and can be photoinhibited uniformly in an aqueous medium. The use of isolated mesophyll cells is aimed at determining the importance of leaf morphology and leaf optical properties in the development of increased resistance to photoinhibition in RH plants. All the photoinhibitory treatments have been done at both 5 and 20°C to assess the importance of low temperature on the overall photosynthetic response to photoinhibition. Recovery from photoinhibition has also been studied in isolated rye cells to see if the kinetics of recovery would help to discern the different mechanisms that could give rise to photoinhibition. Both Chl *a* fluorescence parameters and CO_2 fixation rates have been monitored during photoinhibition and during recovery of isolated rye cells to assess the response of PSII and CO_2 fixation to photoinhibition. This allowed me to assess the validity of F_v/F_m as a tool to estimate the resistance of plants to photoinhibition.

Isolated thylakoids, on the other hand, were used to assess the importance of the structural changes observed at the thylakoid level during cold hardening of rye to the development of increased resistance to photoinhibition. Again, other parameters besides Chl *a* fluorescence were monitored during photoinhibition of isolated rye thylakoids. In addition, an examination of isolated thylakoids has allowed me to assess the importance of protective mechanisms such as O_2 radical scavengers and the zeaxanthin cycle to the development of photoinhibitory resistance in RH plants.

CHAPTER 2

MATERIAL AND METHODS

2.1 Growth conditions

Seeds of *Secale cereale* L. cv. Musketeer were sown in vermiculite and germinated in growth cabinets at 20/16°C (day/night), under a photoperiod of 16 h and a PPF of 250 μmol m^{-2} s^{-1}. After 7 days, seedlings were either kept at 20/16°C (day/night) for an additional 2 weeks or transferred at 5/5°C (day/night) for 8 weeks with all other conditions held constant. Plants were watered with a modified Hoagland's nutrient solution (Huner and Macdowall, 1976) as needed. According to Krol *et al.* (1984), leaves from plants of comparable physiological ages were collected using 21-day old plants acclimated at 20°C and 60-day old plants acclimated at 5°C.

2.2 Cell isolation

Before isolating the mesophyll cells, the plants were kept in the dark for at least 18 h followed by a 2 hour period at a PPF of 250 μmol m^{-2} s^{-1}. Fully expanded leaves were collected (1.5 g) and cut into strips 1 cm long and 1 mm wide in the following incubation medium (10.0 mL): 0.3 M sorbitol, 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.3, 12.5 mM K_2SO_4, 1% (w/v) potassium dextran sulphate (Pharmacia, relative molecular mass 500 000) and 0.00075% (w/v) pectolyase Y23 (Sigma). This enzyme, pectolyase Y23, dissolves the pectic substances of the

middle lamella of rye leaves with high efficiency (Nagata and Ishii, 1979). The leaf segments were infiltrated under vacuum for 1 min and the filtrate discarded. The leaves were then placed in an apparatus similar to the one described by Servaites and Ogren (1977a) and stirred at room temperature, in the dark, with fresh incubation medium (100 mL). This technique used a continuous flow system (5 mL min^{-1}) in order to avoid damaging the freshly isolated cells. Cells were collected on a nylon net of 20 μm mesh-opening housed in a filter unit. Every 15 min, the pump was turned off and the net holding the cells was washed with the following medium to collect the cells: 0.2 M sorbitol, 0.1 M Hepes, 0.1 M *N*-tris(hydroxymethyl)-methylglycine (Tricine) at pH 7.8. The cell suspension was filtered first through a nylon net (100 μm mesh-opening) which retained cell clusters and vascular bundles, and then through a nylon net of 20 μm mesh opening, which separated free cells from chloroplasts and other organelles. The cells were then kept on ice in the dark. Each sample was collected over a 30 min period.

The cells were centrifuged at 1500 g for 5 min in a bench top centrifuge and the pellet resuspended in Percoll of 1.045 mg mL^{-1} (35%, v/v) density for RH cells and of 1.033 mg mL^{-1} (27%, v/v) density for RNH cells (Singh, 1981). The centrifugation was performed at 2500 g for 20 min on a bench top centrifuge and living cells collected as a pellet. Living cells were washed with distilled water and centrifuged at 1500 g for 5 min. The pellet was resuspended in fresh resuspension medium and kept on ice in the dark until photosynthetic activity or Chl *a* fluorescence was measured. The resuspension medium was modified from Larsen *et al.* (1981) and contained: 0.1 M Hepes at pH 7.8, 0.25 M sorbitol, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM KNO_3, 0.5 mM KH_2PO_4, 0.01 mM

$CuSO_4$, and 0.2% (w/v) bovine serum albumin (BSA).

Cell numbers and viability were estimated with an haemocytometer using a phase contrast microscope. Dead and live cells present a different appearance under phase contrast microscopy. Cell viability was estimated also by Evan's blue staining. Cell suspensions were diluted with an equal volume of 0.1% (w/v) Evan's blue solution and examined under the microscope for the exclusion of the dye by the cells (Gaff and Okong'o-Ogola, 1971). Samples were diluted to obtain about 1.6×10^5 viable cells mL^{-1} which represented approximately 15 μg Chl mL^{-1}. Chl concentrations were determined according to Arnon (1949).

All photosynthetic and metabolic rates were based on a viable cell count rather than on Chl content, because the latter does not exclude dead cells. Furthermore, Chl was extracted to a different extent from RH and RNH cells.

Cell areas and perimeters were determined by using a videocamera DAGEMTI 66 (B & W) fitted to a Leitz Dialux microscope. The images were analyzed on an Apple IIe microcomputer using Bioquant II version 8.1 (R & M Biometrics, Nashville, Tennessee).

2.3 Thylakoid isolation from leaves

Thylakoids were isolated from fully expanded rye leaves according to the technique described by Huner (1985a). The leaves were ground in a Waring blender (2 x 5s bursts) in the following buffer: 0.4 M sorbitol, 50 mM Tricine pH 7.8, 10 mM NaCl, cooled to 0°C. The homogenate was then filtered through 2 layers of Miracloth and centrifuged at 3000 g for 2 min at 4°C. The pellet was washed once by centrifuging it at 3000 g for 2 min at 4°C in a low osmolarity buffer to burst the chloroplasts. This buffer contained 50 mM Tricine pH 7.8, 10 mM NaCl and 5 mM $MgCl_2$. The pellet was then resuspended in the following resuspension buffer: 0.1 M sorbitol, 50 mM Tricine pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$ and 1 mM NH_4Cl, and kept on ice in the dark until used. Freshly prepared thylakoids were used in all cases. Chl concentrations were determined according to Arnon (1949). Thylakoid suspensions were diluted with resuspension buffer to a Chl concentration of 150 μg mL^{-1}.

2.4 Thylakoid isolation from cells

Isolated cells were pelleted at 1000 g and resuspended in thylakoid grinding buffer with a minimum number of cells of 2×10^6 for RNH cells and 3.5×10^6 for RH cells. The cells were frozen at -70°C in presence of 1.0 mm glass beads. Thawed samples were agitated with a Mini-Bead beater apparatus (Biospec Products, Barthesville, Oklahoma) for 10 15-s bursts and put back on ice between each burst. When most of the cells appeared damaged, the homogenate was filtered through a nylon net of 20 μm mesh-opening to discard unbroken cells and glass beads. The nylon net

was washed with grinding buffer to collect the thylakoids. The thylakoids were pelleted at 16 000 g for 3 min, washed with wash buffer and pelleted again at 16 000 g at 4°C for 3 min. They were then resuspended in thylakoid resuspension buffer to a final concentration of 50 μg Chl mL^{-1} and kept on ice in the dark until used for measurements.

2.5 Photosynthetic measurements on rye leaves

The room temperature fluorescence induction curves were obtained with a PAM Chlorophyll Fluorometer (Walz, PAM 101) that is described in detail in Schreiber *et al.* (1986). PPF of the modulated beam emitting at 650 nm was 0.06 μmol m^{-2} s^{-1}, and the frequencies used were 1.6 kHz for F_o measurements and 100 kHz when used in conjunction with the saturating PPF beam of white light or the actinic beam. The saturating beam of white light (KL 1500 lamp unit controlled by PAM 103 unit) was adjusted to a PPF of 3450 μmol m^{-2} s^{-1} and the duration of each flash was 400 ms with 10 s between each flash. The actinic light beam (PAM 102 unit) had a PPF of 74 μmol m^{-2} s^{-1} and emitted at 650 nm. The fluorescence signals were recorded on an X-Y recorder Omnigraphic 2000 (Houston Instrument). To standardize the leaf area used for fluorescence, a cuvette was constructed to insure a constant leaf area and a constant distance between the leaf and the fibre optic probe. The 30 min dark adaptation period and the measurements of the samples were done at room temperature. Quenching parameters were estimated after approximately 3 min under actinic light when the leaves had reached steady-state fluorescence emission.

The different fluorescence parameters were measured as shown in Fig. 12 (see also Fig. 3b). F_o was measured in dark adapted samples using the modulated light beam. The pulses of white light were used to determine the F_m values. The ratio F_v/F_m was calculated from $F_v/(F_v + F_o)$. The estimation of the quenching parameters was based on the new F_o values reached at steady-state, F_o'. The q_p, q_N, and q_o values were calculated as followed:

$$q_p = \frac{(F_m' - F)}{(F_m' - F_o')}, \quad q_N = \frac{(F_v - F_v')}{F_v}, \quad q_o = \frac{(F_o - F_o')}{F_o}.$$

I followed the standardized nomenclature recently presented by Van Kooten and Snel (1990).

2.6 Photosynthetic measurements on isolated cells

CO_2 fixation rates were evaluated by measuring the incorporation of $^{14}CO_2$ (supplied as $NaH^{14}CO_3$ in the medium) into 0.2 mL cell samples after a preincubation period of 5 min at the same PPF and temperature as the measurement occurred. The vials were illuminated from the bottom with banks of 30 W reflector lamps and shaken in a temperature controlled water bath. After 10 min in presence of $^{14}CO_2$ (specific activity of 33.3 kBq μmol^{-1}, final concentration of 5 mM), the cells were killed in 6 N acetic acid and dried overnight. After addition of 10 mL of AQUASOL-2 (Dupont) to the samples, radioactivity was measured in a liquid scintillation counter and absolute rates of CO_2 fixation were calculated. The apparent quantum efficiency, ϕ_{app}, and

apparent carboxylation efficiency were estimated by linear regression from the initial linear portion of photon flux and $NaHCO_3$ response curves which represented 5 data points per sample (7 data points for ϕ_{app} at 20°C).

The fluorescence emission spectra were performed at 77 K on samples dark adapted for 10 min, then mixed in 25% (v/v) glycerol and frozen at -70°C. The Chl concentrations of the samples were about 7 $\mu g\ mL^{-1}$. The fluorescence emission spectra were collected using an LS-1 fluorescence spectrometer (PTI Inc., London, Ontario) with 5.6 mm emission and excitation slits and an excitation wavelength of 440 nm. The spectra were corrected for the wavelength sensitivity of the photomultiplier. The ratios F_{685}/F_{740} were calculated from the ratio of the areas under each peak.

Room temperature induction curves of fluorescence were obtained using a PAM Chlorophyll Fluorometer (Walz, PAM 101 unit) with the measuring probe in contact with the side of a plastic spectrophotometric cuvette with a 1 cm pathlength. The conditions were similar to that used for intact leaves (section 2.5). PPF of the modulated beam was 0.26 $\mu mol\ m^{-2}\ s^{-1}$, and the pulse of white light was adjusted to a PPF of 3450 $\mu mol\ m^{-2}\ s^{-1}$ for the first 5 flashes and to a PPF of 1830 $\mu mol\ m^{-2}\ s^{-1}$ for the rest of the measurements. The durations of the flashes were 400 ms with 5 s between each flash. The samples were diluted to 7 μg Chl mL^{-1}, and 5 mM $NaHCO_3$ was added to the dark adapted samples before measurements. Quenching parameters were estimated after approximately 3 min under actinic light when the cells had reached steady-state of fluorescence.

2.7 Photosynthetic measurements of thylakoids

PSII activity (H_2O → 2,6-dichlorophenol-indophenol (DCPIP)) was measured in the resuspension buffer containing 12 μg Chl mL^{-1} and 60 μM DCPIP. The reduction of DCPIP was followed in a Unicam SP1800 UV spectrophotometer at 590 nm in a jacketed cuvette thermoregulated at 25°C as described in Huner (1985a). A light source, filtered through a Cinemoid ruby No. 14 and an orange No. 5 (Strand Electric) filter, was used to excite the thylakoid sample. PPF was varied using neutral density filters. The signal was registered on an ISCO 613 recorder. Correction factors were applied to PSII activities to take into account dark decay of thylakoids at room temperature as a function of time.

PSI activity (ascorbic acid /oxidized DCPIP → methyl viologen (MV)) was measured in the resuspension buffer containing 12 μg Chl mL^{-1} as well as 20 μM DCMU, 5 mM NH_4Cl, 200 μM MV, 60 μM DCPIP, 1 mM NaN_3 and 61 μg mL^{-1} of superoxide dismutase (SOD) (Huner, 1985a). The DCPIP was reduced in the sample cell by adding 1 mM ascorbic acid. The reduction of MV was followed polarigraphically at 20°C with a Hansatech aqueous phase O_2 electrode and recorded on a SE 120 ABB Goerz AG recorder. The reaction was started by turning on a Björkman lamp attached to an optic fibre bundle. PPF was varied using neutral density filters. The apparent quantum efficiency, ϕ_{app}, of PSII and PSI was estimated by linear regression from the initial linear portion of PPF response curves which represented 6 data points per sample.

Chl fluorescence was recorded following the procedure used for the isolated cells. The modulated light beam had a PPF of 0.12 μmol m^{-2} s^{-1} and the pulsed light beam a PPF of 3450 μmol m^{-2} s^{-1}. The Chl concentration was 20 μg mL^{-1}. Correction factors were applied to F_v and to F_m to take into account dark decay of thylakoids at room temperature as a function of time.

Photoacoustic spectroscopy (PAS) measurements were done in collaboration with Dr. R. Carpentier and Dr. R. Leblanc (U.Q.T.R., Trois-Rivières, Qué.). The apparatus used has been described in detail in Carpentier *et al.* (1983). The energy flux of the modulated measuring light beam of 680 nm was varied between 0.16 and 4.15 W m^{-2}, at a frequency of 35 Hz. The non-modulated saturating light beam had an energy flux of 186 W m^{-2}. All measurements were done at room temperature. The thylakoids (240 μg Chl total) were aspirated through a nitrocellulose filter (Millipore Corp., AA type, 25 mm diameter, 0.8 μm pore size) that was then cut to a final diameter of 15 mm and introduced into the cell (Carpentier *et al.*, 1987). A new sample was prepared for each PAS measurement. Correction factors were applied to ϕ'_r to take into account dark decay of thylakoids at room temperature as a function of time.

2.8 Relative absorbance of thylakoids

To determine if these high PPF treatments were photobleaching the Chl pigments, I measured the relative absorbance of the thylakoid preparations following a similar time course as for the photosynthetic measurements. The relative absorbance

was measured at 680 nm in a Shimadzu UV-160 spectrophotometer, using 75 μg Chl in 0.5 mL.

2.9 Atrazine binding studies

Binding of atrazine was carried out according to Tischer and Strotmann (1977). Resuspended thylakoids diluted to 48 μg Chl mL^{-1} were pipetted in an Eppendorf tube and 4 to 40 μl of [ethyl-1-^{14}C]atrazine (925 kBq μmol^{-1}) were added with sufficient volume of 20% (v/v) ethanol solution to maintain a constant ethanol concentration of 4%. The total reaction volume was 1.0 mL. For the studies of binding kinetics, [^{14}C]atrazine concentrations from 0.02 to 20 μM were used. During photoinhibitory studies, a constant concentration of 1 μM of [^{14}C]atrazine was used. The thylakoid mixtures were incubated for 10 min under laboratory light conditions, then centrifuged for 3 min at 16 000 g, at 4°C. The supernatant (0.7 mL) was added to 10 mL of AQUASOL-2 (Dupont) and counted. Controls were carried out the same way but without thylakoids. The amount of labelled atrazine bound to the thylakoids was calculated from the difference between controls and corresponding samples.

2.10 High PPF treatments

Susceptibility to photoinhibition was analyzed at 5°C and 20°C, by submitting the cells to different combinations of high PPFs and incubation times. Cells were exposed to white light from a Fibre-Lite light source (Model 170-D, Dolan-Jenner Inc.) directed

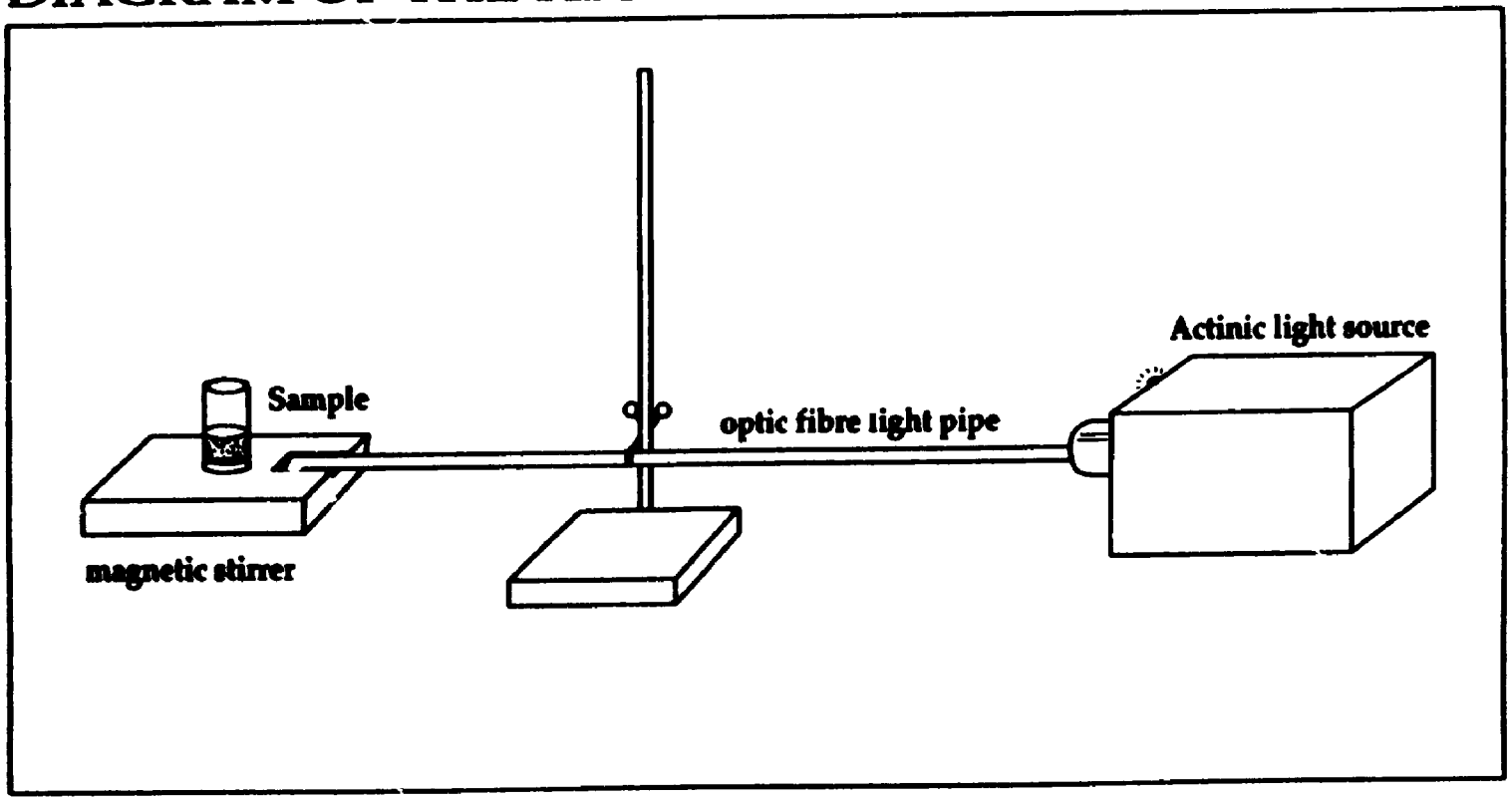
to the sample vial via an optic fibre bundle (Fig. 5). The samples were continuously stirred during the treatment. Recovery occurred in low PPF (30 μmol m^{-2} s^{-1}) at the same temperature as the photoinhibitory treatment. Control samples were kept at either room temperature or 5°C in the dark. For Chl fluorescence measurements, all samples were dark adapted for 10 min following the photoinhibitory treatment prior to measurements of Chl fluorescence. For CO_2 fixation measurements, the samples were submitted to the 5 min preillumination period (see section 2.6) immediately after the treatment.

Photoinhibition of isolated thylakoids was performed using the same device as for the high PPF treatment of isolated cells (Fig. 5). The treatments occurred at room temperature (approx. 20°C) at a PPF of 2600 μmol m^{-2} s^{-1}, unless otherwise mentioned. Some control samples were kept at room temperature and others on ice in the dark until measured. For Chl fluorescence measurements, all samples were dark adapted for 10 min following the photoinhibitory treatment prior to measurements of Chl fluorescence. For PSI, PSII and PAS measurements, the samples were measured immediately after the photoinhibitory treatment.

During photoinhibition and recovery studies, CO_2 fixation rates and fluorescence parameters of rye cells and all photosynthetic measurements of isolated thylakoids were expressed as a percentage of the control values measured on the same samples as followed: (activity of treated sample x 100)/(activity of control sample). The activity of the control samples of isolated rye cells was measured at the beginning and at the end

Figure 5: Diagram of high PPF treatment conditions. The distance between centre of the vial and the end of the optic fibre light pipe was about 2.5 cm and incident PPF was measured at that point.

DIAGRAM OF THE HIGH LIGHT TREATMENT SYSTEM



of the treatment and averaged. Control activities of isolated thylakoids were corrected as described in section 2.7.

Leaf sections floating on distilled water were submitted to 2600 μmol m^{-2} s^{-1} with the same light source used for cell and thylakoid high PPF treatments. A fan was used to circulate the air above the leaf sections to maintain a constant temperature during the treatment. After the treatment (4 h) the leaf sections were kept at room temperature in the dark overnight, then under normal laboratory light conditions for another 12 h to allow recovery to occur. All PPFs were measured with a LI-COR light meter (model LI-185A) from the end of the optic fibre bundles and represent photosynthetically active radiation (PAR).

2.11 Statistical analyses

All data are presented as the average ± SE from 4 replicates unless otherwise indicated. Analysis of variance (1-way or 2-way) was performed where indicated. Statistical data are presented as followed: $F(df_1,df_2)$, p, with F indicating the calculated F value, *df* the number of degrees of freedom, and p the probability level. One-way analysis of variance used as the criterion of classification the population (RH and RNH). Two-way analysis of variance were used here to compare the effect of two factors, population and temperature during the photoinhibitory treatment or during the recovery (20 and 5°C), on the response of rye to photoinhibition. For the analysis of variance of the protein synthesis inhibitor study, the two factors were population and treatment

conditions (absence/chloramphenicol/cycloheximide). For the analysis of variance of the assay PPF effect on the level of photoinhibition and of recovery measured, the two factors were population and PPF during measurements (29/54/250 μmol m^{-2} s^{-1}). Interactions between the simple effects of the two factors were also tested and if significant, least squares differences were calculated to determine which simple effects were significantly different. All analyses of variance were performed on equal number of replicates and on data collected at one time during a time course study. For analysis of variance of F_o and of Q_m, all data points of the time course were used to test the differences between RH and RH and between 5 and 20°C photoinhibitory treatments.

2.12 Protein synthesis inhibitor studies

Protein synthesis inhibitor solutions were added to the cells 10 min before starting the photoinhibitory treatment or at the beginning of the recovery period. Final concentrations of protein synthesis inhibitors were 25 μg mL^{-1} for chloramphenicol and 2 μg mL^{-1} for cycloheximide. The control samples were also treated in the dark with the same protein synthesis inhibitors for similar periods of time as the photoinhibited samples and their photosynthetic activities were usually within 10% of the CO_2 fixation rates measured at the beginning of the treatment.

2.13 L-[^{35}S]methionine uptake and incorporation in isolated cells

For ^{35}S-labelling of proteins, 7 mL of cells containing 15 μg Chl mL^{-1} were illuminated at 100 μmol m^{-2} s^{-1} at 5 or 20°C in a thermoregulated bath. I added 10 mM $NaHCO_3$ and 70 μL of L-[^{35}S]methionine solution (specific activity: 463 MBq μmol^{-1}, 10 mM) to the samples. Aliquots of 0.5 mL were taken during a time course and were washed on Whatman glass fibre filter discs (2.4 cm) with resuspension buffer and dried in a glass scintillation vial. Radioactivity was measured in a Beckman LS6000IC scintillation counter in presence of 5 mL of ECOSCINT (DiaMed).

An additional aliquot (0.5 mL) was taken after 2 h incubation and immediately frozen, to be used later for determination of total trichloroacetic acid (TCA) precipitable material. After thawing the sample, 1.5 mL of 12% (w/v) TCA containing 13 mM methionine was added and the sample was left overnight at 4°C. The protein precipitate was then collected by filtering through 2.4 cm Whatman glass fibre filter disc, washed three times with ice-cold 10% (w/v) TCA, three times with 80% (v/v) ethanol, once with acetone and twice with diethyl ether (DeVilliers and Ashton, 1977). The filter was then placed in a glass scintillation vial, dried under a heat lamp, and counted in presence of 5.0 mL of scintillation fluid. TCA precipitable material was estimated as the fraction of counts per minute (cpm) detected in TCA precipitable material compared with the total uptake of whole cells.

The fraction (15 to 20 mL) of the samples remaining after 2 h of incubation at 20°C was frozen at -70°C in thylakoid grinding buffer with 1 mm glass beads. Cells were

broken as described in section 2.4 and washed thylakoids were resuspended in a minimal volume of thylakoid resuspension buffer for Chl concentration determination. Thylakoids were again centrifuged and diluted in the following solution: 60 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl pH 7.8, 12% (w/v) sucrose, 1 mM Na_2-ethylenediaminetetraacetate (EDTA), 2% (w/v) sodium dodecyl sulfate (SDS), 0.9% (w/v) dithiothreitol (DTT), using sufficient volume of the SDS solution to maintain a ratio SDS/Chl (w/w) of 20. The samples were heated at 60°C for 20 min in the SDS solution and then loaded onto polyacrylamide gels, at about 15 μg Chl per lane. Subsamples were counted to determine the level of radioactivity loaded per lane.

2.14 Polyacrylamide gel electrophoresis and fluorography

Samples were run on urea SDS polyacrylamide gels on Mighty Small II apparatus (Hoefer Scientific Instruments, San Francisco) using the discontinuous buffer system of Laemmli (1970). A polyacrylamide solution was prepared containing 12% (w/v) acrylamide (0.4% (w/v) bisacrylamide), 0.46 M Tris-HCl pH 8.8, and 4 M urea, and degassed for 10 min. Then, 0.12% (w/v) SDS, N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate were added and the solution was poured in the mini-gel casting apparatus. The stacking gel solution contained 5% (w/v) acrylamide (0.13% (w/v) bisacrylamide), 0.13 M Tris-HCl pH 6.8, TEMED and ammonium persulfate. The running buffer contained 0.05 M Tris-HCl pH 8.8, 0.38 M glycine and 0.1% (w/v) SDS. Polypeptides were electrophoresed on a 1.5-mm thick gel at an initial current of 10 mA (200 V). Once the sample had migrated into the resolving gel, the

current was increased to 15 mA (200 V). The gel system was run at 4°C for approximately 3 h. Molecular mass standards (Bio-Rad) used were lysozyme, 14 400; soybean trypsin inhibitor, 21 500; carbonic anhydrase, 31 000; ovalbumin, 42 699; BSA, 66 200; phosphorylase B, 97 400 daltons.

Once the free pigment front reached the bottom of the gel, the gel was stained with the following Coomassie Blue solution: 0.1% (w/v) Coomassie Blue R, 40% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained in 20% (v/v) methanol and 0.075% (v/v) acetic acid. For fluorography, the destained gel was impregnated for 1 h with Amplify (Amersham) to which I added 10% (v/v) glycerol. The gel was then dried using a Gel Drying kit (Promega, Madison Wisconsin). The dried gel was placed in contact with X-Omat AR Diagnostic film (Kodak) and kept in the dark at -70°C for the required exposure time.

CHAPTER 3

RESULTS

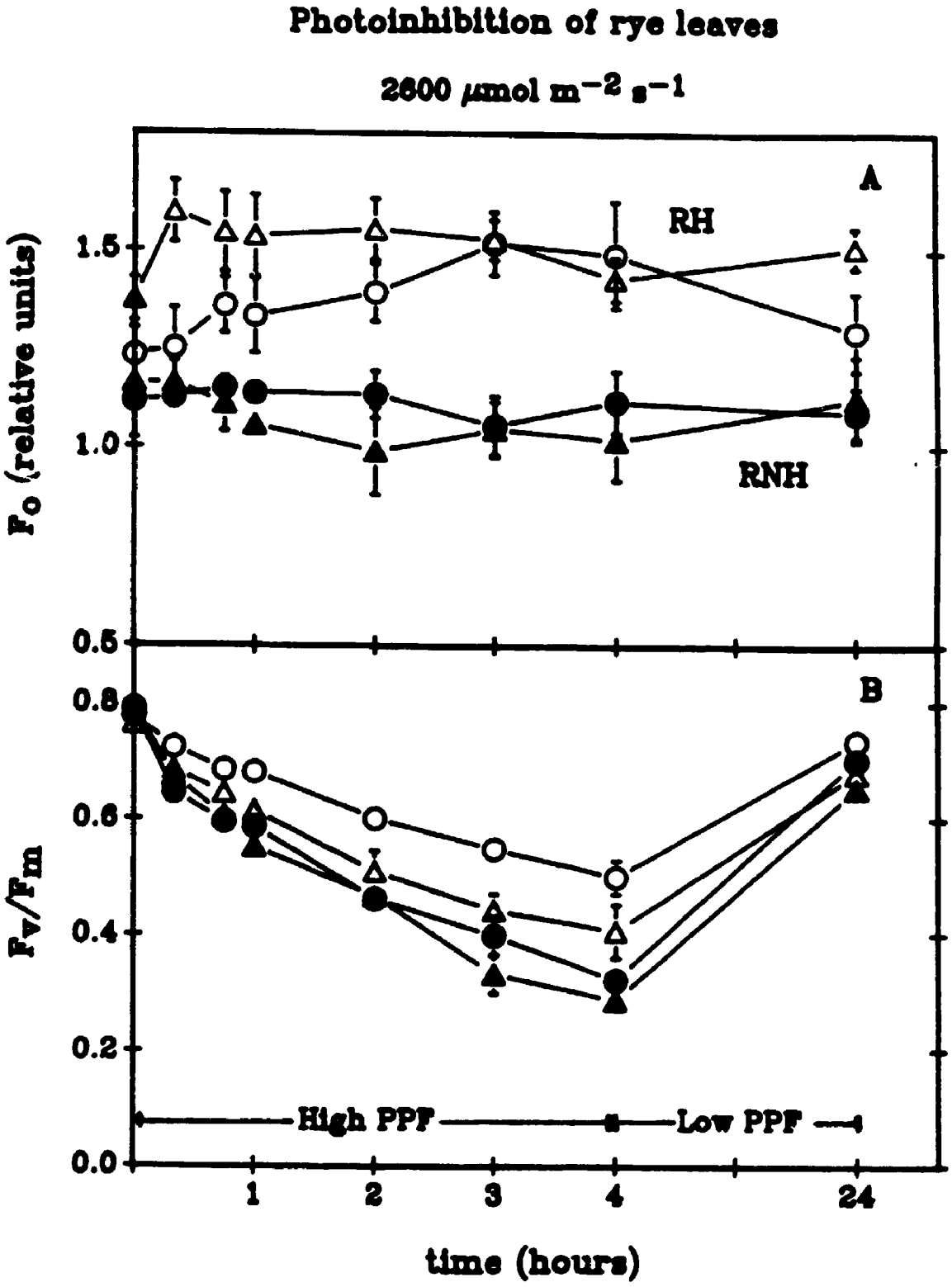
3.1 Photoinhibition and recovery of rye leaves

Even though the response of RH and RNH leaves to photoinhibition had already been studied (Öquist and Huner, 1991), further measurements were done under the same conditions as used for isolated cells and for isolated thylakoids. This was necessary in order to compare the photoinhibitory response of isolated cells and thylakoids with that of leaves. Furthermore, fluorescence quenching mechanisms were measured in rye leaves during and following photoinhibition to estimate the photosynthetic activity under steady-state conditions.

3.1.1 Fluorescence measurements under non steady-state conditions

F_o values were initially higher for RH than for RNH leaves (Fig. 6A). During high PPF treatments at 20 or at 5°C, F_o values of RNH did not change while F_o values of RH leaves increased over the first hour of treatment, then stayed high for the next 3 h. Once standardized to the initial F_o values, RH showed higher F_o values than RNH leaves during the photoinhibitory treatment ($F(1,156) = 34.32$, $p \leq 0.01$). After 20 h of recovery, the F_o values were still higher than control values for RH and did not change for RNH leaves.

Figure 6: F_o values (A) and F_v/F_m (B) during exposure of RH and RNH leaves to 2600 μmol m^{-2} s^{-1} (High PPF) at 5 and at 20°C. The extent of recovery after 20 h at 20°C is also presented (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○, △) RH, (●, ▲) RNH. All measurements were done at room temperature.

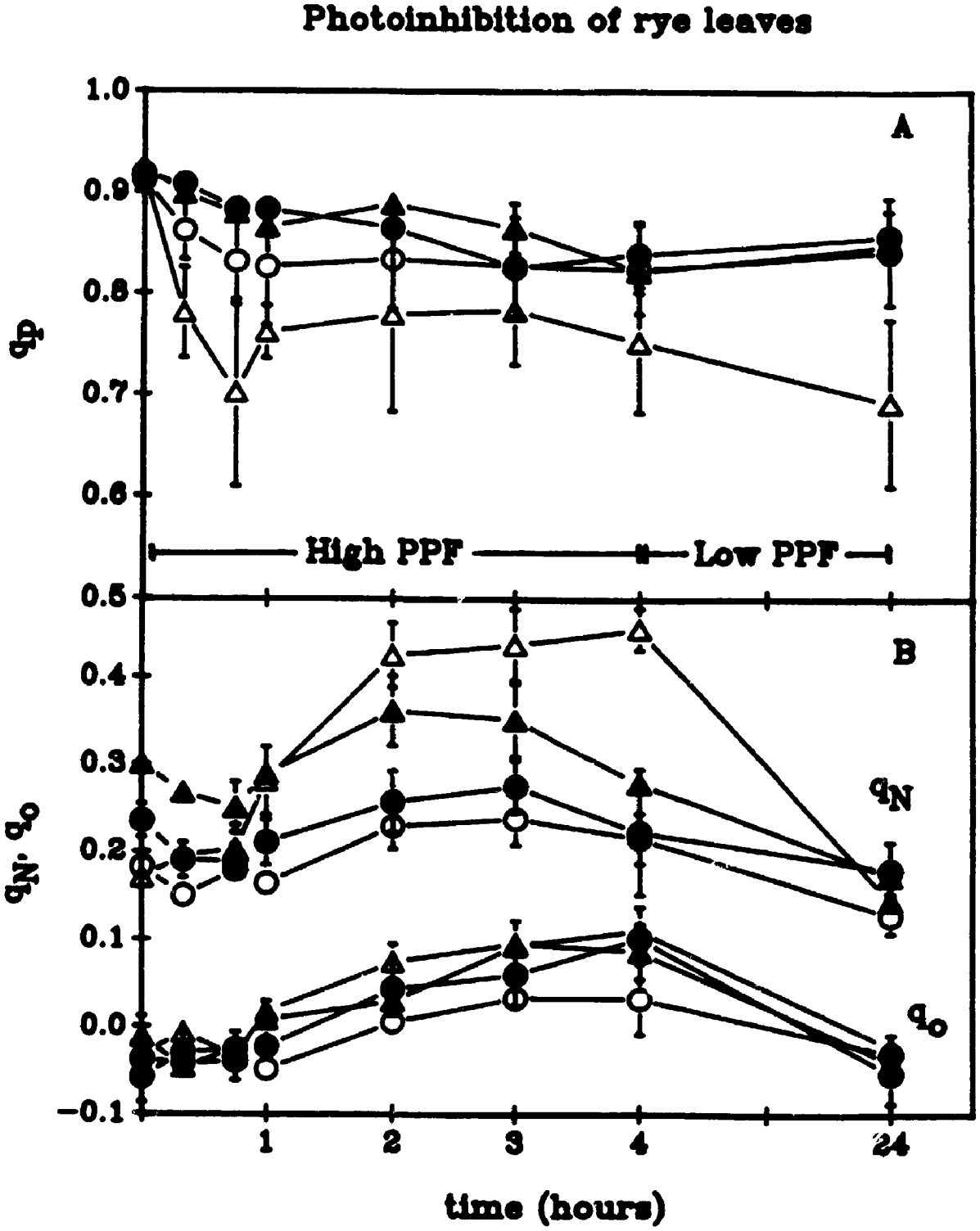

Photoinhibition of rye leaves
2600 μmol m⁻² s⁻¹
A
RH
RNH
B
Fo (relative units)
Fv/Fm
1.5
1.0
0.5
0.8
0.6
0.4
0.2
0.0
High PPF
Low PPF
1
2
3
4
24
time (hours)

Initial F_v/F_m ratios were 0.80 for RNH and 0.76 for RH; those differences were small but consistent ($F(1,14)= 11.25$, $p\leq 0.01$). Somersalo and Krause (1989) have also shown slightly lower F_v/F_m for hardened spinach, but Boese and Huner (1990) have not seen any difference between F_v/F_m of hardened and non-hardened spinach. High PPF treatments decreased substantially the F_v/F_m ratios of both RH and RNH leaves (Fig. 6B), but RNH were significantly more affected than RH leaves after both 5 and 20°C treatments ($F(1,12)= 22.72$, $p\leq 0.01$). The difference between 5 and 20°C treatments were not significant after 4 h of treatment. After 20 h of recovery at room temperature at low PPF, leaves recovered their F_v/F_m ratios to around 0.7. Recovery of F_v/F_m did not show significant difference between RH and RNH leaves, but for both RH and RNH, leaves photoinhibited at 20°C recovered to a slightly greater extent than leaves photoinhibited at 5°C ($F(1,12)= 11.52$, $p\leq 0.01$).

3.1.2 Fluorescence measurements under steady-state conditions

Control RH and RNH leaves showed similar q_p values of 0.92 while q_N values were higher for RNH leaves. During high PPF treatment at 5 and at 20°C, RNH q_p values slowly decreased over the 4 h of treatment (Fig. 7A). At 20°C, RH q_p values decreased from 0.91 to 0.83 while RNH q_p decreased from 0.92 to only 0.88 over the first hour. However, both RH and RNH leaves exhibited similar q_p values after 4 h of photoinhibition at 20°C. At 5°C, q_p values of RH leaves decreased from 0.92 down to 0.7 within 1 h, then increased slightly, but were maintained at lower values than for the 20°C treated leaves. In contrast, q_p of RNH leaves photoinhibited at 5°C was similar to

Figure 7: q_p values (A) and q_N and q_o (B) during exposure of RH and RNH leaves to 2600 μmol m^{-2} s^{-1} (High PPF) at 5 and at 20°C. The extent of recovery after 20 h at 20°C is also presented (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○, △) RH, (●, ▲) RNH. All measurements were done at room temperature.


Photoinhibition of rye leaves
A
B
High PPF
Low PPF
q_p
q_N, q_o
q_N
q_o
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
−0.1
1
2
3
4
24
time (hours)

that observed for RNH leaves at 20°C. Interestingly, after 20 h of recovery at low PPF, q_p values of RNH leaves treated at 20 and at 5°C and of RH leaves treated at 20°C returned to their original values (0.85 as opposed to 0.92 for control values), but q_p values of RH leaves treated at 5°C remained much lower at about 0.7.

Prior to high PPF exposure, q_N of RNH leaves (0.27) was slightly higher than that of RH leaves (0.18). At 20°C, q_N values of RNH and RH leaves changed little during the photoinhibitory treatment, increasing slowly between the first and third hour of treatment (Fig. 7B). At 5°C q_N values increased much more than at 20°C, especially in RH leaves. After 4 h of treatment at 5°C, q_N values of RH leaves were 2.7 times higher, but RNH leaves were 7% lower than control values. Following 20 h of recovery at low PPF, q_N decreased to values still lower than control values (57 to 85% of control values), with q_N values of RNH leaves (0.18) still higher than q_N values of RH leaves (0.14).

In control samples, q_o was actually negative at around -0.03 ± 0.01 in both RH and RNH leaves and became positive only after 1 h of photoinhibition. During the following 3 h of high PPF treatment, q_o increased slowly to reach 0.1 in RNH and 0.08 in RH leaves at both 5 and 20°C. After recovery at low PPF the q_o values were similar to the initial q_o values.

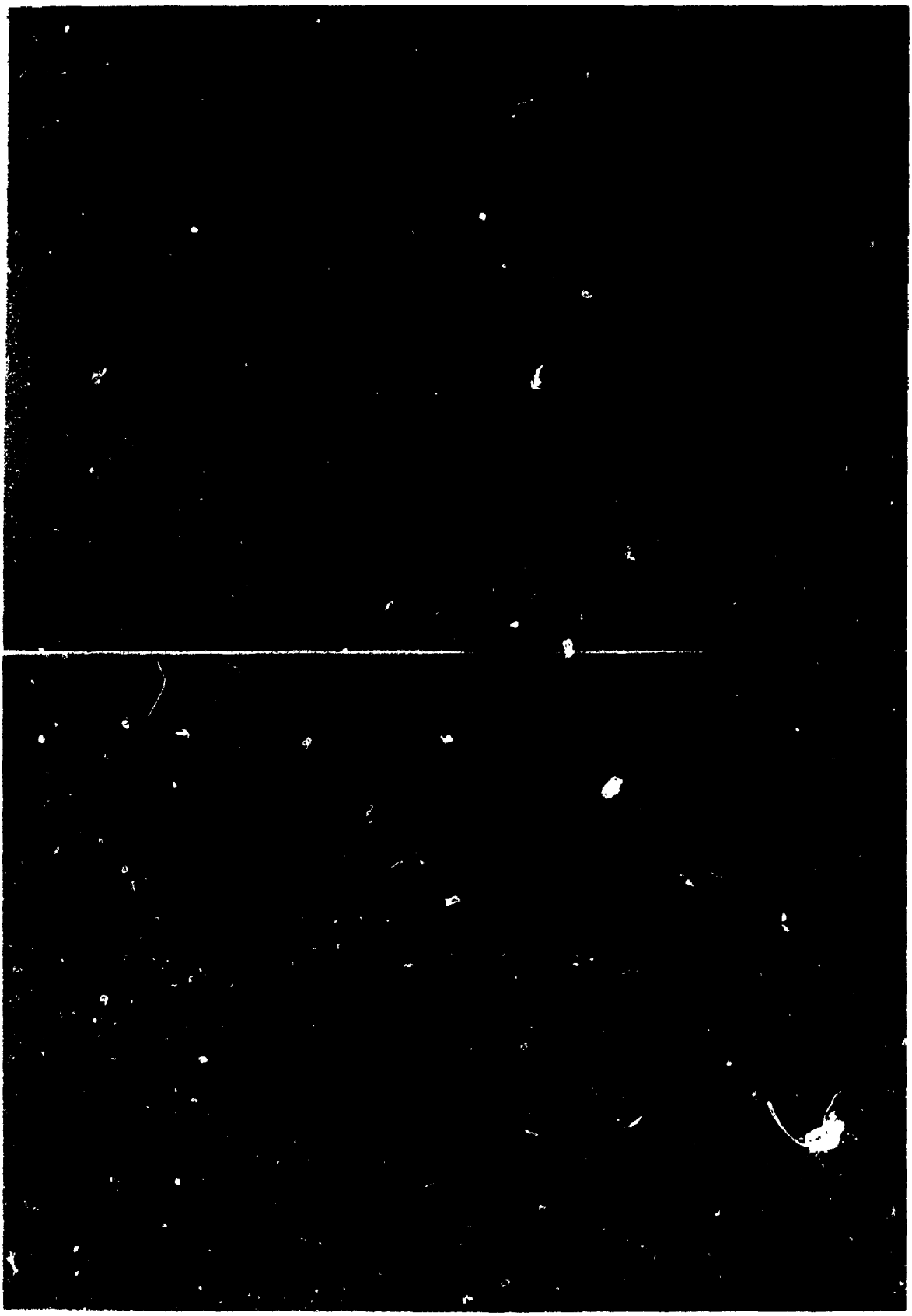
3.2 Characterisation of isolated rye mesophyll cells

In order to assess the competence of both RH and RNH cells after isolation, viability, photosynthetic and metabolic parameters were measured. Furthermore optimum conditions for CO_2 fixation rates were estimated.

3.2.1 Cell viability

The presence of high relative molecular mass (M_r) potassium dextran sulfate during cell isolation was essential for good yield. However, it was still necessary to eliminate dead cells using a Percoll gradient. The Percoll gradient purification step caused a 1.3-fold increase in the percentage of viable rye cells. This purification step also resulted in a 1.5-fold increase in the rate of CO_2 fixation of both RH and RNH cells Therefore, Percoll eliminates not only dead cells but also other components that inhibit cell activity. The average viability of purified cells, as measured by the appearance of the cells under phase contrast microscopy, was 80 ±6% for RH cells and 73 ±8% for RNH cells (Table II). Preliminary experiments comparing Evan's blue staining (Fig. 8) and phase contrast microscopy gave viability estimates that were not significantly different, 55 ±4% *vs.* 65 ±5% for RNH cells and 84 ±5% *vs.* 81 ±3% for RH cells. Therefore, viability of isolated cells was estimated by phase contrast microscopy for each sample used in this study. The yield of isolated mesophyll cells based on Chl content varied between 5% (RNH) and 10% (RH); this represented 3.3 x 10^6 (RNH) to 6.7 x 10^6 (RH) cells per g of fresh weight of leaves.

Figure 8: Photograph of RNH (A) and RH (B) mesophyll isolated cells. The samples were stained with Evan's blue and photographed under bright field.



To obtain cells with high photosynthetic activities it was necessary to submit the plant to a 24 h dark period followed by 2 h of illumination before collecting the leaves for cell isolation (Servaites and Ogren, 1977a). The osmolarity of the medium was also important: in a medium of low osmolarity, 0.25 M sorbitol, the cells had CO_2 fixation rates 1.4 times higher than in the high osmolarity solutions of 0.6 to 0.8 M used by many authors (Aono *et al.*, 1974). In addition, the presence of BSA in the medium was important to preserve the photosynthetic activity of rye cells (Larsen *et al.*, 1981). Preliminary work showed that the photosynthetic activity of rye cells was not affected by the pH of the resuspension buffer between a pH of 7 and 8.6. At pH lower than 7.0, the photosynthetic activity of rye cells was reduced.

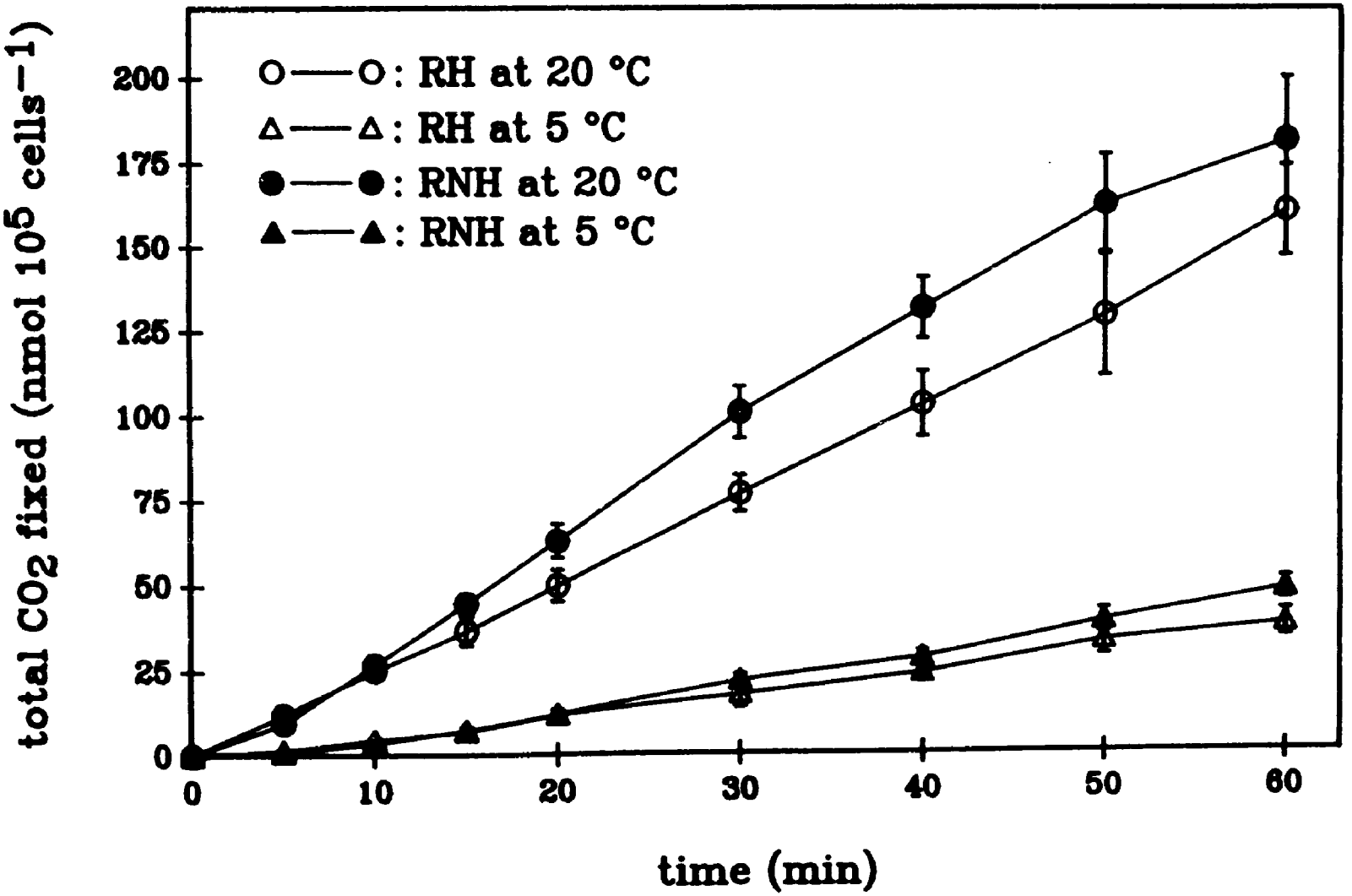
Cell areas have been estimated to be 1420 ± 30 μm^2 for RH cells and 1660 ± 30 μm^2 for RNH cells. Their perimeters were 160 ± 2 μm for RH and 187 ± 3 μm for RNH cells. Size variation between cells was high (standard deviations values represented 28% of the means values for n= 250), but in general, populations of RNH cells showed a large range of sizes from small cells (area < 1150 μm^2) to very big cells (area > 2470 μm^2), while RH cells were more uniform in size.

3.2.2 CO_2 fixation rates

The amount of CO_2 fixed increased linearly within the first hour of measurement for both groups of cells (Fig. 9). After 5 min in presence of $Na^{14}CO_3$ the rates were constant; therefore I used 5 min of preillumination for all the other measurements. The

Figure 9: Total CO_2 fixed as a function of time at 20 and at 5°C for RH and RNH cells. CO_2 fixation rates were light saturated, CO_2 saturated and measured at the incubation temperature. Triangles and circles represent measurements at 5°C and at 20°C respectively. (○, △) RH, (●, ▲) RNH.

TOTAL CO_2 FIXED VS. INCUBATION TIME



rate of CO_2 fixed was about 5 times faster at 20°C than at 5°C for both RH and RNH cells. Because these rates were CO_2 saturated (5 mM $NaHCO_3$, see Fig. 11), the effect of temperature on photorespiratory rates can be neglected. Dark CO_2 fixation represented 2% at 20°C and 3% at 5°C of the total CO_2 fixed in the light in both RH and RNH cells (data not shown); these dark CO_2 fixation rates were subtracted from the light dependent rates to determine net CO_2 fixation rates.

The highest CO_2 fixation rates were recorded at 35°C for RNH cells and at 30°C for RH cells (Fig. 10). From 5 to 20°C, RH and RNH cells showed similar CO_2 fixation rates. Over 25°C, RNH cells increased their photosynthetic activity to a greater extent with an increase in temperature than RH cells. Both groups of cells showed inhibition at 40°C, and no activity was detected at 45°C.

The curves in Figure 11 show no difference between the $NaHCO_3$ concentrations required to saturate the photosynthetic activities of RH and RNH cells. I chose 5 mM as the saturating $NaHCO_3$ concentration for all other measurements. Relative carboxylation efficiency, represented by the initial slope of the curves was 3.8 (RH) to 7.2 (RNH) times higher at 20°C than at 5°C (Table II). RNH cells showed higher relative carboxylation efficiency at 20°C than RH cells, but both had similar relative carboxylation efficiencies at 5°C.

Rye cells reached their light saturated rates of CO_2 fixation at 250 μmol m^{-2} s^{-1} at 20°C, but needed only 50 (RNH) to 75 (RH) μmol m^{-2} s^{-1} at 5°C to attain light saturated

Figure 10: CO_2 fixation rates as a function of temperature of incubation for RH and RNH cells. CO_2 fixation rates were light saturated, CO_2 saturated and measured at the incubation temperature. Triangles and circles represent measurements at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH.

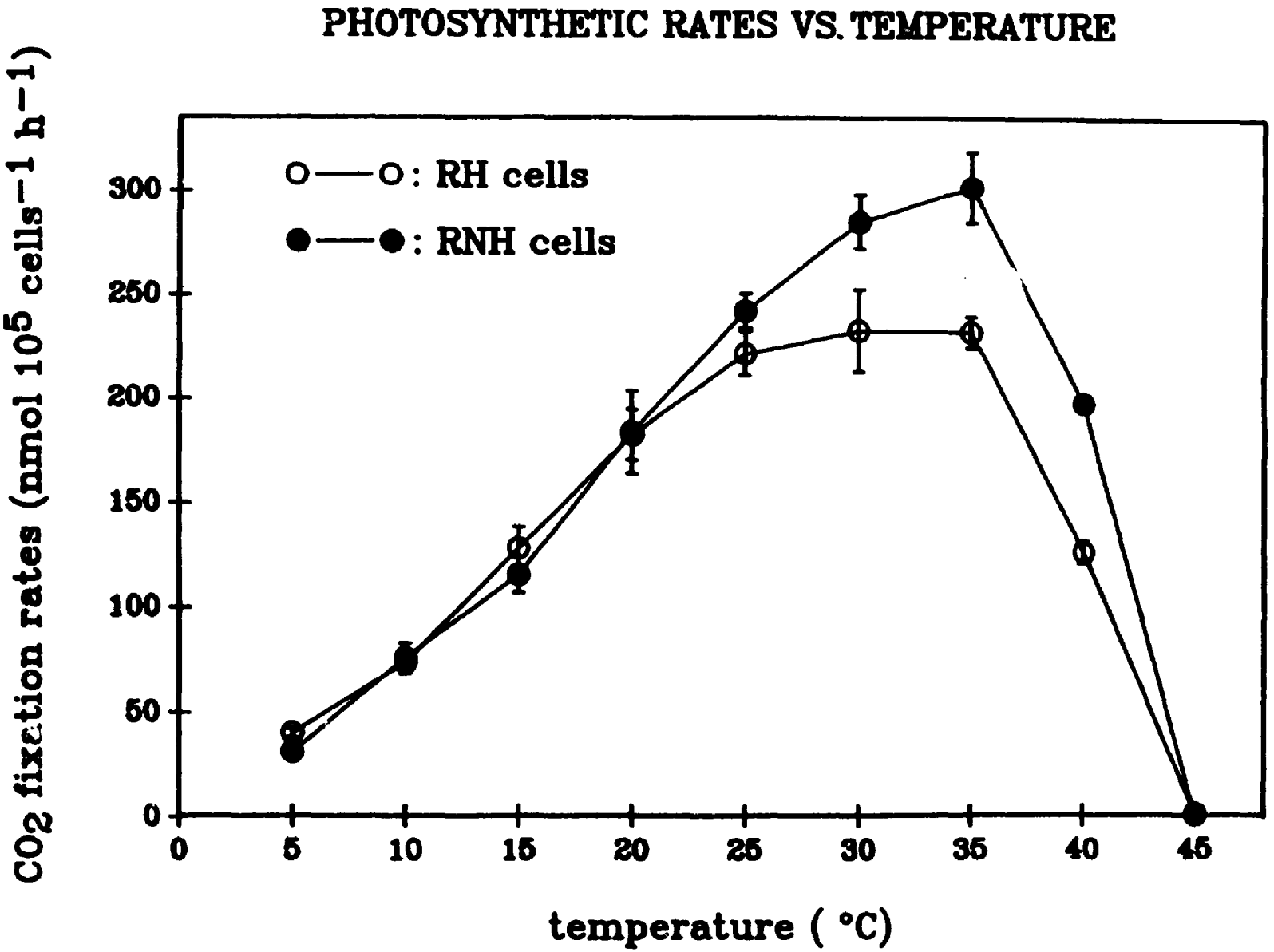
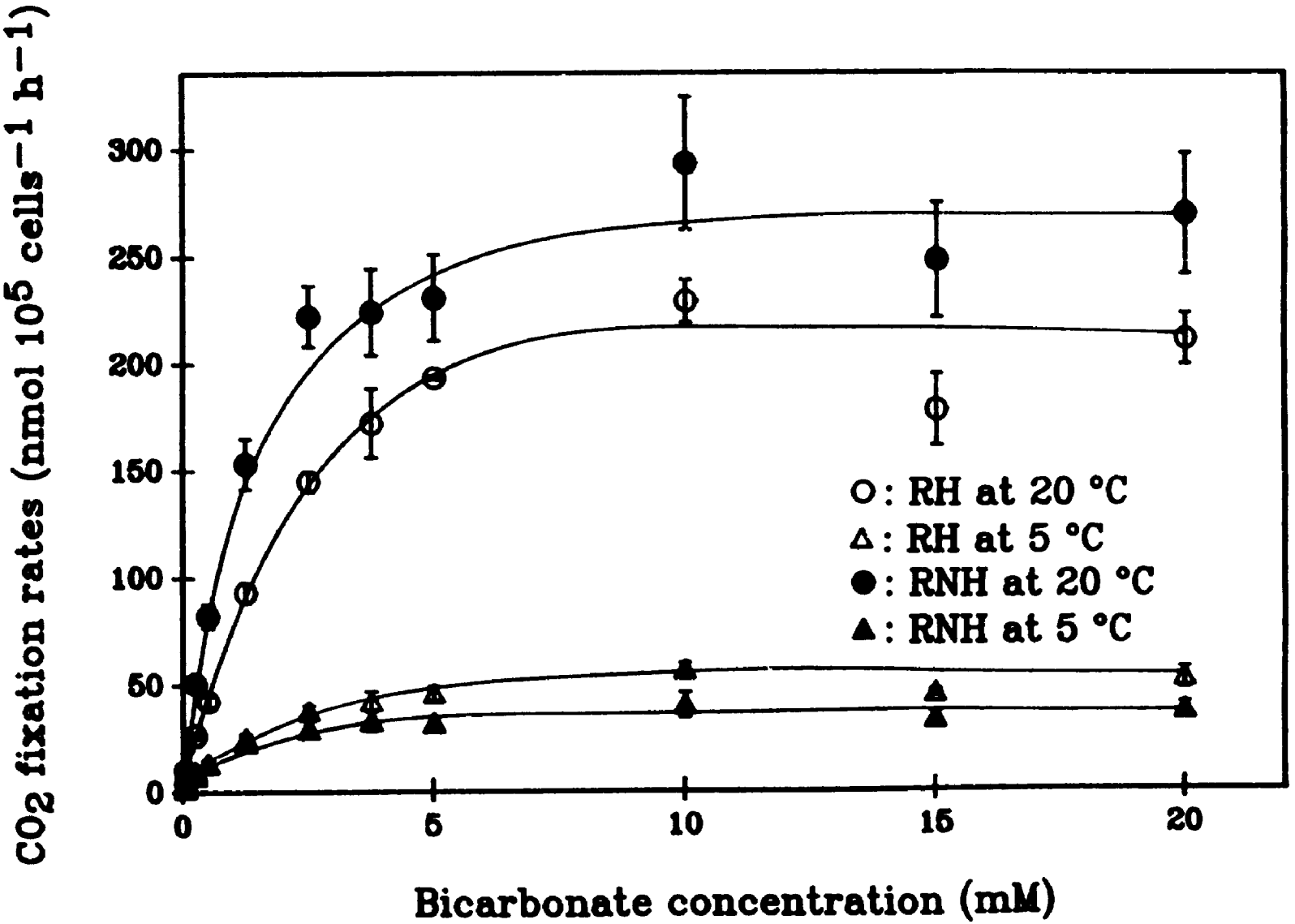

PHOTOSYNTHETIC RATES VS. TEMPERATURE
O——O : RH cells
●——● : RNH cells
CO_2 fixation rates (nmol 10^5 cells^{-1} h^{-1})
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
temperature (°C)

Figure 11: $NaHCO_3$ response curves at 20 and at 5°C for RH and RNH cells. CO_2 fixation rates were light saturated and measured at the incubation temperature. Triangles and circles represent measurements at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH.

PHOTOSYNTHETIC RATES VS. BICARBONATE CONCENTRATION



rates (Fig. 12). A PPF of 250 μmol m^{-2} s^{-1} was used in all other measurements where light saturated CO_2 fixation rates were measured. The ϕ_{app} was 1.4 (RH) to 2.2 (RNH) times higher at 20°C than at 5°C for rye cells (Table II). As observed with the relative carboxylation efficiency, the ϕ_{app} of RNH cells was higher than the ϕ_{app} of RH cells at 20°C, but both showed similar ϕ_{app} at 5°C. According to the data presented in figures 11 and 12, RNH cells showed significantly higher light saturated rates than RH cells at 20°C ($F(1,10)= 7.94$, $p\leq 0.05$) while at 5°C, RH cells had significantly higher light saturated rates ($F(1,10)= 19.40$, $p\leq 0.01$).

Table II summarizes the principal photosynthetic characteristics of isolated RH and RNH cells. CO_2 fixation rates on a Chl basis are also presented to compare with intact leaf photosynthetic rates and with previous studies on isolated cells. RH cells showed higher light saturated CO_2 fixation rates per mg of Chl than RNH cells at both 20 and 5°C; this is probably related to the differential extraction of Chl by the usual pigment extraction methods used. RH cells retained more Chl than RNH cells after extraction with 90% acetone. Two other extraction techniques were tried without success: extraction with a methanol:acetone mixture (1:1 v/v) for 1 h at -20°C (Bowles *et al.*, 1985), or with 1% Triton X-100, stirred for 15 min and then diluted with 90% acetone (Oliver *et al.*, 1979). F_v/F_m ratios of rye cells are also included (see section 3.2.4) and are similar to the F_v/F_m of rye leaves. Therefore, both RH and RNH cells appeared to be photosynthetically competent after isolation and purification.

Figure 12: PPF response curves at 20 and at 5°C for P.H and RNH cells. CO_2 fixation rates were CO_2 saturated and measured at the incubation temperature. Triangles and circles represent measurements at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH.

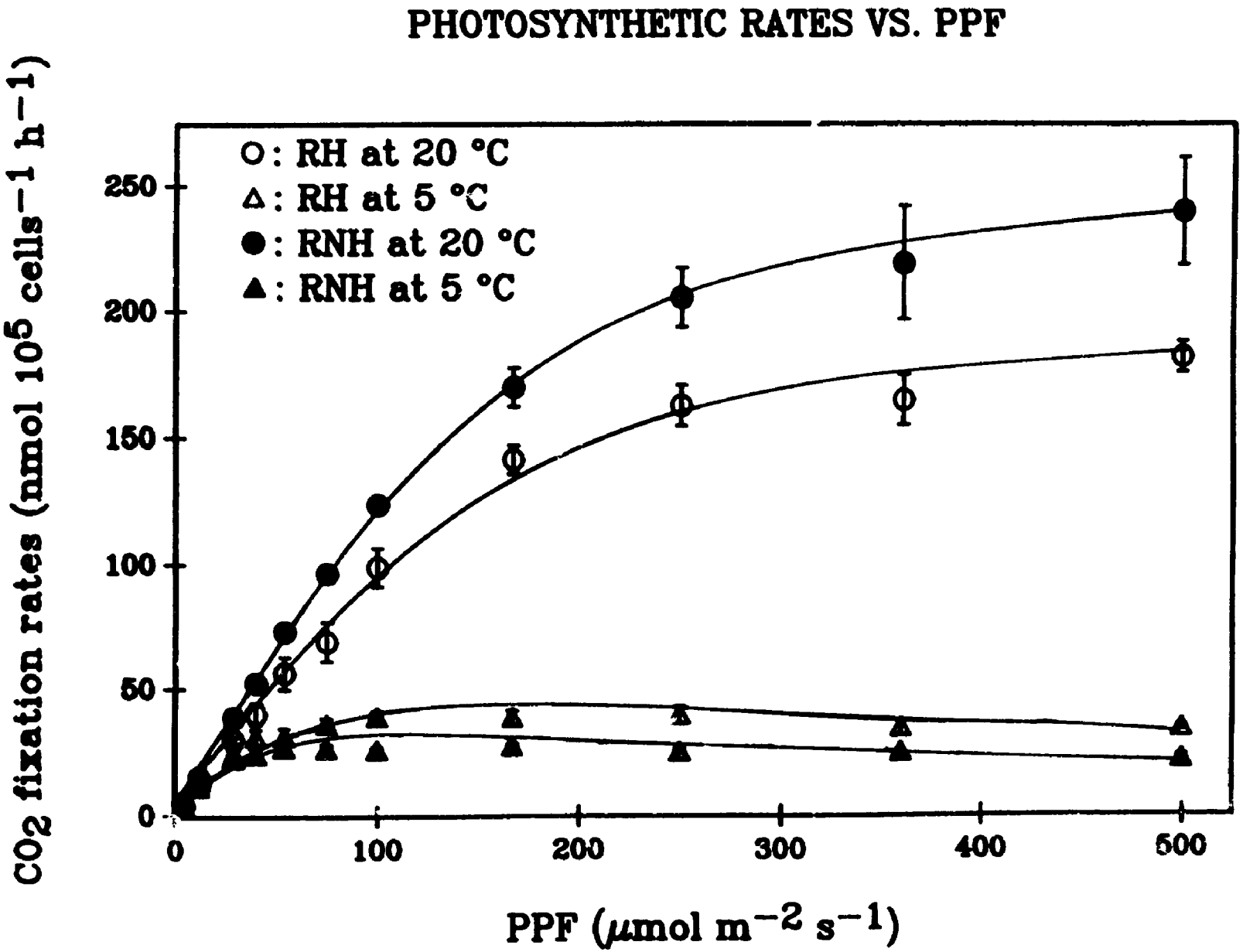

PHOTOSYNTHETIC RATES VS. PPF
O: RH at 20 °C
Δ: RH at 5 °C
●: RNH at 20 °C
▲: RNH at 5 °C
CO2 fixation rates (nmol 10^5 cells^-1 h^-1)
PPF (μmol m^-2 s^-1)
0
50
100
150
200
250
0
100
200
300
400
500

TABLE II: CO_2 fixation rates expressed on a cell basis, and on a Chl basis, ϕ_{app} and carboxylation efficiency, F_v/F_m ratios and percent of viability of RH and RNH cells. CO_2 fixation rates have been measured at 5 and at 20°C, under light saturated, CO_2 saturated conditions (± SE).

	RNH		RH	
parameter	20°C	5°C	20°C	5°C
CO_2 fixed 10^{-5} cells h^{-1}	218 ±12	28 ±2	178 ±8	43 ±3
CO_2 fixed mg Chl^{-1} h^{-1}	28 ±2	4 ±1	53 ±3	12 ±2
ϕ_{app}[1]	1.27 ±0.02	0.59 ±0.05	0.98 ±0.08	0.69 ±0.06
carboxylation efficiency[2]	98 ±7	14 ±2	62 ±1	17 ±1
viability	73 ±8	___	80 ±6	___
F_v/F_m	0.71 ±0.04	___	0.71 ±0.01	___

1 units: μmol CO_2 10^5 viable cells^{-1} h^{-1} μmol photons^{-1} m^2 s^1
2 units: μmol CO_2 10^5 viable cells^{-1} h^{-1} mM CO_2^{-1}

3.2.3 Fluorescence emission spectra

RH and RNH cells exhibited similar fluorescence emission spectra at 77K (Fig. 13). Fluorescence emission spectra showed a large absorption peak at 740 nm and a much lower peak of absorption at 680 nm and a shoulder at 685 nm. The emission peaks at 685 nm and 695 nm have been assigned to PSII core antennae (Briantais *et al.*, 1986), while the emission peak at 735 nm has been assigned to PSI. The PSII peaks are thus slightly blue shifted in rye cells. Their ratios F_{680}/F_{740}, which estimate the relative fluorescence emission of PSII and its antennae in relation to PSI, were 0.367 ±0.018 for RNH cells and 0.361 ±0.020 for RH cells.

3.2.4 Chl *a* fluorescence induction curves

RH and RNH cells exhibited different fluorescence induction curves (Fig. 14). The maximal fluorescence of RNH cells was quenched more rapidly than that of RH cells. However, at steady-state RNH cells exhibited higher fluorescence yield than RH cells. F_v/F_m was similar on average in RNH (0.71 ±0.04) and in RH cells (0.71 ±0.01), but the highest F_v/F_m values were measured in RNH cells. F_o values were variable since the samples were diluted as a function of cell number and the Chl concentration of the samples varied.

At steady-state, RH showed higher q_p values (0.65 ±0.01) than RNH cells (0.43 ±0.05) but similar q_N values at around 0.4. The quenching of F_o, q_o, was also different between RH and RNH cells. While RH cells quenched F_o slightly during photosynthesis

Figure 13: Corrected fluorescence emission spectra at 77K of RH and RNH cells. Samples contained 7 μg Chl ml^{-1}. The excitation wavelength was 440 nm.

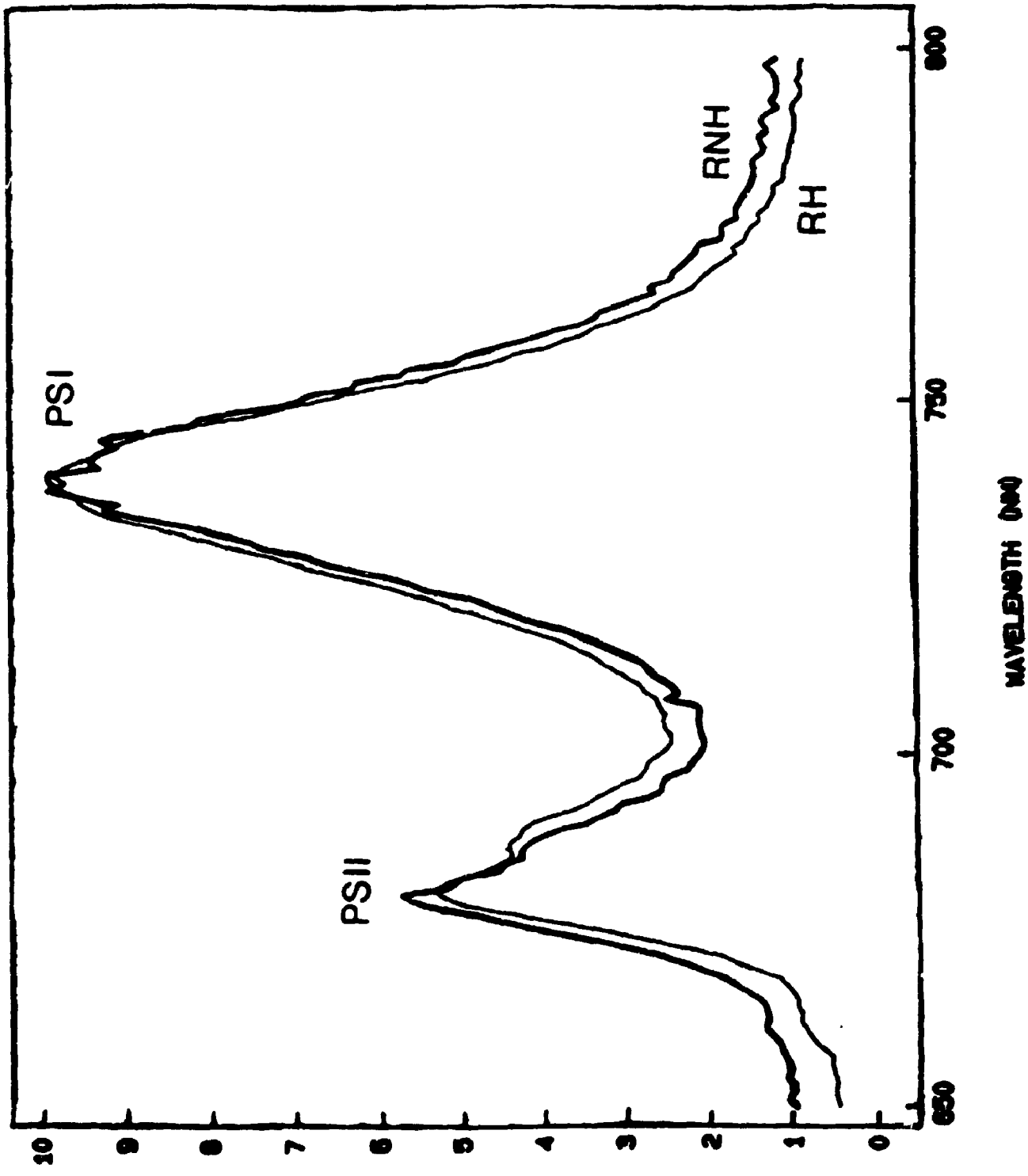
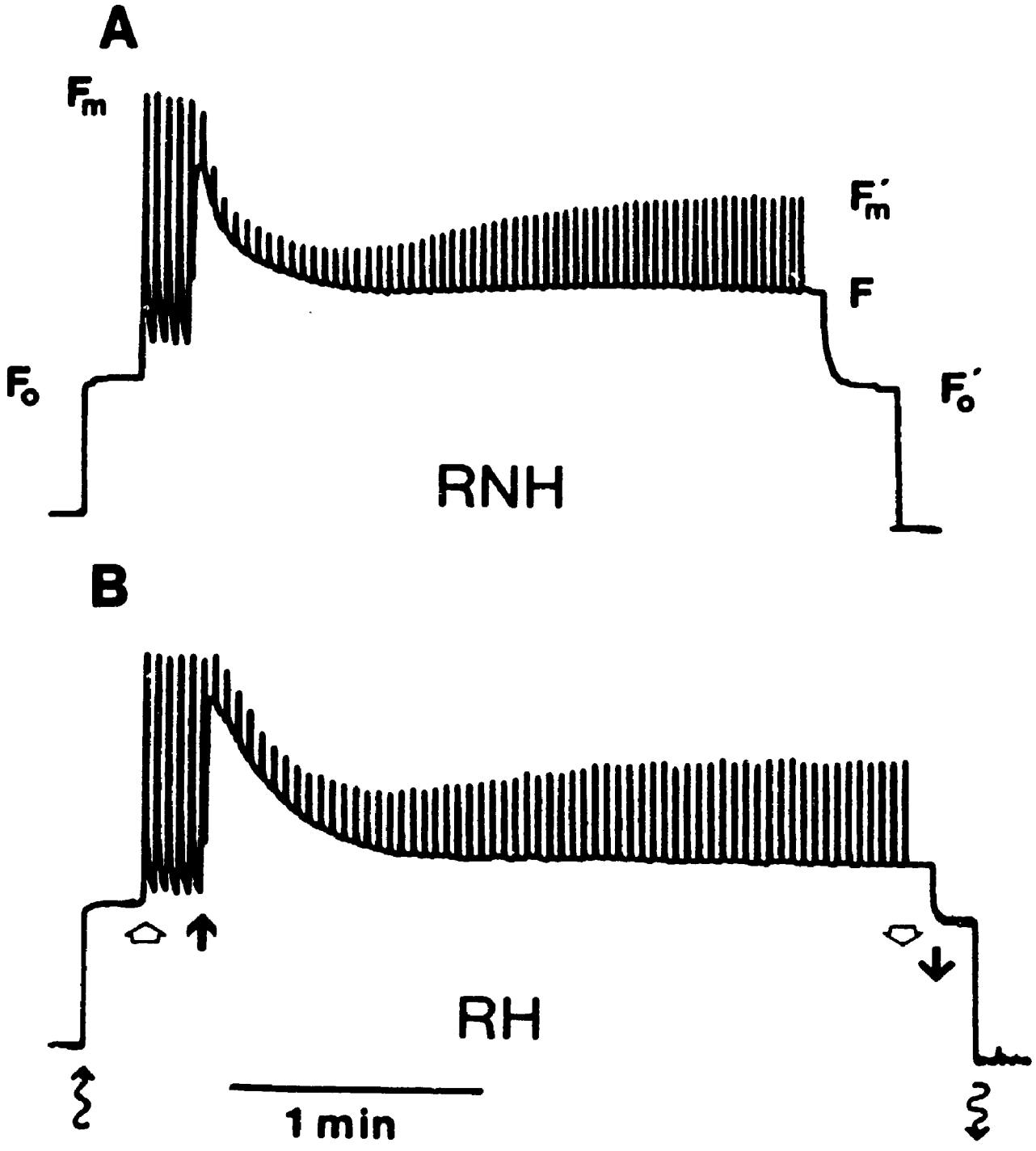

PSI
PSII
RNH
RH
SCALED FLUORESCENCE INTENSITY
WAVELENGTH (nm)

Figure 14: Room temperature Chl *a* fluorescence induction curves of A) RNH and B) RH cells. Modulated light source on ≀, off ≀. Saturating light pulses on ⌓, off ⌓. Non-modulated 650 nm actinic beam on ↑, off ↓.


A
F_m
F_m'
F
F_o
F_o'
RNH
B
RH
1 min

(0.08 ±0.01), RNH cells showed higher F_o under steady-state conditions than after dark adaptation (-0.07 ±0.01). The q_p, q_N and q_o values are relative values obtained from the calculations presented in Chapter 2.

3.2.5 L-[^{35}S]methionine labelling

To assess the integrity of these isolated mesophyll cells I also measured their capacity to import and use amino acids. The uptake of L-[^{35}S]methionine by rye cells was linear during the 2 h incubation period at 20°C, but reached a plateau within the first hour of incubation at 5°C (Fig. 15). The uptake of L-[^{35}S]methionine was much lower at 5°C and RNH took up significantly more of L-[^{35}S]methionine at both 20 ($F(1,94) = 15.7$, $p \leq 0.01$) and 5°C ($F(1,62) = 18.926$, $p \leq 0.01$) than RH cells. TCA precipitable material showed the same trend: more L-[^{35}S]methionine had been incorporated at 20°C than at 5°C and RNH cells had incorporated 1.4 times more of the L-[^{35}S]methionine present in the cells than RH cells (Table III).

3.2.6 Polypeptide profile of isolated rye cells

Figure 16 presents the polypeptide composition of thylakoids isolated from RH (B) and RNH (C) cells as well as the fluorograms of these same samples (D and E). Thylakoid proteins were preferred to total soluble protein content to assess that the cells were capable of synthesizing complete and functional chloroplast proteins. The SDS polyacrylamide gel showed no difference in the thylakoid composition between RH

Figure 15: Uptake of L-[^{35}S]methionine at 20 and at 5°C as a function of incubation time for RH and RNH cells. Triangles and circles represent incubation at 5°C and at 20°C respectively. (○, △) RH, (●, ▲) RNH.

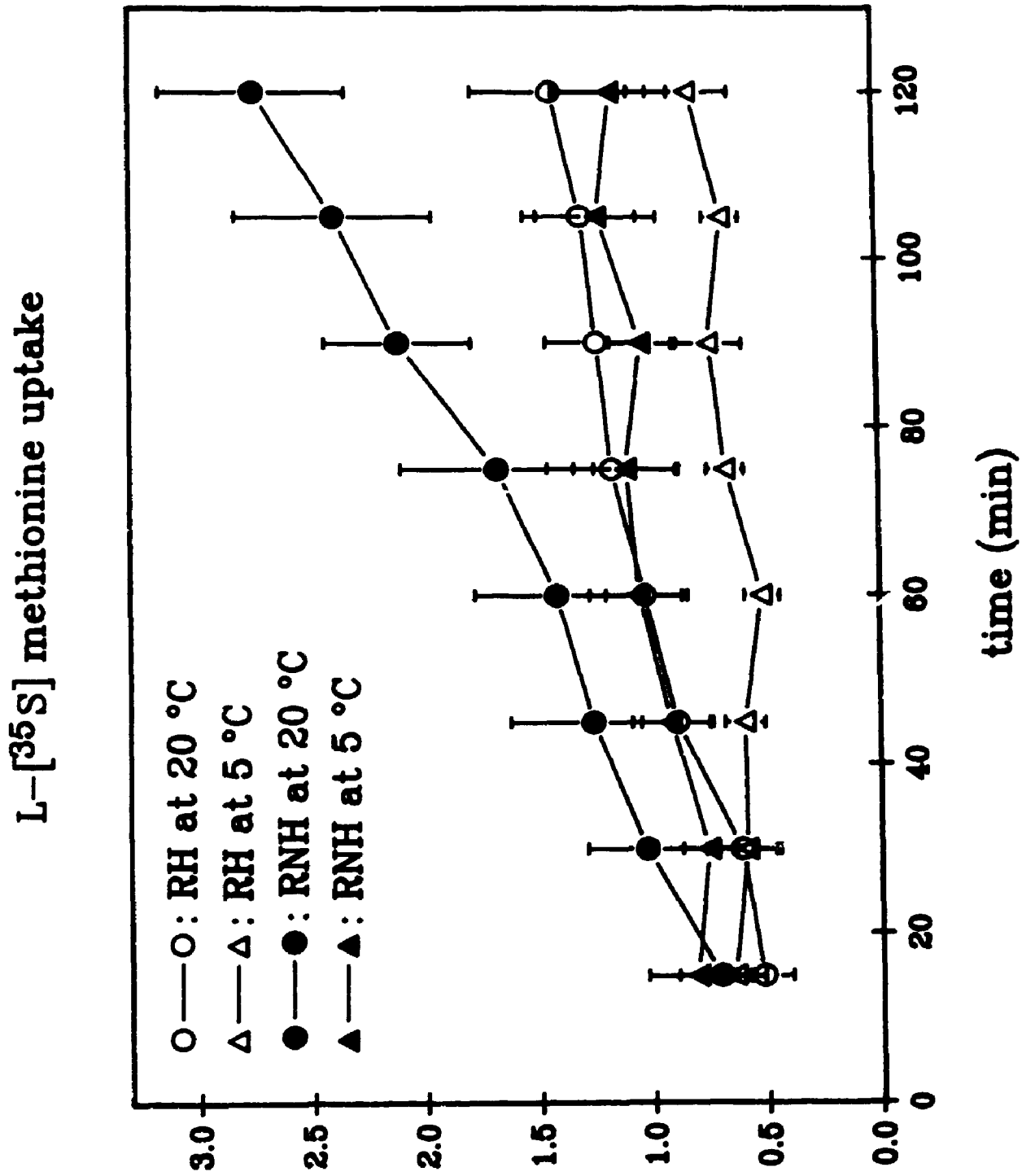
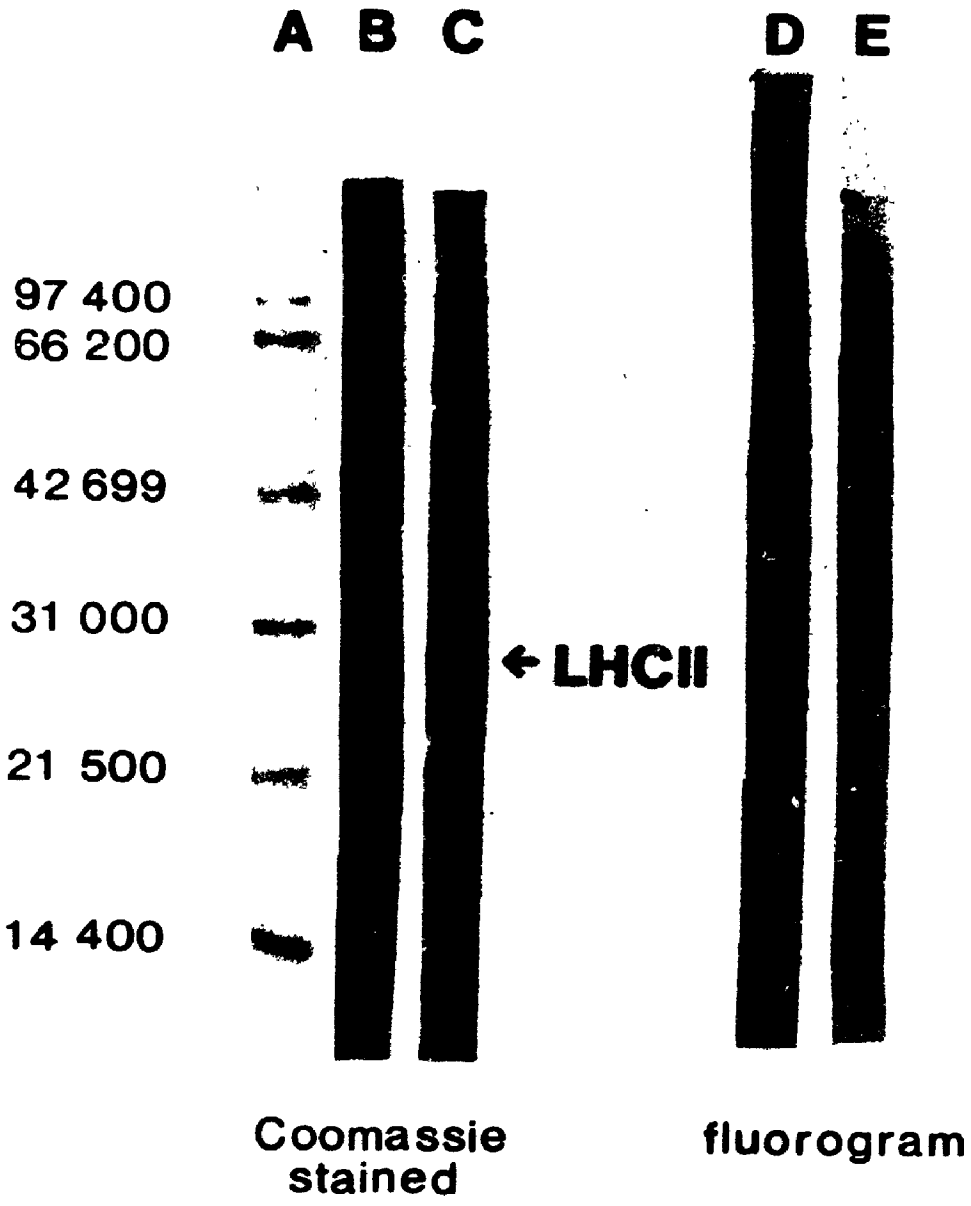

L-[35S] methionine uptake
μmol methionine 10⁵ cells⁻¹ (x10⁻⁶)
time (min)
O—O: RH at 20 °C
Δ—Δ: RH at 5 °C
●—●: RNH at 20 °C
▲—▲: RNH at 5 °C
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
120

TABLE III: Percentage of total L-[^{35}S]methionine uptake that precipitated with TCA in RH and RNH cells after 2 h of incubation in presence of L-[^{35}S]methionine at 5 and at 20°C.

	Temperature during L-[^{35}S]methionine uptake	
sample	20°C	5°C
	% of total incorporation	
RNH	42.1	32.1
RH	31.4	21.8

The values represent the percentage of total uptake incorporated into TCA precipitable material and are the means of two replicates.

Figure 16: Polypeptide profile of rye cells. Coomassie Blue stained gels of RNH (B) and RH (C) isolated thylakoids. Fluorogram from the same gels of RNH (D) and RH (E) isolated thylakoids. Samples containing about 15 μg of Chl and 35 500 cpm were loaded on each lane. Molecular mass standards were loaded in lane A and stained with Coomassie Blue.


A B C
D E
97 400
66 200
42 699
31 000
21 500
14 400
← LHCII
Coomassie stained
fluorogram

and RNH cells. The fluorograms showed that many polypeptides were synthesized during the 2 h incubation period of rye cells at 20°C in presence of L-[^{35}S]methionine. Most of the polypeptides synthesized during that period were of M_r between 43 000 and 100 000. Another major thylakoid polypeptide synthesized in rye cells had a M_r slightly higher than 31 000 and is barely apparent on Coomassie Blue-stained gel (B and C). It is probably the 32 kDa polypeptide, also called the Q_B protein (Ohad *et al.*, 1984). Interestingly, the polypeptides synthesized to a greater extent during the 2 h period were not the major polypeptides of rye thylakoids. These abundantly synthesized polypeptides might be turned over rapidly and therefore maintained at a low concentration in thylakoids.

The only band that differed between RH and RNH fluorograms was around 43 000 M_r but in another experiment this difference did not show up. Therefore the difference seen in this gel is probably related to a differential separation or solubilization of the polypeptides in the two lanes. Mature RH and RNH cells synthesized the same polypeptides during short term pulse-labelled experiments, but RNH cells synthesized more of them as shown by the L-[^{35}S]methionine uptake (Fig. 15) and the TCA precipitable material experiments (Table III).

3.3 Photoinhibition of isolated rye mesophyll cells

The same parameters were measured in isolated rye cells as had been measured for rye leaves, F_o, F_v/F_m, q_o, q_p, and q_N during photoinhibition. Both high photon flux

(2600 μmol m^{-2} s^{-1}) and moderate photon flux (400 μmol m^{-2} s^{-1}) were used to induce photoinhibition in rye cells to compare with the present study on rye leaves and the study of Öquist and Huner (1991) where rye leaves were photoinhibited at 400 μmol m^{-2} s^{-1}. Furthermore, the effect of photoinhibition on the overall photosynthetic activity of rye cells was also monitored by light limited CO_2 fixation rates.

3.3.1 Fluorescence measurements under non steady-state conditions

F_v/F_m ratios of rye cells decreased linearly as a function of time when exposed to 2600 μmol m^{-2} s^{-1} (Fig. 17A). No significant difference in photoinhibitory resistance to high PPF treatment was observed between RH and RNH cells. However, F_v/F_m ratios of RH cells were always slightly higher than RNH F_v/F_m ratios. The 5 and 20°C treatments caused similar reduction of F_v/F_m ratios of rye cells.

I also measured F_v/F_m during a time course at 400 μmol m^{-2} s^{-1} to see if a lower PPF would differentiate the photoinhibitory response of RH and RNH cells at 5 and at 20°C (Fig. 17B). RH cells retained 90 to 95% of their initial F_v/F_m ratios even after 3 h at 400 μmol m^{-2} s^{-1}. In contrast, RNH cells were significantly more affected than RH cells ($F(1,12)= 32.90$, $p\leq 0.01$), and lost 20% of their initial F_v/F_m ratios after 3 h at 400 μmol m^{-2} s^{-1}. The 5 and 20°C treatments induced the same level of inhibition of F_v/F_m.

F_o values were plotted for both 400 and 2600 μmol m^{-2} s^{-1} treatments (Fig. 18). Treatments at 2600 μmol m^{-2} s^{-1} (solid lines) maintained, on average, F_o values close to

Figure 17: F_v/F_m ratios of RH and RNH cells during exposure to 2600 μmol m^{-2} s^{-1} (A) and to 400 μmol m^{-2} s^{-1} (B) at 5 and at 20°C. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (O , △) RH, (● , ▲) RNH. Control F_v/F_m values were 0.71 ±0.04 for RNH and 0.71 ±0.01 for RH cells. All measurements were done at room temperature.

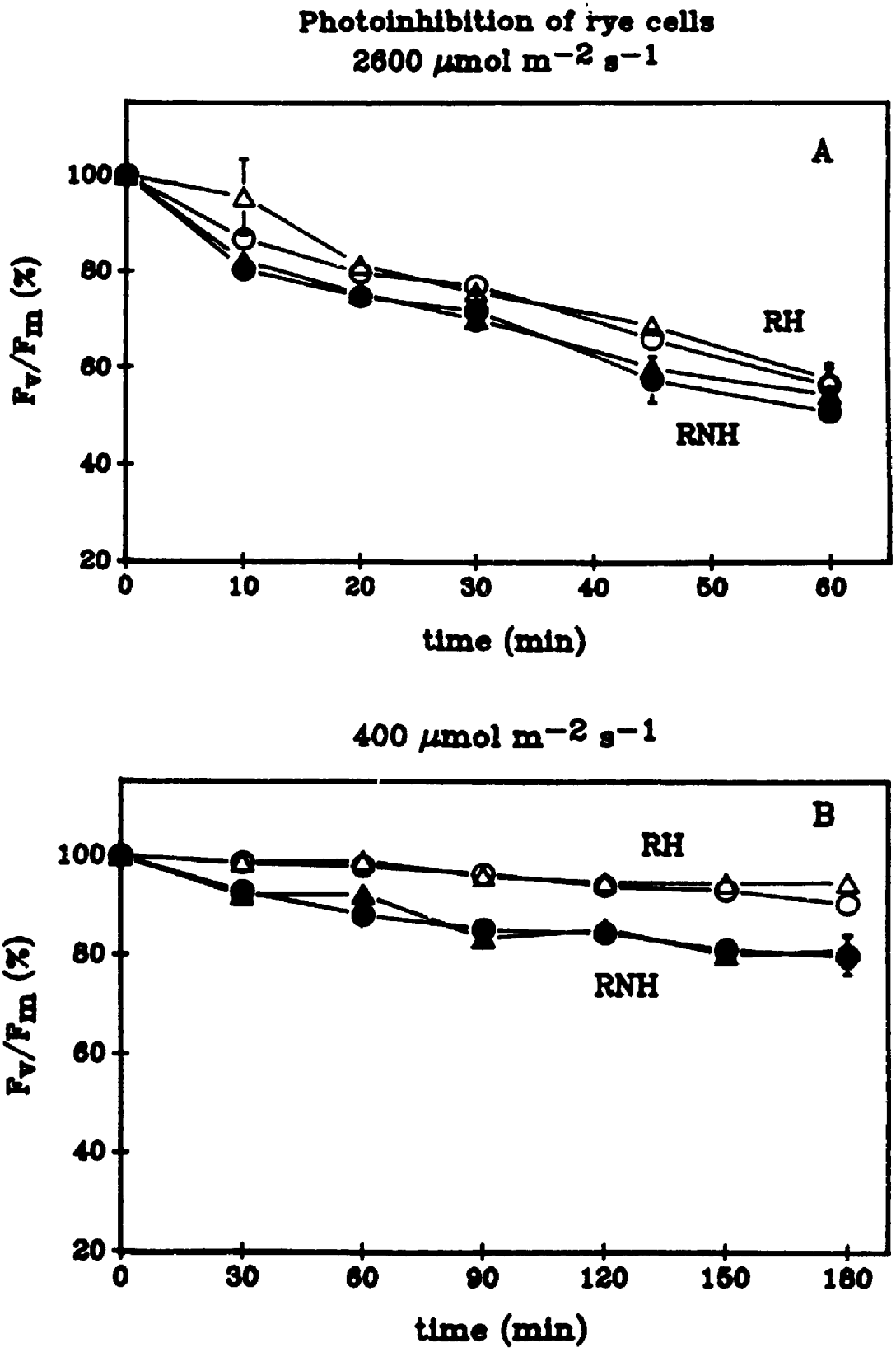
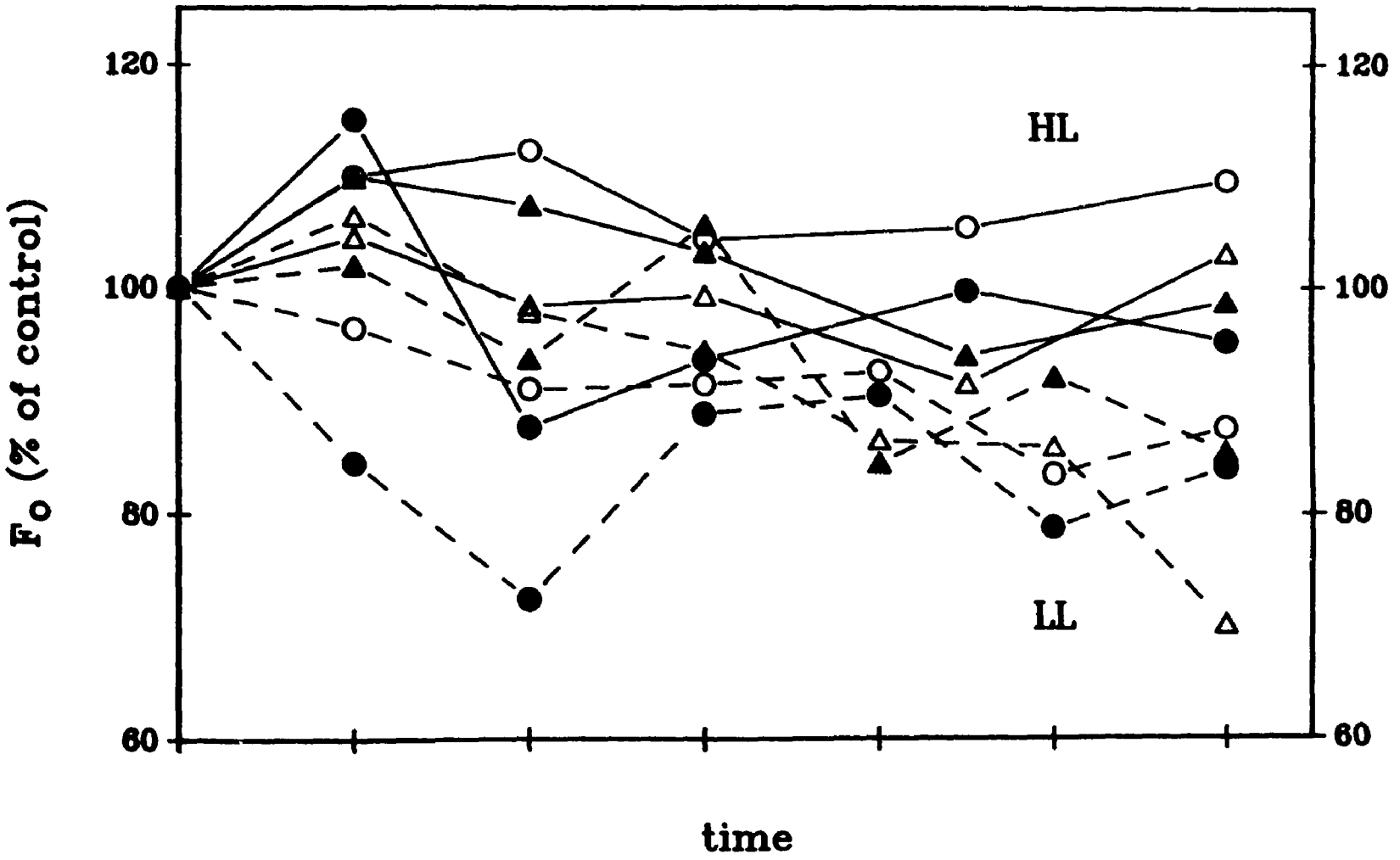

Photoinhibition of rye cells
2600 μmol m⁻² s⁻¹
A
RH
RNH
100
80
60
40
20
Fv/Fm (%)
0
10
20
30
40
50
60
time (min)
400 μmol m⁻² s⁻¹
B
RH
RNH
100
80
60
40
20
Fv/Fm (%)
0
30
60
90
120
150
180
time (min)

Figure 18: F_o values of RH and RNH cells during exposure to both 2600 (solid lines, HL) and to 400 (broken lines, LL) μmol m^{-2} s^{-1} treatments at 5 and at 20°C. Time course was from 0 to 30 min during photoinhibitory treatments at 2600 μmol m^{-2} s^{-1} and from 0 to 3 h during photoinhibitory treatments at 400 μmol m^{-2} s^{-1}. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. All measurements were done at room temperature.

Photoinhibition of rye cells



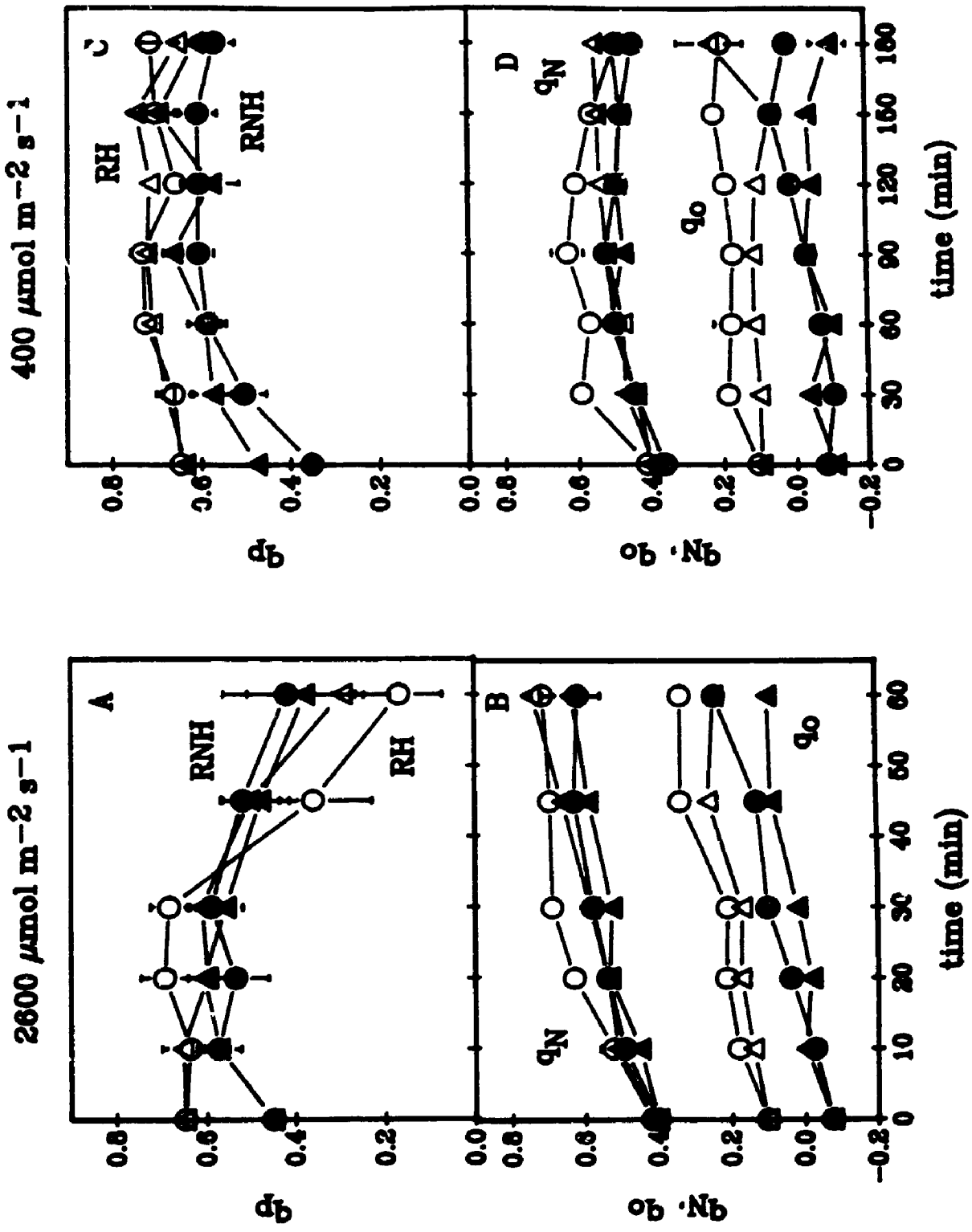
the control values, with no difference between 20 and 5°C treatment nor between RH and RNH cells. Treatments at 400 μmol m^{-2} s^{-1} (broken lines) induced a general decrease of about 20% in F_o. At 20°C the F_o values of rye cells were significantly lower during the 400 μmol m^{-2} s^{-1} treatment than during the 2600 μmol m^{-2} s^{-1} treatment ($F(1,76)= 15.40$, $p\leq 0.01$), but no significant difference was measurable at 5°C.

3.3.2 Photosynthetic measurements under steady-state conditions

3.3.2.1 Fluorescence quenching

Control RH cells showed higher q_p values (0.65 $\pm$0.01) than RNH cells (0.43 $\pm$0.05). During photoinhibition the parameter q_p exhibited a different response at 2600 than at 400 μmol m^{-2} s^{-1} (Fig. 19A & C). During the first 30 min at 2600 μmol m^{-2} s^{-1}, q_p was stable or increased slightly (Fig. 19A). During the following 30 min of treatment RH cells showed a drastic decrease in q_p down to 45% of the initial q_p values at 5°C and down to 26% of the initial q_p values at 20°C. RNH cells also showed a decrease in their q_p after 30 min, but the final q_p values were only slightly lower than the initial q_p values. Both 5 and 20°C treatments induced similar changes in q_p at 2600 μmol m^{-2} s^{-1}. During the treatment at 400 μmol m^{-2} s^{-1}, q_p increased for up to 1.5 h and then remained stable (Fig. 19C). RH cells showed 1.13 increase in q_p at both 5 and 20°C and RNH cells showed 1.72 (20°C) and 1.4 (5°C) increase over the first 1.5 h of high PPF treatment. At the end of the treatment at 400 μmol m^{-2} s^{-1} RH and RNH cells showed similar q_p values.

Figure 19: q_p values of RH and RNH cells during exposure to 2600 μmol m^{-2} s^{-1} (A) and to 400 μmol m^{-2} s^{-1} (C) at 5 and at 20°C. q_N and q_o values of RH and RNH cells during exposure to 2600 μmol m^{-2} s^{-1} (B) and to 400 μmol m^{-2} s^{-1} (D) at 5 and at 20°C. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. All measurements were done at room temperature.


2600 μmol m−2 s−1
400 μmol m−2 s−1
A
B
C
D
RNH
RH
qp
qN, qo
time (min)

Photoinhibition at 2600 μmol m^{-2} s^{-1} induced an increase in q_o in both groups of cells at both 5 (138 to 232% increase) and 20°C (230 to 424% increase) (Fig. 19B). The treatment at 400 μmol m^{-2} s^{-1} induced a lower increase in q_o (0 to 138% increase) than the treatment at 2600 μmol m^{-2} s^{-1} (Fig. 19D). Under both PPFs, the 20°C treatment induced higher q_o than the 5°C treatment and RH cells maintained higher q_o during photoinhibition than RNH cells even though the increase was more substantial in RNH cells But q_o data of rye cells are difficult to interpret because initial q_o values were positive in RH cells (0.08 ±0.01) and negative in RNH cells (-0.07 ±0.01).

Along with an increase in q_o, q_N increased during high PPF treatments in rye cells. The treatment at 2600 μmol m^{-2} s^{-1} induced an important increase in q_N from 1.6 (RNH) to 1.8 (RH) times the initial q_N values after 60 min of treatment (Fig. 19B). Both the 5 and 20°C treatment induced a similar increase in q_N, but RH had significantly higher q_N values after photoinhibition than RNH cells ($F(1,12)= 4.80$, $p\leq 0.05$). At 400 μmol m^{-2} s^{-1} rye cells increased their q_N for the first 1.5 h of treatment up to 1.5 times the initial q_N values then decreased it slightly for the next 1.5 h (Fig. 19D). No difference was seen between RH and RNH increases in q_N nor between 5 and 20°C treatment at 400 μmol m^{-2} s^{-1}.

3.3.2.2 Light limited CO_2 fixation rates

Because photoinhibition has been usually monitored as a decrease in the ϕ_{app}, light limited rates of CO_2 fixation of rye cells were measured as an estimate of overall

photosynthetic ϕ_{app}. A single assay temperature (25°C) was used to compare directly the 20 and the 5°C photoinhibitory treatments and to allow direct comparison with the room temperature fluorescence parameters. The time course of high PPF treatment of rye cells at 2600 μmol m^{-2} s^{-1}, when measured as light limited CO_2 fixation rates (29 μmol m^{-2} s^{-1}), showed a linear decrease of photosynthetic activity (Fig. 20A). No significant difference was found between RH and RNH cells even after 30 min of treatments (60% photoinhibition), nor between the 20 and the 5°C treatment.

When the photoinhibitory treatment was done at 400 μmol m^{-2} s^{-1}, the decrease in photosynthetic activity was much slower and almost 90% of the initial activity was retained after 2.5 h of treatment at 20°C (Fig. 20B). This treatment could not differentiate the response of RH and RNH cells, but the 5°C photoinhibitory treatment induced a significantly greater decrease in CO_2 fixation rates of both RH and RNH cells than the 20°C treatment (at t= 3 h, $F(1,20)= 5.1$, $p\leq 0.05$).

3.3.3 Light limited CO_2 fixation rates *vs.* F_v/F_m

Light limited CO_2 fixation rates of control and photoinhibited samples were plotted against F_v/F_m of samples photoinhibited for the same length of time (Fig. 21). Many studies have shown a linear relationship between F_v/F_m and ϕ_{app} as estimated by O_2 evolution or by CO_2 fixation rates. In some of these studies, the plot passed through the origin indicating a 1:1 relationship (Genty *et al.*, 1989; Adams *et al.*, 1990b), while in other studies it crossed the abscissa (Somersalo and Krause, 1989; Tyystjärvi *et al.*, 1989).

Figure 20: Light limited CO_2 fixation rates of RH and RNH cells during exposure to 2600 μmol m^{-2} s^{-1} (A) and to 400 μmol m^{-2} s^{-1} (B) at 5 and at 20°C. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (O , Δ) RH, (● , ▲) RNH. Control light limited CO_2 fixation rates for the 2600 μmol m^{-2} s^{-1} treatment were 38 ± for RH and 47 ±6 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. Control light limited CO_2 fixation rates for the 400 μmol m^{-2} s^{-1} treatment were 44 ±3 for RH and 46 ±3 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. All measurements were done at 25°C.

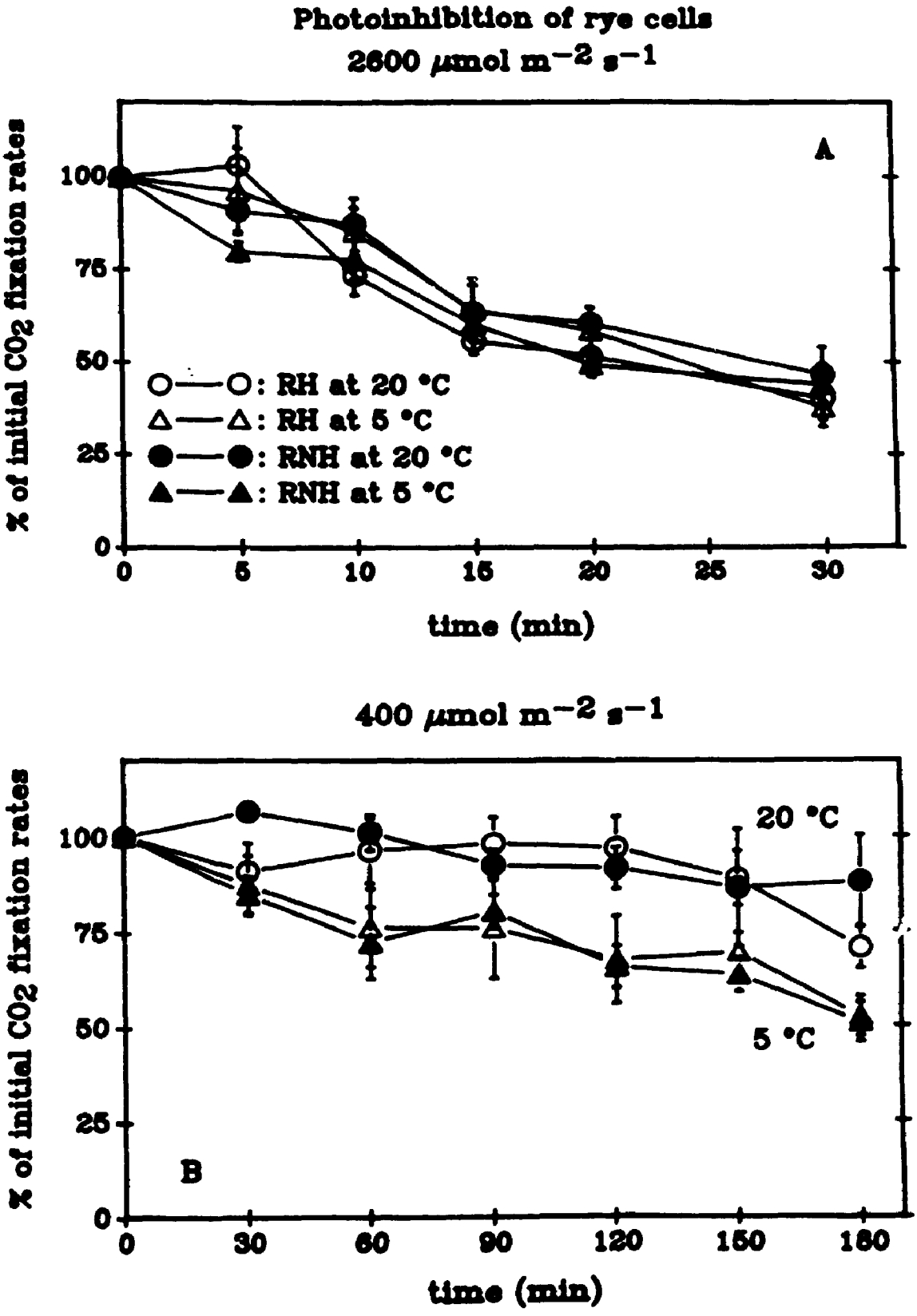
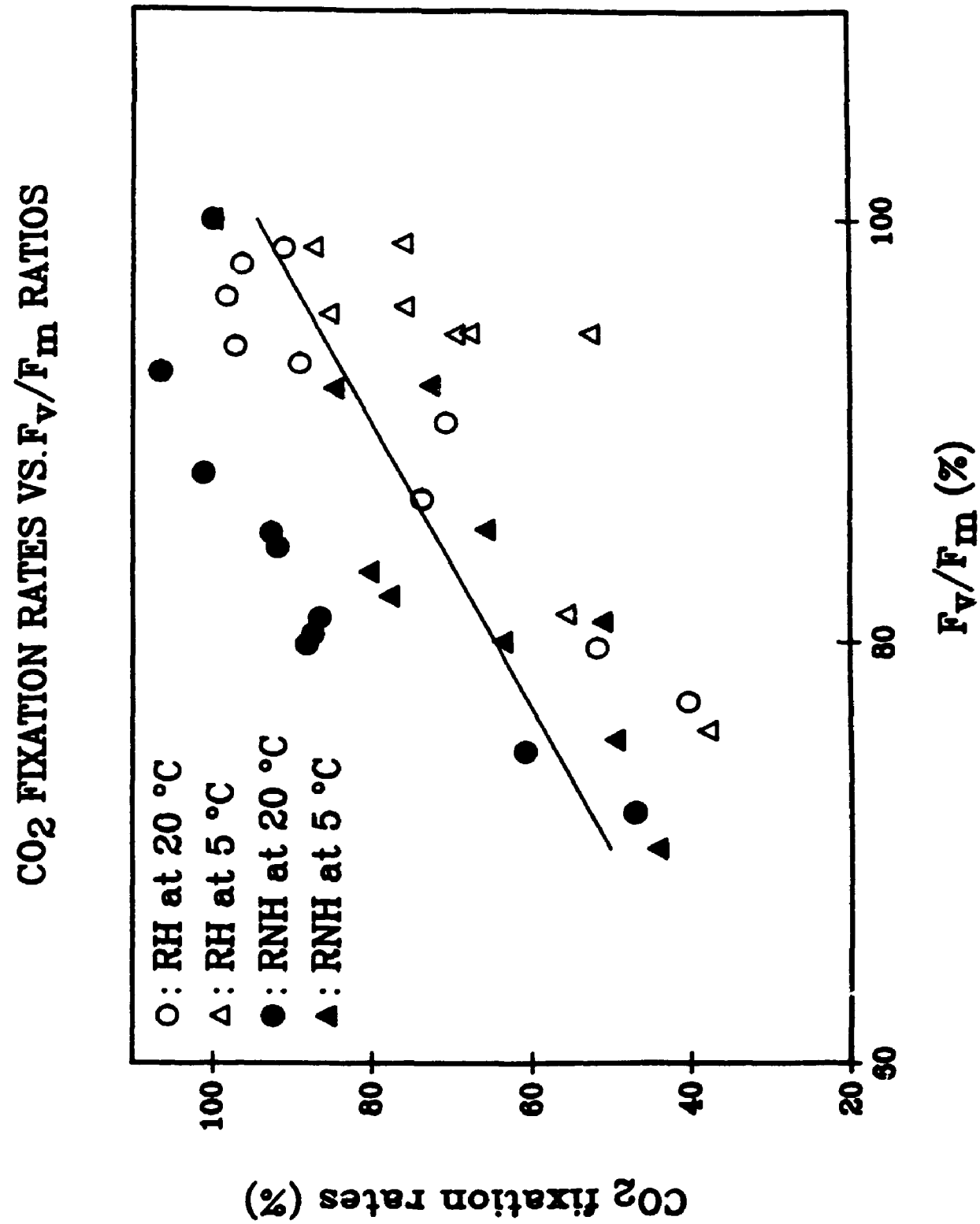

Photoinhibition of rye cells
2600 μmol m⁻² s⁻¹
A
% of initial CO2 fixation rates
100
75
50
25
0
0
5
10
15
20
25
30
time (min)
○—○: RH at 20 °C
△—△: RH at 5 °C
●—●: RNH at 20 °C
▲—▲: RNH at 5 °C
400 μmol m⁻² s⁻¹
B
20 °C
5 °C
% of initial CO2 fixation rates
100
75
50
25
0
0
30
60
90
120
150
180
time (min)

Figure 21: Light limited CO_2 fixation rates *vs.* F_v/F_m for RH and RNH cells photoinhibited at 2600 and at 400 μmol m^{-2} s^{-1} at 5 and at 20°C. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Data are from Figure 13 and 16 exposed to the same PPF, at the same temperature, for the same exposure time. The correlation parameter has been estimated for all data together.


CO_2 FIXATION RATES VS. F_v/F_m RATIOS
○: RH at 20 °C
△: RH at 5 °C
●: RNH at 20 °C
▲: RNH at 5 °C
CO_2 fixation rates (%)
100
80
60
40
20
60
80
100
F_v/F_m (%)

In rye cells, CO_2 fixation rates were affected to a greater extent than F_v/F_m especially when photoinhibited at 5°C. Furthermore RH cells maintained higher F_v/F_m than RNH cells for the same level of photoinhibition of their light limited CO_2 fixation rates. Therefore, F_v/F_m is not a good estimate of ϕ_{app} for CO_2 fixation in isolated rye cells.

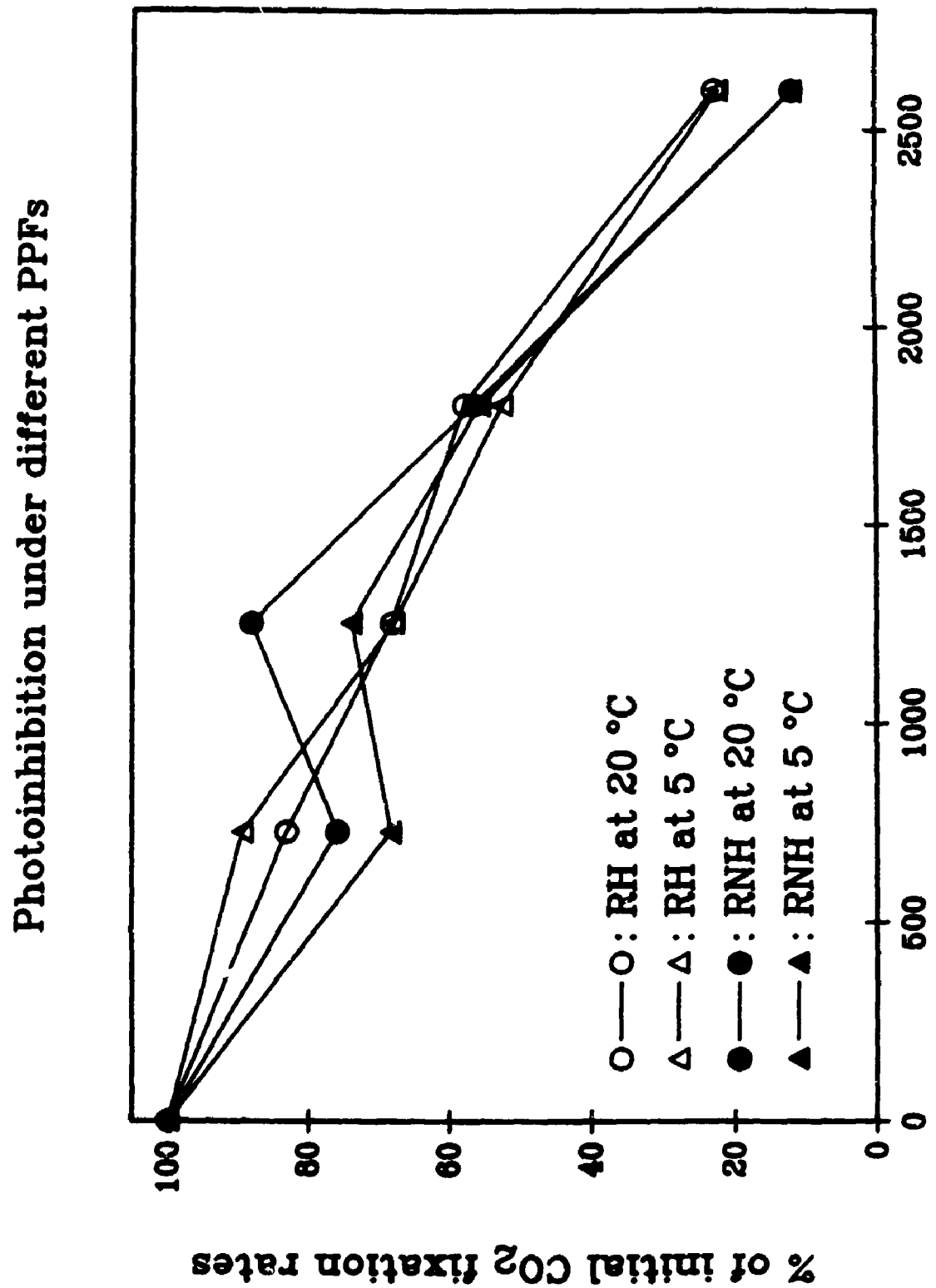
3.4 Factors affecting the photosynthetic response of isolated rye cells during photoinhibition

Many factors can affect the rate and extent of photoinhibition; I assessed two of these, photon flux levels and the presence of protein synthesis inhibitors in combination with the two photoinhibitory treatment temperatures. However, the measuring conditions can also affect the estimation of the extent of photoinhibition. Two measuring conditions were also varied, PPF and the assay temperature during measurements of CO_2 fixation rates. The effect of these factors was estimated by CO_2 fixation rates in RH and RNH cells.

3.4.1 Photoinhibition under different PPFs

The extent of photoinhibition was dependent upon PPF; inhibition of the rate of CO_2 fixation in rye cells increased from 11 to 32% at 750 μmol m^{-2} s^{-1} to about 80% at 2600 μmol m^{-2} s^{-1} (Fig. 22). PPF of 725 μmol m^{-2} s^{-1} could induce some photoinhibition of rye cells after 45 min, but only about 20% of the light saturated rates were lost. No difference was noticed between the response of RH and RNH cells to the different

Figure 22: Light saturated CO_2 fixation rates of RH and RNH cells after 45 min of high PPF treatment at different PPFs at 5 and at 20°C. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Control light saturated CO_2 fixation rates were 425 ±72 for RH and 311 ±77 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells. All measurements were done at 25°C. Each point is the mean of two replicates.


Photoinhibition under different PPFs
% of initial CO2 fixation rates
100
80
60
40
20
0
0
500
1000
1500
2000
2500
PPF (μmol m−2 s−1)
O—O: RH at 20 °C
Δ—Δ: RH at 5 °C
●—●: RNH at 20 °C
▲—▲: RNH at 5 °C

photoinhibitory PPFs.

3.4.2 The effects of protein synthesis inhibitors on the photoinhibitory response of isolated rye cells

The level of photoinhibition measured should reflect the extent of damage and concomitant recovery to PSII occurring during the high PPF treatment (Greer *et al.*, 1986). Therefore, to assess the extent of recovery that occurred during photoinhibition of rye cells, I subjected them to photoinhibition in presence of protein synthesis inhibitors. Cycloheximide blocks cytoplasmic protein synthesis and chloramphenicol blocks protein synthesis on 70S ribosomes. No significant effect was noticed when protein synthesis inhibitors were added during the photoinhibitory treatment compared with photoinhibitory treatment without protein synthesis inhibitors (Table IV). This was tested during a 5 and a 20°C photoinhibitory treatment, and the photosynthetic activities were measured at 29 μmol m^{-2} s^{-1} at 25°C. Because these protein synthesis inhibitors had an effect during recovery of rye cells from photoinhibition (see section 3.6.2), they were able to penetrate into the cells. There was thus no apparent repair mechanism based on protein synthesis that occurred during the 30 min photoinhibitory treatment. RNH and RH cells responded the same way to the presence of protein synthesis inhibitors during photoinhibition.

TABLE IV: Effect of the presence of cyclohexamide (CH) and chloramphenicol (CAP) on the extent of photoinhibition of rye cells CO_2 fixation rates after 30 min of high PPF treatment at 2600 μmol m^{-2} s^{-1} at 20 and at 5°C. Light limited CO_2 fixation rates are expressed as a percentage of control.

	RNH		RH	
treatment	20°C	5°C	20°C	5°C
control	31 ± 4	32 ± 6	35 ± 10	32 ± 8
CAP	23 ± 4	34 ± 5	45 ± 3	35 ± 6
CH	33 ± 2	39 ± 2	31 ± 7	40 ± 7

Control light limited CO_2 fixation rates were 38 ±5 for RH and 47 ±6 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells.

3.4.3 Photoinhibitory effect on light limited *vs.* light saturated rates of CO_2 fixation

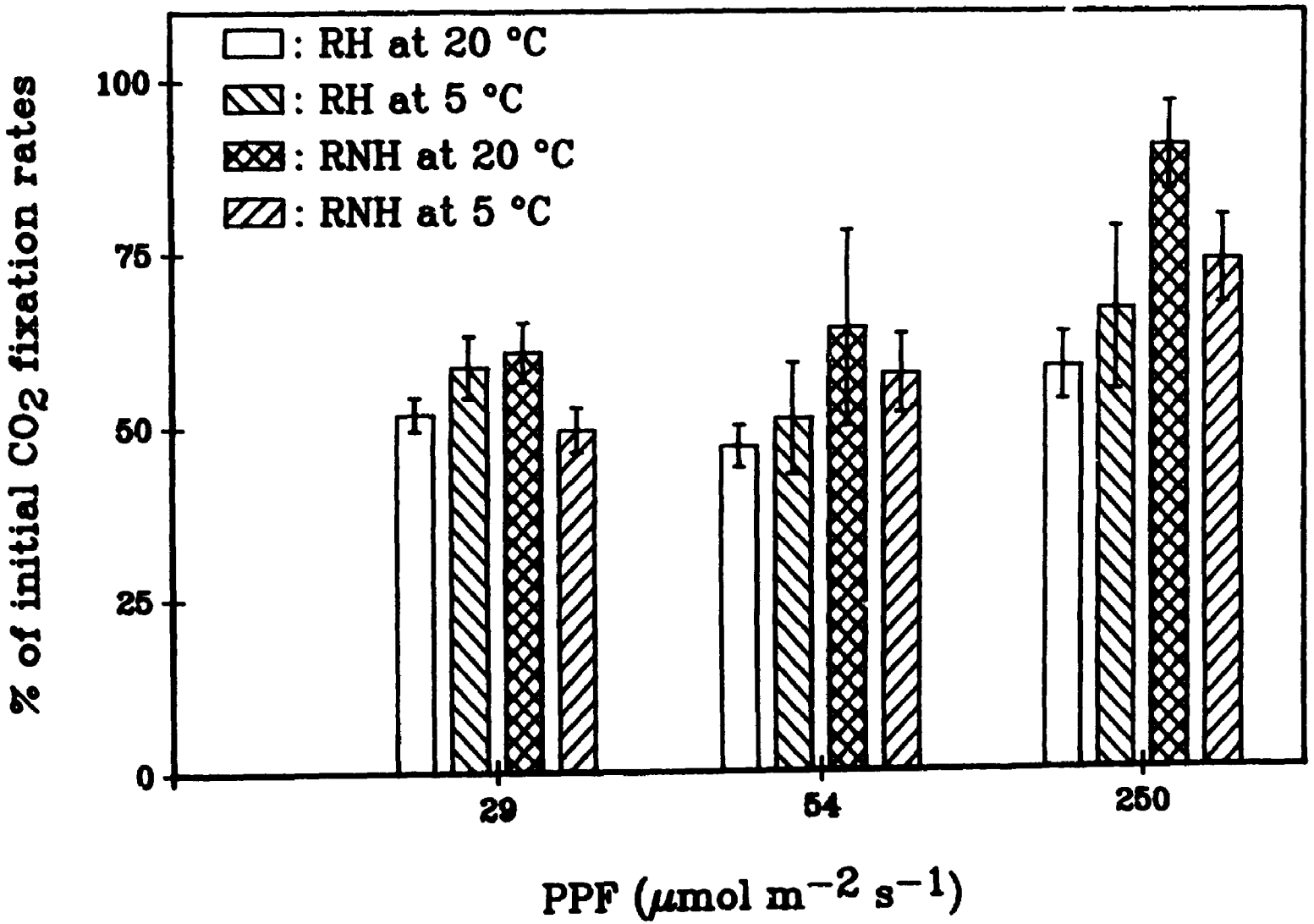
I monitored the effect of photoinhibition on the capacity of rye cells to photosynthesize by measuring light saturated rates. PPF of 250 μmol m^{-2} s^{-1} represents the usual PPF rye cells have experienced during growth. Light limited rates of CO_2 fixation of RNH cells showed more extensive photoinhibition than their light saturated photosynthetic rates at both 5 ($F(1,12)= 6.95$, $p\leq 0.05$) and 20°C ($F(1,12)= 21.30$, $p\leq 0.01$), while RH cells showed similar level of photoinhibition under both PPF conditions (Fig. 23). Under saturating PPF conditions RNH cells were significantly more resistant than RH cells to photoinhibition at 20°C ($F(1,12)= 7.18$, $p\leq 0.05$), but exhibited similar degree of photoinhibition at 5°C. Light limited rates of CO_2 fixation showed that RNH cells were significantly more resistant to the 20°C than to the 5°C treatment ($F(1,60)= 4.7$, $p\leq 0.05$); that difference was detected using a larger number of replicates ($n= 16$) than used in the time course study (Fig. 20A). Light limited rates of RH cells were similar after a 20 min high PPF treatment at 20 and at 5°C, and RH and RNH cells showed similar level of photoinhibition of their light limited CO_2 fixation rates at both temperature treatments.

3.4.4 Effect of the assay temperature on the photoinhibitory response of isolated rye cells

All the previous CO_2 fixation data presented in the photoinhibition study were measured at 25°C. However, when photoinhibition occurs at 5°C, it is important to consider how CO_2 fixation of rye cells is affected at 5°C. To address this point, CO_2

Figure 23: Effect of 20 min at 2600 μmol m^{-2} s^{-1} at 5 and at 20°C on the light limited and light saturated CO_2 fixation rates of RH and RNH cells. Control CO_2 fixation rates measured at 29 μmol m^{-2} s^{-1} were 46 ±4 for RH and 42 ±7 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. Control CO_2 fixation rates measured at 54 μmol m^{-2} s^{-1} were 81 ±5 for RH and 79 ±11 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. Control CO_2 fixation rates measured at 250 μmol m^{-2} s^{-1} were 277 ±16 for RH and 198 ±26 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. All measurements were done at 25°C.

Photoinhibition of light saturated vs. light limited rates



fixation rates were measured at the same temperature as the treatment temperature. Photoinhibition was time dependent for RH and RNH cells when the treatment occurred at 20°C with a 50% decrease of photosynthetic activity after about 20 min of photoinhibitory treatment at 2600 μmol m^{-2} s^{-1} (Fig. 24). These results are similar to what was found when light saturated rates of CO_2 fixation were measured at 25°C (Fig. 23). The decrease in photosynthetic activities of RH and RNH cells was at least biphasic, being faster during the first 30 min of treatment than during the subsequent 30 min. When the photoinhibitory treatment was performed at 5°C and the CO_2 fixation rates were measured at 5°C, there was an initial increase in the light saturated CO_2 fixation rates which was particularly pronounced in RNH cells (50% increase), followed by a decrease in activity after 30 min at 2600 μmol m^{-2} s^{-1}. After 60 min of high PPF treatment, the cells treated at 5°C still showed higher rates of CO_2 fixation than the cells treated at 20°C ($F(1,12)= 25.07$, $p\leq 0.01$). In both 5 and 20°C treatments, RNH and RH cells showed a similar susceptibility to photoinhibition.

To see if the 5°C assay temperature was responsible for the difference observed between the 20 and the 5°C treatment, I also measured light limited and light saturated rates of CO_2 fixation at 5°C following high PPF treatments of rye cells at 20 and at 5°C (Fig. 25). After 20 min of treatment at 5 or at 20°C, the 5°C assay temperature showed no photoinhibition of the light saturated rates of CO_2 fixation for RH and "activated" rates of CO_2 fixation for RNH cells (Fig. 25). These results at the 5°C assay temperature confirm the previous results obtained with the time course experiment (Fig. 24) and show that the assay temperature is a determinant factor in the level of

Figure 24: Effect of assay temperature on the extent of photoinhibition of light saturated rates of CO_2 fixation of RH and RNH cells during exposure to 2600 μmol m^{-2} s^{-1} at 5 and at 20°C. The CO_2 fixation rates were measured at the same temperature as the treatment. Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Control light saturated CO_2 fixation rates at 20°C were 129 ±8 for RH and 164 ±20 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. Control light saturated CO_2 fixation rates at 5°C were 30 ±6 for RH and 31 ±3 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells.

Assay temperature effect

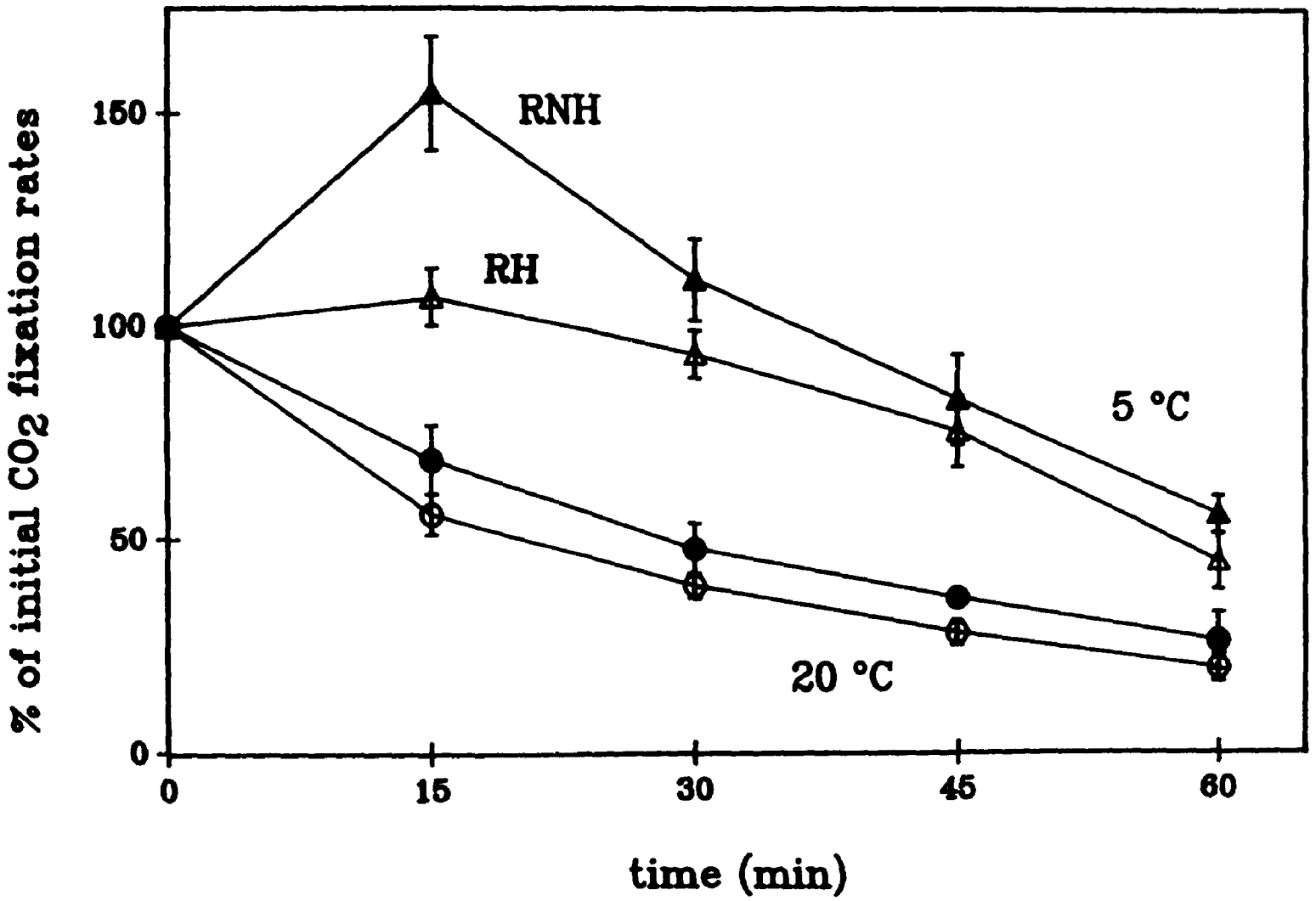
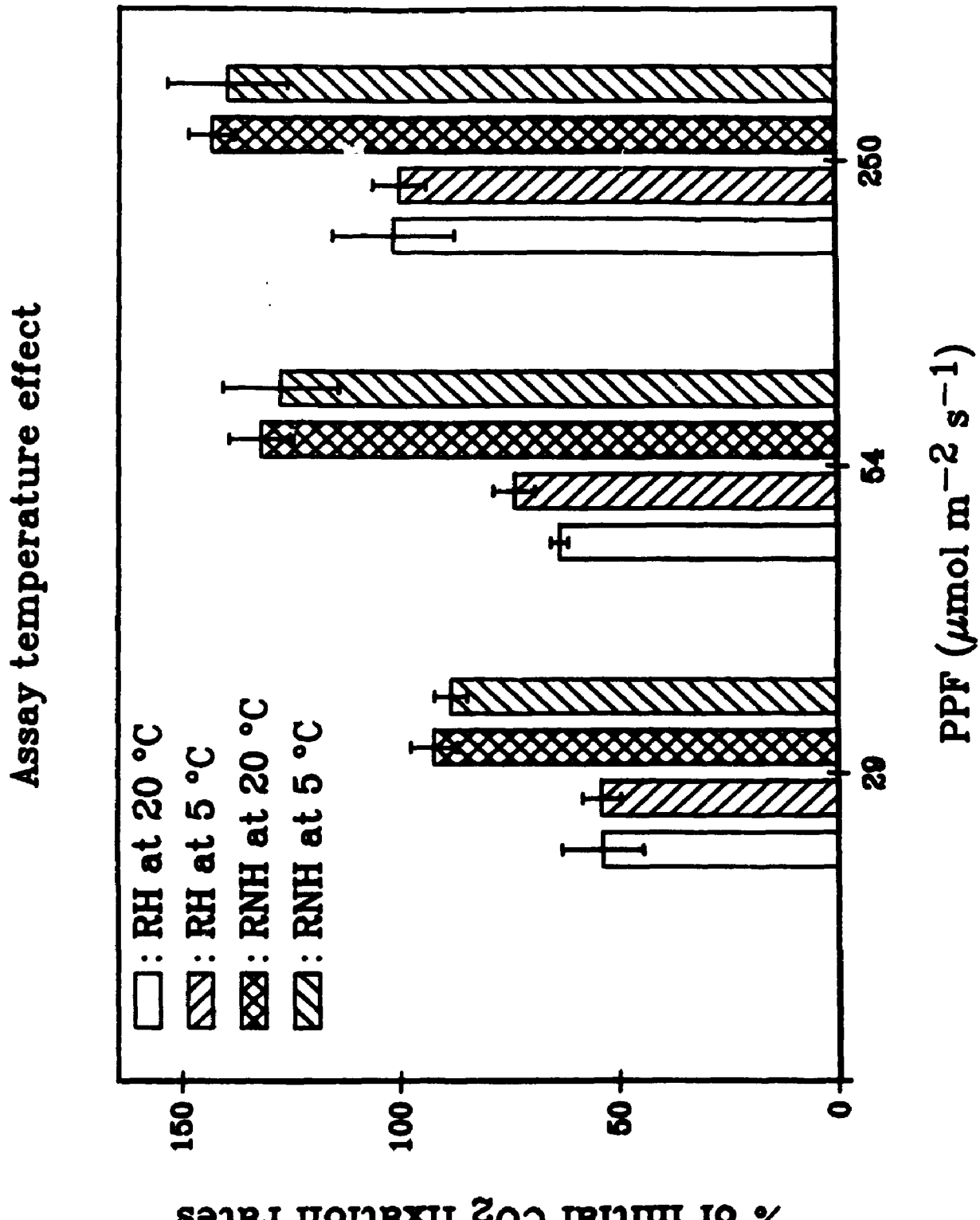


Figure 25: Light limited and light saturated rates of CO_2 fixation of RH and RNH cells measured at 5°C after 20 minutes at 2600 μmol m^{-2} s^{-1} at 5 and at 20°C. Control CO_2 fixation rates measured at 29 μmol m^{-2} s^{-1} were 40 ±3 for RH and 23 ±2 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells. Control CO_2 fixation rates measured at 54 μmol m^{-2} s^{-1} were 53 ±4 for RH and 25 ±1 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells. Control CO_2 fixation rates measured at 250 μmol m^{-2} s^{-1} were 62 ±5 for RH and 26 ±1 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells.


Assay temperature effect
□: RH at 20 °C
▨: RH at 5 °C
▩: RNH at 20 °C
▨: RNH at 5 °C
150
100
50
0
% of initial CO_2 fixation rates
29
54
250
PPF (μmol m^{-2} s^{-1})

photoinhibition measured.

Photosynthetic rates measured at 29 μmol m^{-2} s^{-1} did show photoinhibition at the assay temperature of 5°C, while the light saturated rates did not show any photoinhibition. A greater inhibition of light limited rates of CO_2 fixation as opposed to light saturated rates has also been noticed at the assay temperature of 25°C (Fig. 23). The photosynthetic rates measured at 54 μmol m^{-2} s^{-1} at 5°C showed some photoinhibition for RH cells and some activation of the CO_2 fixation rates for RNH cells after both the 5 and the 20°C treatments. Light limited and light saturated rates of CO_2 fixation measured at 5°C showed that RNH cells were more resistant to photoinhibition than RH cells after both the 5 ($F(1,12)= 20.08$, $p\leq 0.01$) and the 20°C ($F(1,12)= 18.88$, $p\leq 0.01$) treatments.

3.5 Recovery from photoinhibition in isolated rye cells

The capacity to recover from photoinhibition could assess the integrity of isolated rye mesophyll cells. Furthermore, the kinetics of recovery could help to understand the process of photoinhibition in rye cells. During recovery, I monitored the same parameters as during photoinhibition of rye cells. Recovery was followed after 20 min at 2600 μmol m^{-2} s^{-1}. Recovery was not studied in cells photoinhibited at 400 μmol m^{-2} s^{-1} because the level of photoinhibition was too low.

3.5.1 Fluorescence measurements under non steady-state conditions

Rye cells showed some recovery of their F_v/F_m ratios during exposure at 30 μmol m^{-2} s^{-1} (low PPF) after high PPF treatment (Fig. 26). The recovery was significantly faster at 20°C than at 5°C for both RH and RNH cells ($F(1,12)= 7.74$, $p\leq 0.05$) and the two groups of cells presented similar recovery rates. F_v/F_m ratios recovered faster during the first 30 to 60 min and at a slower rate for the next 1 h at 20°C. During recovery the F_o values of RH and RNH cells did not change (data not shown) and were maintained close to the F_o values obtained after photoinhibition at both 5 and 20°C (see Fig. 18).

3.5.2 Photosynthetic measurements under steady-state conditions

3.5.2.1 Fluorescence quenching

During recovery at low PPF, q_p increased in both RH and RNH cells and at both 5 and 20°C (Fig. 27A). In RNH cells q_p was higher after photoinhibition than in control samples, and kept increasing during recovery to a level about 1.7 times higher than the initial steady-state q_p. In RH cells the photoinhibited samples had a much lower q_p than the control samples and even though q_p increased during recovery, it was still 14 (5°C) to 20% (20°C) lower than the initial q_p after 2 h of recovery. Therefore RNH cells maintained higher q_p values than RH cells during recovery; the differences were significant after 2 h of recovery ($F(1,12)= 29.21$, $p\leq 0.01$). No significant difference was seen between the recovery at 5 and at 20°C.

Figure 26: Time course of recovery of F_v/F_m ratios of RH and RNH cells after 45 min of high PPF treatment at 2600 μmol m^{-2} s^{-1} at 5 and at 20°C. Recovery occurred at 5 or at 20°C under 30 μmol m^{-2} s^{-1} (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Control F_v/F_m ratios were 0.69 ±0.01 for RH and 0.71 ±0.04 for RNH cells. All measurements were done at room temperature.

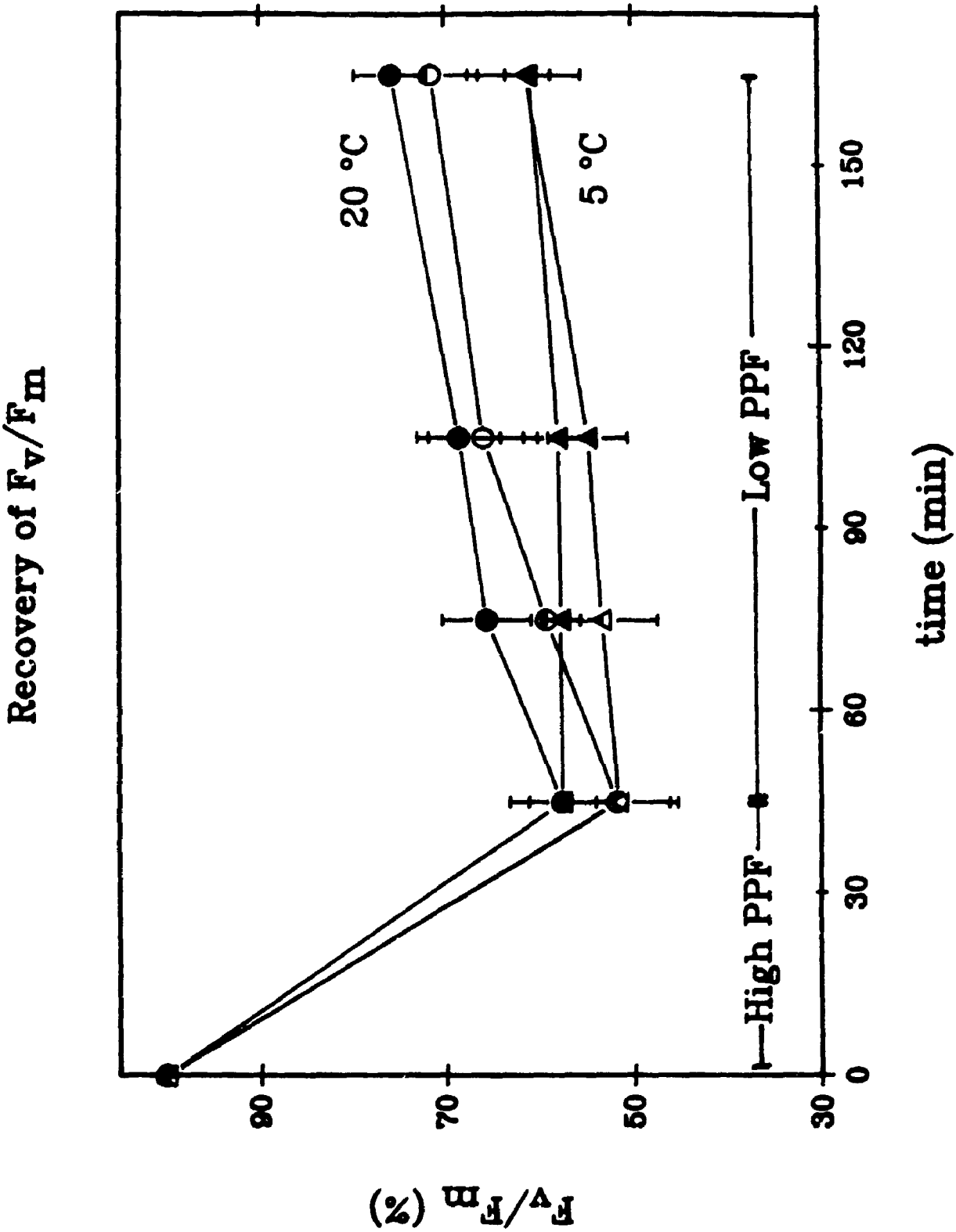
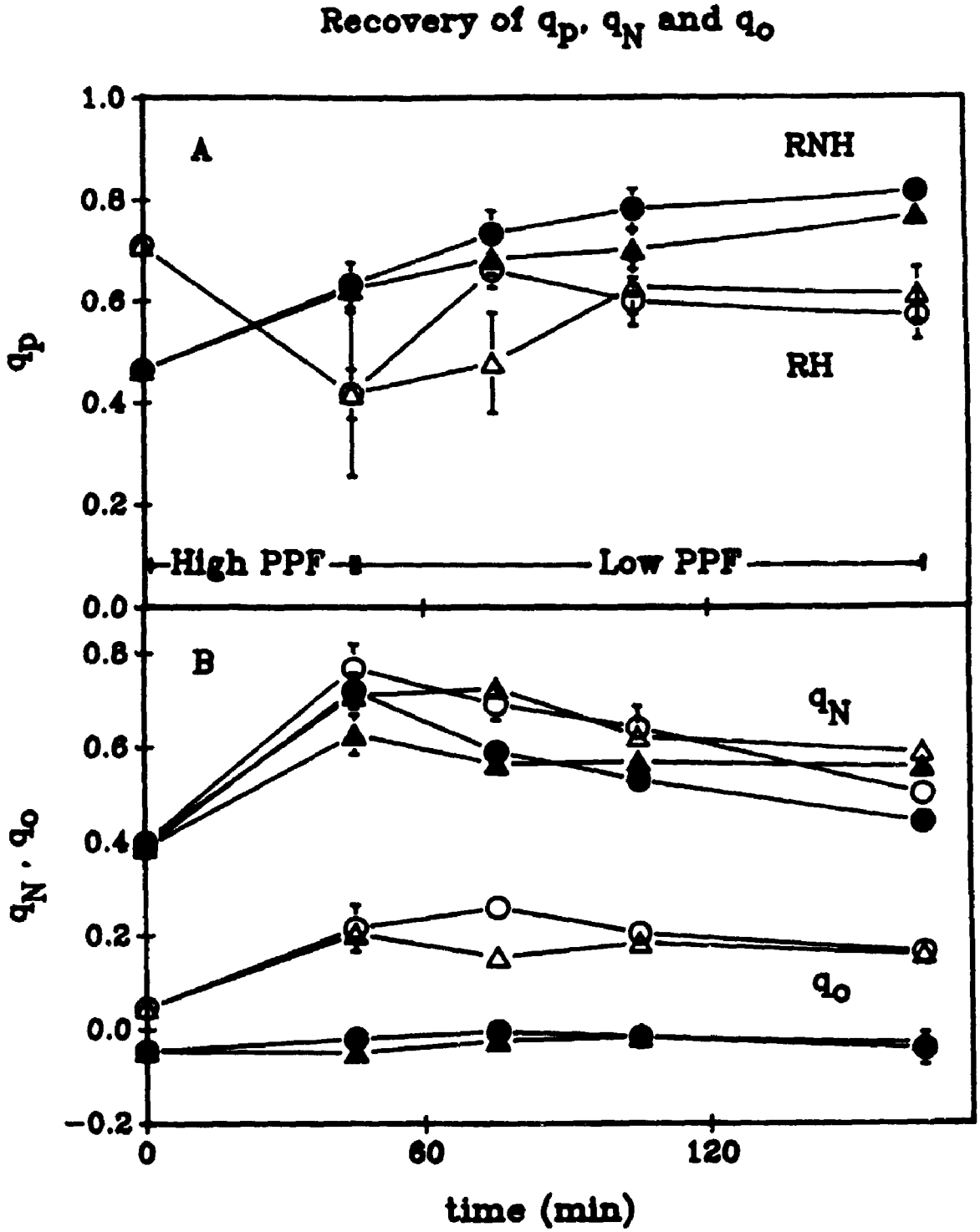

Recovery of F_v/F_m
F_v/F_m (%)
90
70
50
30
0
30
60
90
120
150
time (min)
20 °C
5 °C
High PPF
Low PPF

Figure 27: q_p (A), q_N and q_o (B) during recovery of RH and RNH cells after 45 min of high PPF treatment at 2600 μmol m^{-2} s^{-1} at 5 and at 20°C. Recovery occurred at 5 or at 20°C under 30 μmol m^{-2} s^{-1} (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. All measurements were done at room temperature.


Recovery of q_p, q_N and q_o
A
RNH
RH
High PPF
Low PPF
q_p
B
q_N
q_o
q_N , q_o
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
60
120
time (min)

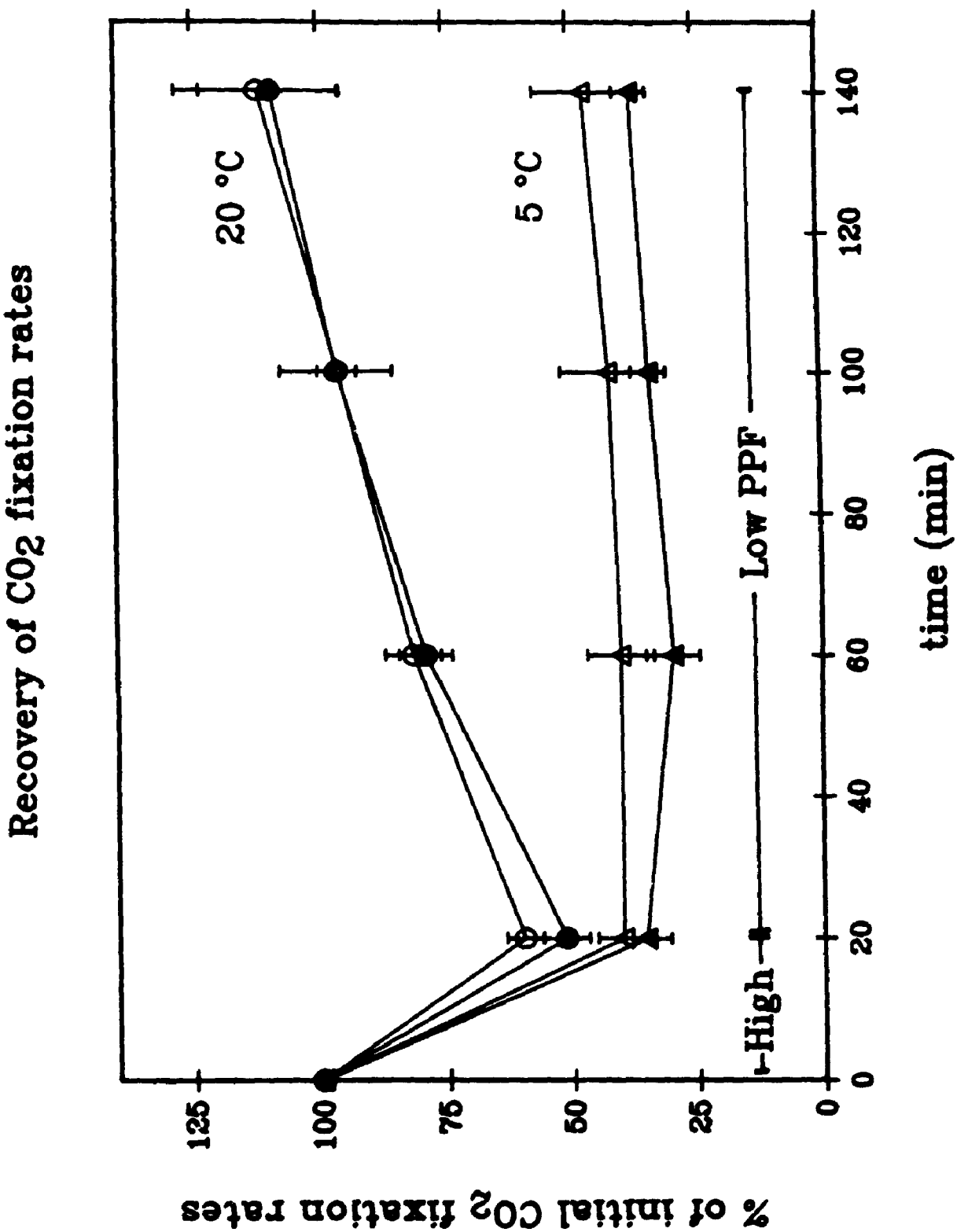
During recovery q_o changed little in both RH and RNH cells (Fig. 27B). It was maintained at the photoinhibitory level, with RH cells having a higher q_o than RNH cells. The 5 and 20°C recovery affected q_o level similarly.

The q_N values decreased slowly during recovery in both RH and RNH cells (Fig. 27B). After 2 h of recovery at low PPF, q_N values were still 1.13 (RNH 20°C) to 1.48 (RH 5°C) times higher than the initial q_N values. q_N values after 2 h of recovery at 20°C were significantly lower than q_N values (15 to 21% lower) after recovery at 5°C ($F(1,12)= 37.33$, $p\leq 0.01$), but RH and RNH cells had similar q_N values at the end of the recovery period.

3.5.2.2 Light limited CO_2 fixation rates

Rye cells showed recovery of their light limited rates of CO_2 fixation after photoinhibition (Fig. 28). The recovery rates at low PPF were faste. 'uring the first 40 min at 20°C, then slower for the next 80 min, to reach the initial CO_2 fixation rates after 2 h. A two phase recovery was also noticeable during the F_v/F_m recovery of rye cells (Fig. 26). The recovery rates were significantly slower at 5°C than at 20°C (at t= 2 h, $F(1,12)= 21.2$, $p \leq 0.01$) for both RNH and RH cells. No significant difference was seen between RH and RNH recovery rates. Preliminary work has shown that recovery occurred faster under low PPF than in the dark, but some recovery was even seen in the dark (data not presented).

Figure 28: Time course of recovery of light limited CO_2 fixation rates of RH and RNH cells after 20 min of high PPF treatment at 2600 μmol m^{-2} s^{-1} at 5 and at 20°C. Recovery occurred at 5 or at 20°C under 30 μmol m^{-2} s^{-1} (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Control light limited CO_2 fixation rates were 35 ±4 for RH and 19 ±1 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. All measurements were done at 25°C.


Recovery of CO_2 fixation rates
% of initial CO_2 fixation rates
125
100
75
50
25
0
20 °C
5 °C
High
Low PPF
0
20
40
60
80
100
120
140
time (min)

At 5°C, recovery of F_v/F_m (Fig. 26) and of light limited rates of CO_2 fixation (Fig. 28) were very similar. But at 20°C, the extent of recovery after 2 h was greater for the CO_2 fixation rates than for the F_v/F_m ratios. Following a 50% decrease in photosynthetic activity, CO_2 fixation rates were recovered back to the initial rates after 2 h, while F_v/F_m ratios were still at 73% of the initial F_v/F_m ratios. Therefore similar high PPF treatments induced higher level of photoinhibition of light limited CO_2 fixation rates than of F_v/F_m ratios (Fig. 17 & 20), but the cells recovered to a greater extent their CO_2 fixation rates.

3.6 Factors affecting the recovery rates of isolated rye cells following photoinhibition

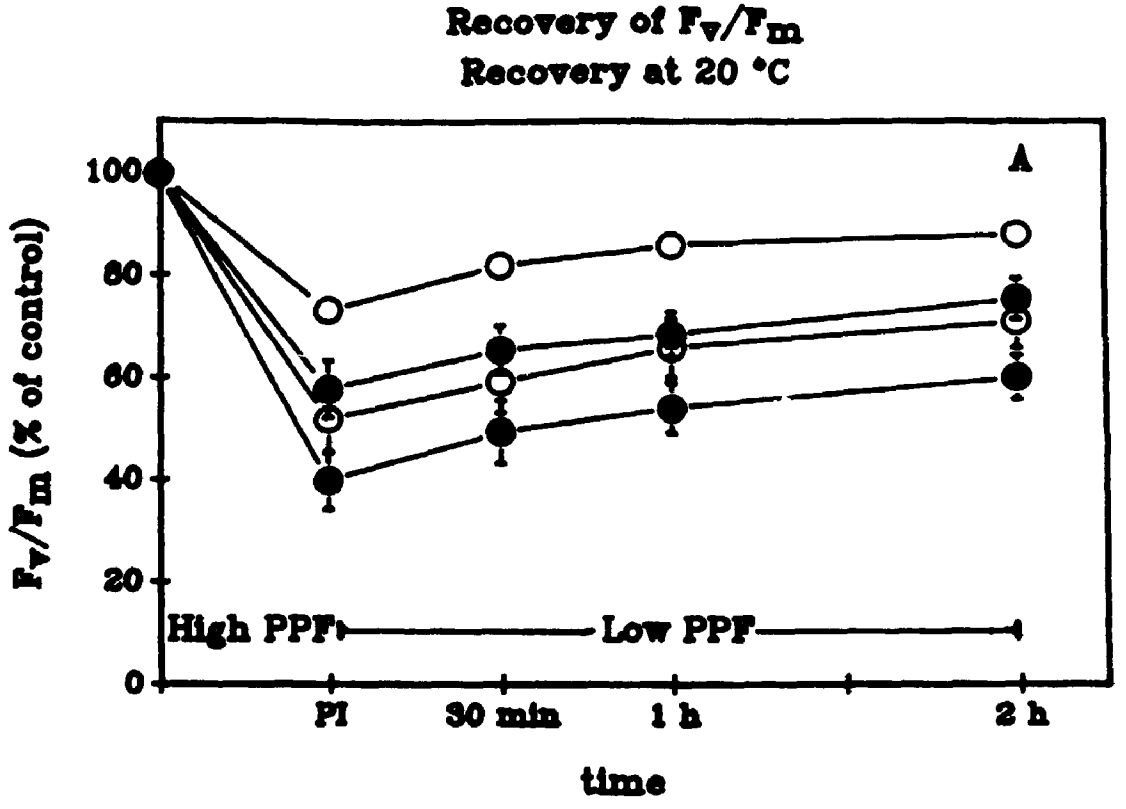
As I showed in previous sections, several factors can affect the rate and extent of photoinhibition of rye cells. Some of these factors were tested again during recovery: presence of protein synthesis inhibitors during recovery and the photon flux levels during CO_2 fixation measurements. The extent of photoinhibition of rye cells appeared as an other important factor to test during recovery to see whether there were different patterns of recovery depending on the extent of initial photoinhibition.

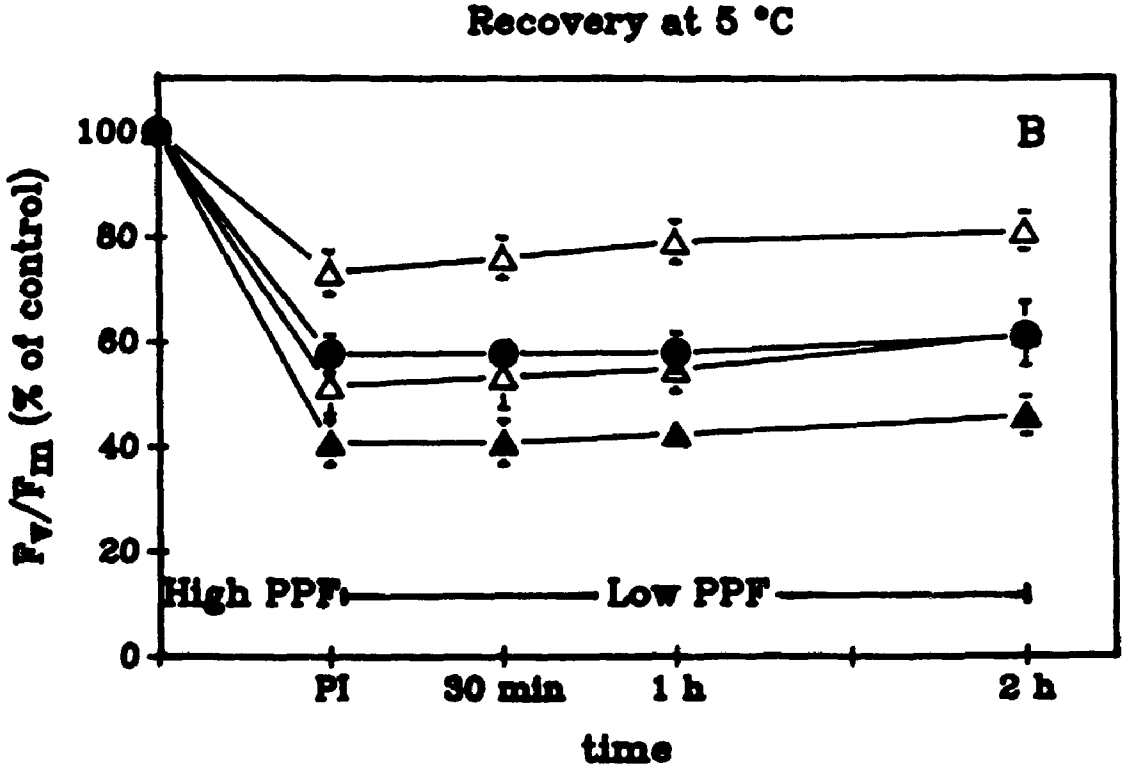
3.6.1 Effect of the extent of photoinhibition on the recovery rates

3.6.1.1 Recovery measured as F_v/F_m

I plotted the recovery of F_v/F_m of rye cells photoinhibited to different extents (Fig. 29). Different levels of photoinhibition of rye cells induced similar rates of

Figure 29: Effect of the extent of photoinhibition at 2600 μmol m^{-2} s^{-1} (High PPF) on the rate and level of recovery of F_v/F_m ratios of RH and RNH cells at 20°C (A) and at 5°C (B). Recovery occurred at 5 or at 20°C under 30 μmol m^{-2} s^{-1} (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Control F_v/F_m ratios were 0.69 ±0.01 for RH and 0.71 ±0.04 for RNH cells. All measurements were done at room temperature.


Recovery of F_v/F_m
Recovery at 20 °C
A
100
80
60
40
20
0
F_v/F_m (% of control)
High PPF
Low PPF
PI
30 min
1 h
2 h
time


Recovery at 5 °C
B
100
80
60
40
20
0
F_v/F_m (% of control)
High PPF
Low PPF
PI
30 min
1 h
2 h
time

recovery of F_v/F_m ratios (Fig. 29). This is inconsistent with the results of Greer and Laing (1988) who have reported different recovery rates in kiwifruit following different extents of photoinhibition. At 5°C, the recovery of F_v/F_m ratios was very slow for all samples independent of their level of photoinhibition (Fig. 29B).

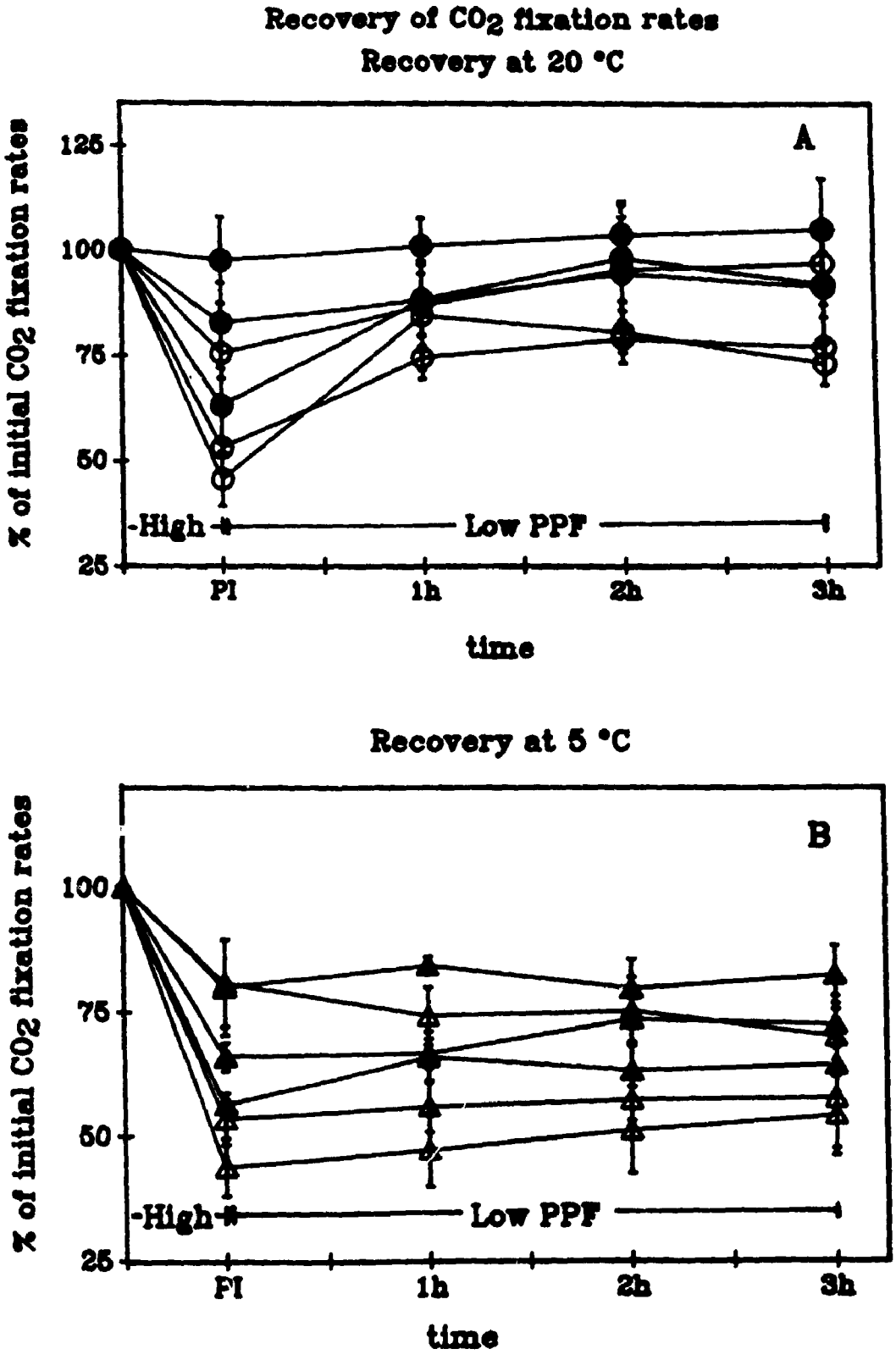
3.6.1.2 Recovery measured as light limited CO_2 fixation rates

I also tested the effect of the extent of photoinhibition on the rate and extent of recovery of CO_2 fixation. Recovery of light limited CO_2 fixation rates at 20°C was affected by the extent of photoinhibition (Fig. 30A). Samples that were most severely photoinhibited showed faster initial rates of recovery than less photoinhibited samples. After 1 h of recovery at low PPF all samples showed similar level of CO_2 fixation rates at about 70 to 80% of control rates. During the subsequent 2 h of recovery, the two most photoinhibited samples appeared to slow down their recovery while the other samples recovered almost to 100%. At 5°C, the recovery rates were almost nil for all samples independent of their level of photoinhibition (Fig. 30B). Because recovery of F_v/F_m showed a quite different pattern, I conclude that recovery of F_v/F_m and recovery of CO_2 fixation rates are limited and regulated by different factors in isolated rye cells.

3.6.2 Effects of protein synthesis inhibitors on recovery of CO_2 fixation

Although protein synthesis inhibitors did not affect the extent of photoinhibition, I tested their effect during recovery to see if recovery was dependent upon protein

Figure 30: Effect of the extent of photoinhibition at 2600 μmol m^{-2} s^{-1} (High PPF) on the rate and level of recovery of light limited CO_2 fixation rates of RH and RNH cells at 20°C (A) and at 5°C (B). Recovery occurred at 5 or at 20°C under 30 μmol m^{-2} s^{-1} (Low PPF). Triangles and circles represent photoinhibition at 5°C and at 20°C respectively. (○ , △) RH, (● , ▲) RNH. Control light limited CO_2 fixation rates were 51 ±5 for RH and 70 ±9 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. All measurements were done at 25°C.


Recovery of CO_2 fixation rates
Recovery at 20 °C
A
% of initial CO_2 fixation rates
125
100
75
50
25
-High
Low PPF
PI
1h
2h
3h
time
Recovery at 5 °C
B
% of initial CO_2 fixation rates
100
75
50
25
-High
Low PPF
PI
1h
2h
3h
time

synthesis. During the first 40 min of recovery following a 20 min photoinhibitory treatment, the presence of protein synthesis inhibitors showed no significant effect (Fig. 31A & B). After 40 min ($F(2,18)= 9.19$, $p\leq 0.01$) of recovery at 20°C, both cycloheximide and chloramphenicol almost completely inhibited recovery in RH and RNH cells. Cycloheximide significantly decreased the extent of recovery of both RNH ($F(1,12)= 5.60$, $p\leq 0.05$) and RH cells ($F(1,12)= 6.20$, $p\leq 0.05$) by 29 to 36% after 2 h of recovery. Chloramphenicol affected the extent of recovery of RNH cells by 50% ($F(1,12)= 10.50$, $p\leq 0.01$) and the extent of recovery of RH cells by 26% (NS). Since the recovery rates were practically nil at 5°C the effect of protein synthesis inhibitors could not be satisfactory tested with that method.

3.6.3 Recovery of light limited *vs.* light saturated CO_2 fixation rates

This experiment was aimed at testing whether the photosynthetic efficiency and photosynthetic capacity of rye cells recovered similarly from photoinhibition. Both light saturated and light limited rates of CO_2 fixation showed recovery after 2 h in low PPF at 20°C (Fig. 32). The light limited rates of photosynthesis showed greater recovery than the light saturated rates at 20°C, while at 5°C the recovery was slow at both PPF conditions (data not shown). The extent of recovery has been compared after the data have been transformed as follows: (% of initial CO_2 fixation rates after recovery)/(% of initial CO_2 fixation rates after photoinhibition). No significant difference was found between the recovery level of RH and RNH cells in any conditions tested. However, light limited ($F(1,44)= 34.7$, $p\leq 0.01$) and light saturated ($F(1,12)= 8.81$, $p\leq 0.05$) rates

Figure 31: Effect of protein synthesis inhibitors on the rate and level of recovery of light limited CO_2 fixation rates of RNH (A) and RH (B) cells after 20 min of photoinhibition at 2600 μmol m^{-2} s^{-1}, 20°C. Recovery occurred at 20°C under 30 μmol m^{-2} s^{-1}. Circles, triangles and diamonds represent recovery in absence of protein synthesis inhibitors (control), in presence of chloramphenicol (CAP) and in presence of cycloheximide (CH) respectively. Control light limited CO_2 fixation rates were 35 ±4 for RH and 19 ±1 nmol 10^5 viable $cells^{-1}$ h^{-1} for RNH cells. All measurements were done at 25°C.

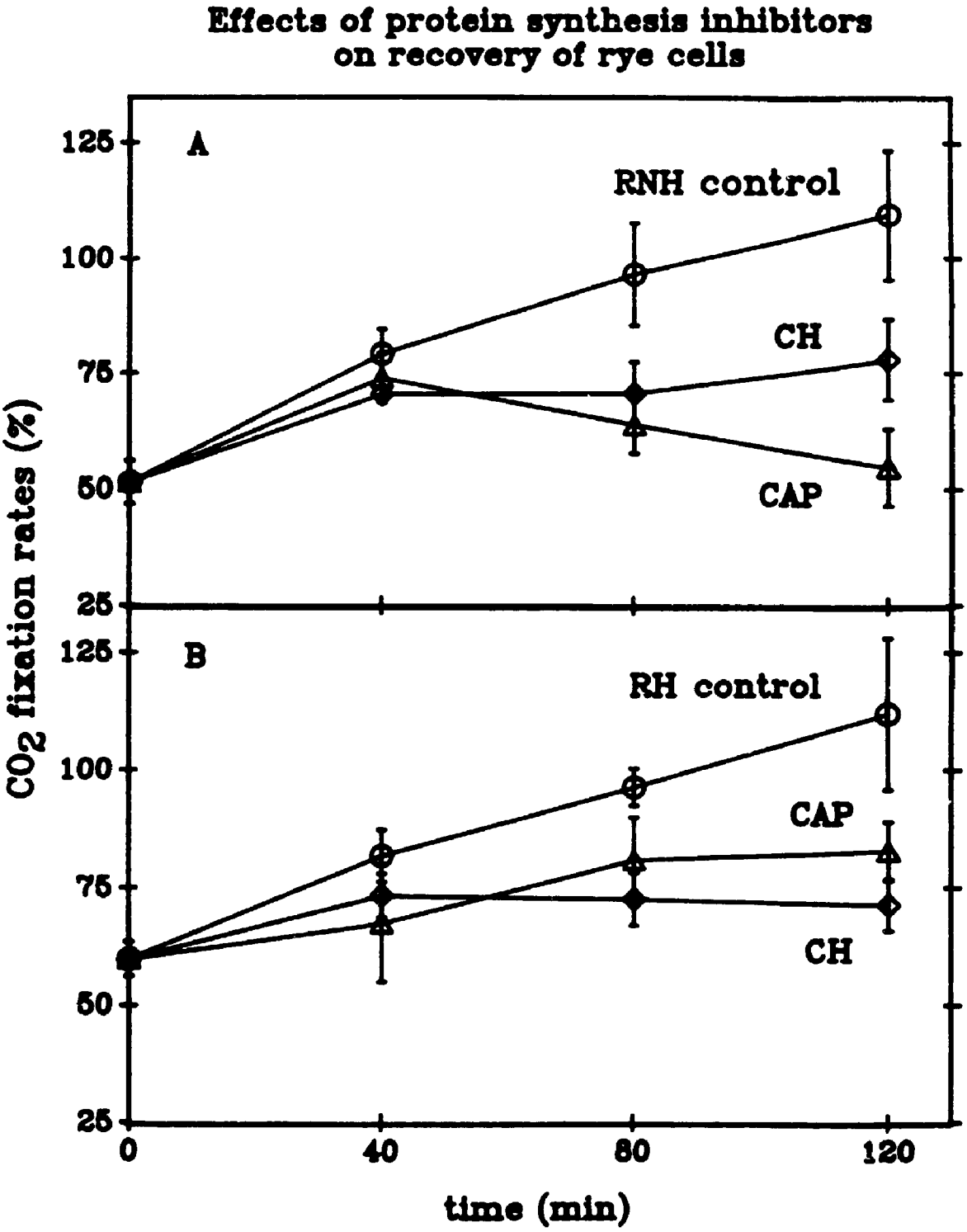
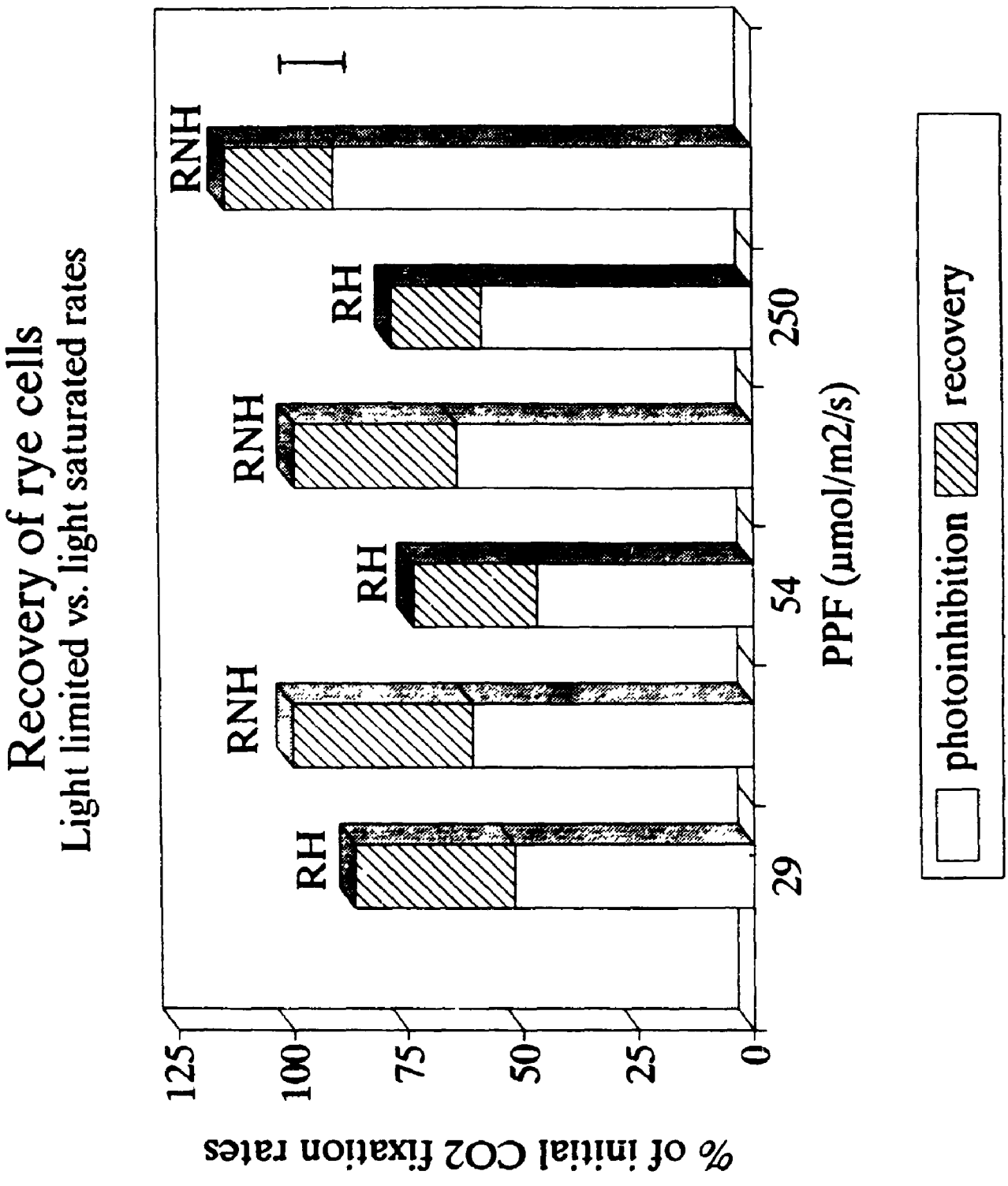

Effects of protein synthesis inhibitors
on recovery of rye cells
A
RNH control
CH
CAP
B
RH control
CAP
CH
125
100
75
50
25
0
40
80
120
CO_2 fixation rates (%)
time (min)

Figure 32: Extent of recovery of light limited and light saturated CO_2 fixation rates of RH and RNH cells after 20 min of high PPF treatment at 2600 μmol m^{-2} s^{-1} at 20°C. Recovery occurred at 20°C under 30 μmol m^{-2} s^{-1} for 2 h. Open bars represent photoinhibition levels and crossed bars, the CO_2 fixation rates reached after 2 h of recovery. Control CO_2 fixation rates measured at 29 μmol m^{-2} s^{-1} were 46 ±4 for RH and 42 ±7 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells. Control CO_2 fixation rates measured at 54 μmol m^{-2} s^{-1} were 81 ±5 for RH and 79 ±11 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells. Control CO_2 fixation rates measured at 250 μmol m^{-2} s^{-1} were 277 ±16 for RH and 198 ±26 nmol 10^5 viable cells^{-1} h^{-1} for RNH cells. All measurements were done at 25°C. The largest SE for photoinhibited and recovered samples is presented.


Recovery of rye cells
Light limited vs. light saturated rates
% of initial CO2 fixation rates
125
100
75
50
25
0
RH
RNH
RH
RNH
RH
RNH
29
54
250
PPF (µmol/m2/s)
photoinhibition
recovery

of CO_2 fixation recovered significantly faster at 20°C than at 5°C.

3.7 Photoinhibition of rye thylakoids

Several recent reports on photoinhibition have implied that some protective mechanisms reside at the level of the thylakoid membrane (Demmig *et al.*, 1987b; Schöner and Krause, 1990). If modifications in thylakoid membrane organization observed during hardening of rye (see section 1.1) impart resistance to photoinhibition, then isolated RH thylakoids should exhibit more resistance to the photoinhibitory induced decrease in PSII efficiency than RNH thylakoids. This hypothesis will be tested in this section. As previously done with isolated rye cells, photoinhibition of isolated rye thylakoids was monitored with different parameters and under different conditions. Two different PPFs and two temperatures were used to induce photoinhibition in isolated rye thylakoids and their response was monitored with room temperature Chl *a* fluorescence, *in vitro* electron transport activity, photoacoustic spectroscopy and [^{14}C]atrazine binding. All photoinhibitory treatments of rye thylakoids were done in the presence of uncouplers (NH_4Cl) to eliminate the effect of non-photochemical quenching caused by the build-up of a pH gradient across the membranes (Krause *et al.*, 1983). However, a preliminary study of photoinhibition of rye thylakoids showed the same trends in the presence or absence of this uncoupler.

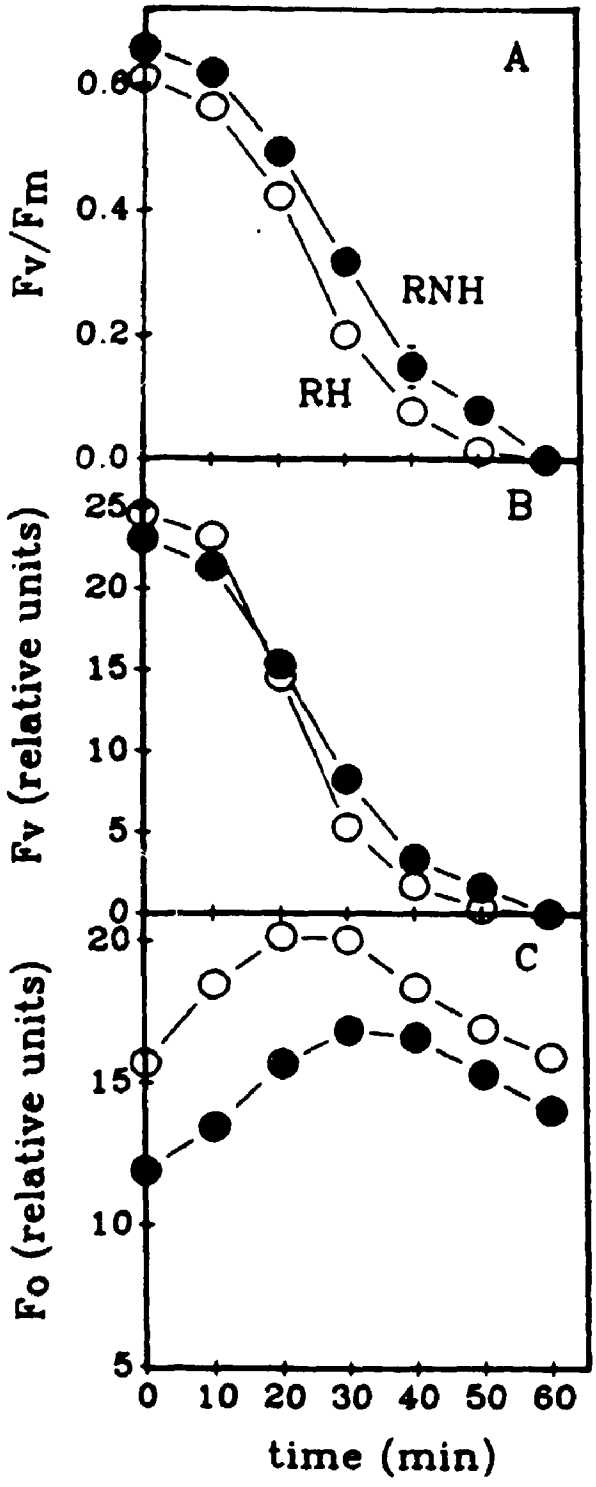
3.7.1 Chl a fluorescence

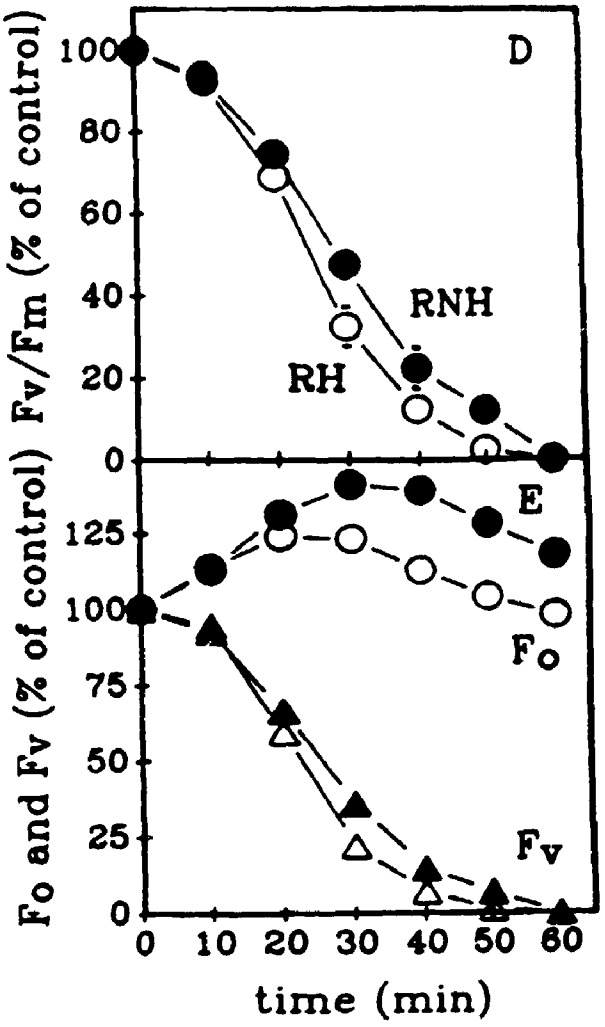
3.7.1.1 Photoinhibition of isolated rye thylakoids at 20°C

F_v/F_m ratios of control samples taken from different experiments varied between 0.65 and 0.74 for RNH thylakoids and from 0.61 to 0.64 for RH thylakoids. This is consistent with *in vivo* determinations of F_v/F_m for RH and RNH leaves (Öquist and Huner, 1991) and with earlier F_v/F_m measurements on isolated rye thylakoids (Griffith *et al.*, 1984b). The lower F_v/F_m ratios in RH thylakoids were essentially due to 30% higher F_o values while their F_v values were similar to those of RNH thylakoids (Fig. 33B & C) as also shown in intact leaves (Fig. 6). Regardless, exposure of RH and RNH thylakoids to 2600 μmol m^{-2} s^{-1} at 20°C for 1 h caused a similar but somewhat larger decrease in F_v/F_m of RH than of RNH thylakoids (Fig. 33D). After 60 min, F_v (Fig. 33B) could not longer be detected and, consequently, F_v/F_m had decayed to zero (Fig. 33A).

An examination of the fluorescence parameters indicated that the decrease in F_v/F_m was due to a gradual loss of F_v over the 60 min treatment period (Fig. 33B) coupled with a substantial increase in F_o during the first 30 min (Fig. 33C). After 90 min of high PPF treatment F_o values were still higher than initial F_o values for RNH thylakoids and slightly lower than initial values for RH thylakoids. On a percent basis, RH thylakoids exhibited a 25% increase in F_o whereas RNH thylakoids exhibited a 40% increase in F_o after 30 min of photoinhibitory treatment (Fig. 33E); the maximal increase in F_o was significantly higher in RNH than in RH thylakoids ($F(1,6)= 34.01$, $p \leq 0.01$). Yet, the F_v of RH thylakoids decreased more rapidly than that of RNH

Figure 33: The effect of photoinhibitory treatment at 20°C on the fluorescence characteristics of RH and RNH thylakoids. (A,B,C) relative values of F_v/F_m, F_v, and F_o. (D,E) normalized data. Thylakoids were exposed to 2400 μmol m^{-2} s^{-1}. (○ , △) RH, (● , ▲) RNH. All measurements were done at room temperature. SE were equal to or smaller than the symbol size.


A
B
C
RNH
RH
Fv/Fm
Fv (relative units)
Fo (relative units)
0.6
0.4
0.2
0.0
25
20
15
10
5
0
20
15
10
5
0 10 20 30 40 50 60
time (min)


D
E
RNH
RH
Fo
Fv
Fo and Fv (% of control) Fv/Fm (% of control)
100
80
60
40
20
0
125
100
75
50
25
0
0 10 20 30 40 50 60
time (min)

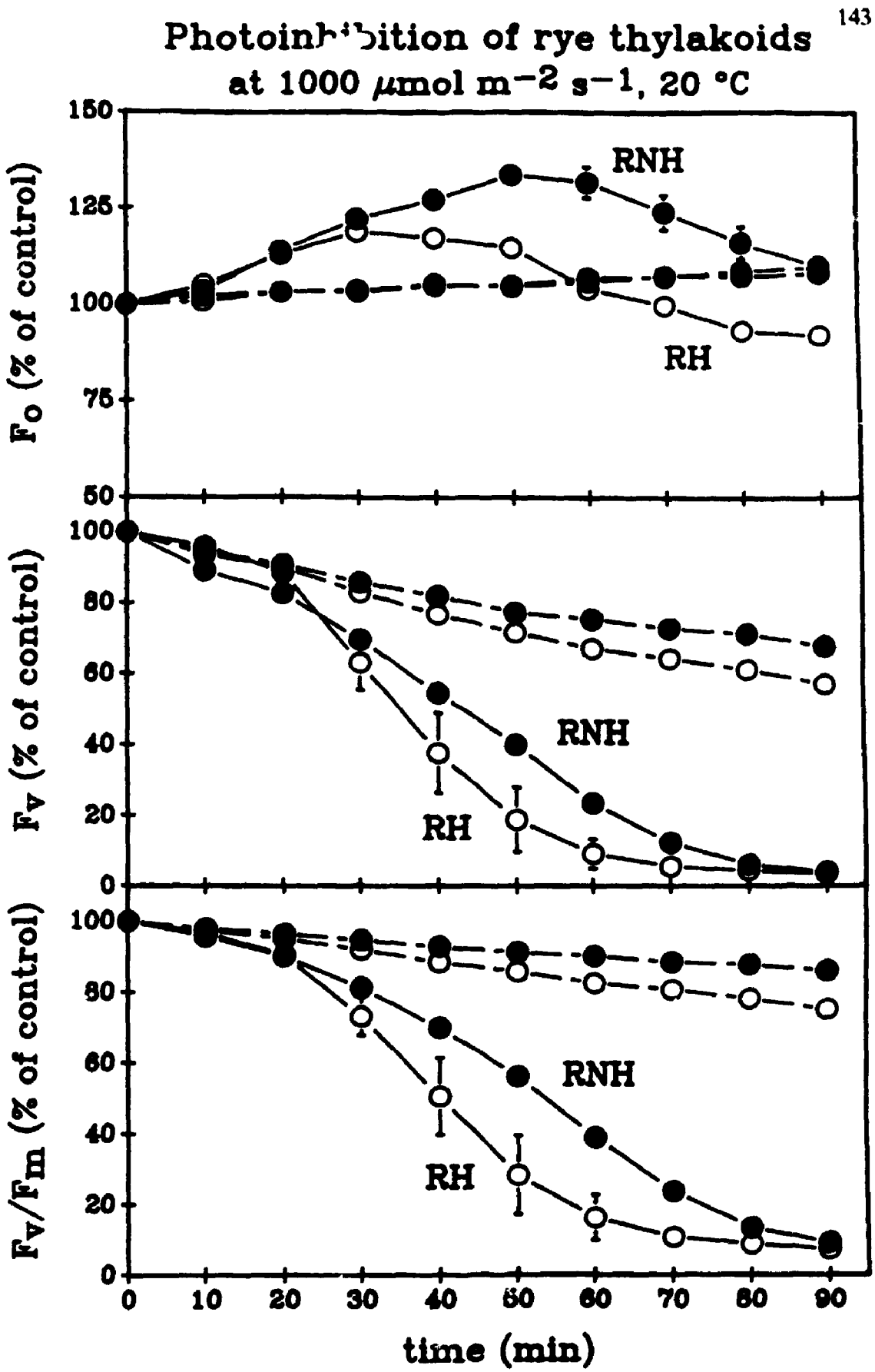
thylakoids (Fig. 33E). Therefore F_v/F_m of RH thylakoids was significantly more sensitive to photoinhibitory treatment than F_v/F_m of RNH thylakoids (between t=30 and t=50 min, $F(1,6)= 6.47$, $p \leq 0.05$) (Fig. 33D).

3.7.1.2 Photoinhibition under lower PPF and dark controls

I also treated rye thylakoids under lower PPF (Fig. 34) as for rye cells. F_o, F_v and F_v/F_m exhibited changes similar to what was found with the higher PPF treatment (Fig. 33D & E), but the rate of change in these parameters was slower at 1000 μmol m^{-2} s^{-1}. The initial F_o increase extended for up to 50 min for RNH thylakoids while RH F_o values increased for the first 30 min only (Fig. 34A). F_v values decreased steadily during the treatment to reach almost zero after 90 min at 1000 μmol m^{-2} s^{-1}. The difference between RH and RNH thylakoids fluorescence parameters was accentuated by lower PPF treatment; this treatment clearly showed that RNH thylakoids were more resistant to high PPF treatment than RH thylakoids.

Figure 34 presents also the dark decay of fluorescence parameters of rye thylakoids at room temperature. While F_o values increased slowly but consistently over 90 min (Fig. 34A), both F_v (Fig. 34B) and F_v/F_m (Fig. 34C) decreased. After 90 min at room temperature, RH thylakoids had F_o values 10% higher than at the beginning of the treatment and RNH F_o values had increased by 8% during the same period. F_v/F_m ratios decreased by 14% (RNH) and 25% (RH) respectively over 90 min. RNH thylakoids were therefore more stable than RH thylakoids at room temperature though

Figure 34: The effect of lower PPF during photoinhibitory treatment at 20°C on F_o (A), F_v (B) and F_v/F_m (C) of RH and RNH thylakoids (solid lines). Thylakoids were exposed to 1000 μmol m^{-2} s^{-1}. Fluorescence characteristics of control samples kept in the dark at 20°C are also presented (broken lines). (○) RH, (●) RNH. Control F_v/F_m were 0.64 ±0.01 for RH and 0.73 ±0.01 for RNH thylakoids. All measurements were done at room temperature.


Photoinhibition of rye thylakoids
at 1000 μmol m−2 s−1, 20 °C
Fo (% of control)
Fv (% of control)
Fv/Fm (% of control)
RNH
RH
time (min)
150
125
100
75
50
80
60
40
20
0
10
30
50
70
90

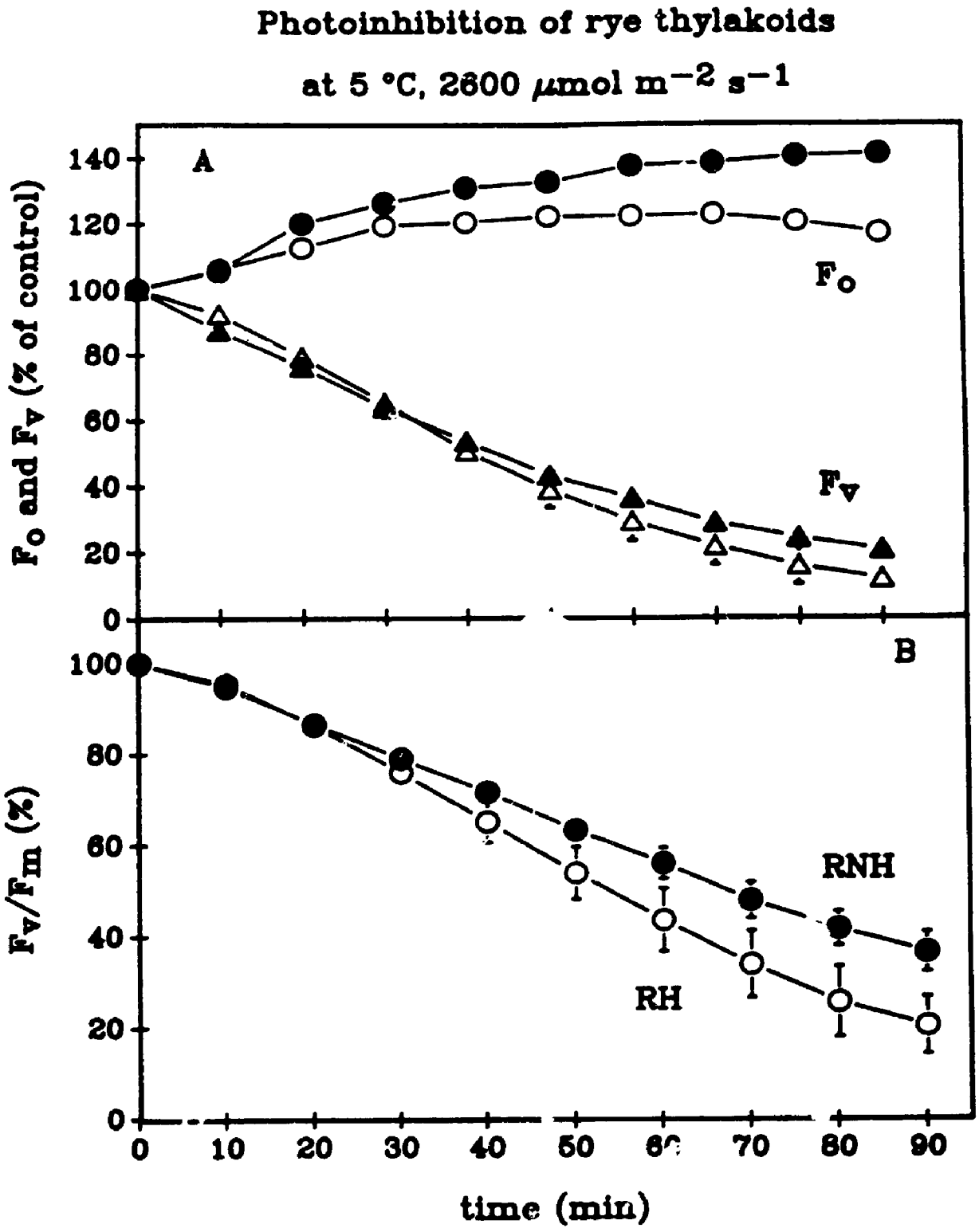
their stability on ice were comparable (data not shown). I corrected the F_v values and F_v/F_m ratios to account for the dark decay of those parameters to separate the effect of high PPF treatment from the dark decay component.

3.7.1.3 Photoinhibition of isolated rye thylakoids at 5°C

In vivo exposure of rye to moderate PPF and low temperature markedly increased susceptibility to photoinhibition (Öquist and Huner, 1991). In contrast to *in vivo* observations, isolated rye thylakoids appeared to be less sensitive to photoinhibition at 5°C than at 20°C as indicated by a slower rate of change of F_o, F_v and F_v/F_m during photoinhibition at the lower temperature (Fig. 35). The F_v/F_m of RH thylakoids was more sensitive to low temperature photoinhibition than RNH thylakoids F_v/F_m (Fig. 35B). The differential increase in F_o observed at 5°C (Fig. 35A) is consistent with the results obtained during photoinhibition at 20°C (Fig. 33E).

F_o reached similar maximal values independently of PPF or temperature during the treatment in both RH and RNH thylakoids. However, this maximum was reached at a different time with respect to the decrease in F_v depending on the temperature treatment. Maximum F_o values were reached when F_v values had decreased by 62% (RNH) or by 39% (RH) during room temperature high PPF treatment. During 5°C treatment, F_o reached maximum values only after F_v values had decreased by 82% in RH and RNH thylakoids. Thus the F_o increase and F_v decrease appeared to be partially independent phenomenon during the photoinhibition of isolated rye thylakoids.

Figure 35: The effect of photoinhibitory treatment at 5°C on F_o, F_v (A) and F_v/F_m (B) of RH and RNH thylakoids. Thylakoids were exposed to 2600 μmol m^{-2} s^{-1}. (○ , △) RH, (● , ▲) RNH. Control F_v/F_m were 0.64 ±0.01 for RH and 0.74 ±0.01 for RNH thylakoids. All measurements were done at room temperature.


Photoinhibition of rye thylakoids
at 5 °C, 2600 μmol m⁻² s⁻¹
A
B
Fo
Fv
RNH
RH
Fo and Fv (% of control)
Fv/Fm (%)
time (min)
0
20
40
60
80
100
120
140
0
10
20
30
40
50
70
80
90

3.7.1.4 Photoinhibition of isolated rye thylakoids in higher osmoticum

To determine if the lower susceptibility to photoinhibition in RNH thylakoids was caused by the instability of RH thylakoids in low osmoticum as previously shown by Huner and Hopkins (1985), I resuspended and photoinhibited both RH and RNH thylakoids in 0.8 M sorbitol buffer (Fig. 36). The same trend was observed in high as in low osmolarity buffer: F_o values increased more in RNH thylakoids and F_v/F_m was less affected in RNH than in RH thylakoids.

3.7.2 Loss of relative absorbance during photoinhibition

Since photoinhibition occurred rapidly in isolated thylakoids, it was important to separate photoinhibition from photooxidation of photosynthetic pigments. This was monitored by following the changes in relative absorbance at 680 nm of thylakoid samples during high PPF treatment. During photoinhibition of rye thylakoids some loss of Chl was observed (Fig. 37). The loss of Chl was greater in RH than in RNH thylakoids. After 60 min of high PPF treatment when F_v had reached zero, 85% (RNH) and 70% (RH) of the initial amount of Chl was still present in the samples. Clearly, the initial decrease in F_v was essentially caused by photoinhibition rather than by photooxidation. The slow loss of Chl was also noticed as a decrease in F_o after 30 min of photoinhibition at 20°C (Fig. 33C).

Figure 36: Photoinhibition of RH and RNH thylakoids in higher osmoticum solution at 2600 μmol m^{-2} s^{-1}, 20°C. (○ , △) RH, (● , ▲) RNH. Control F_v/F_m were 0.60 ±0.01 for RH and 0.69 ±0.02 for RNH thylakoids. All measurements were done at room temperature. Each point is the mean of two replicates.

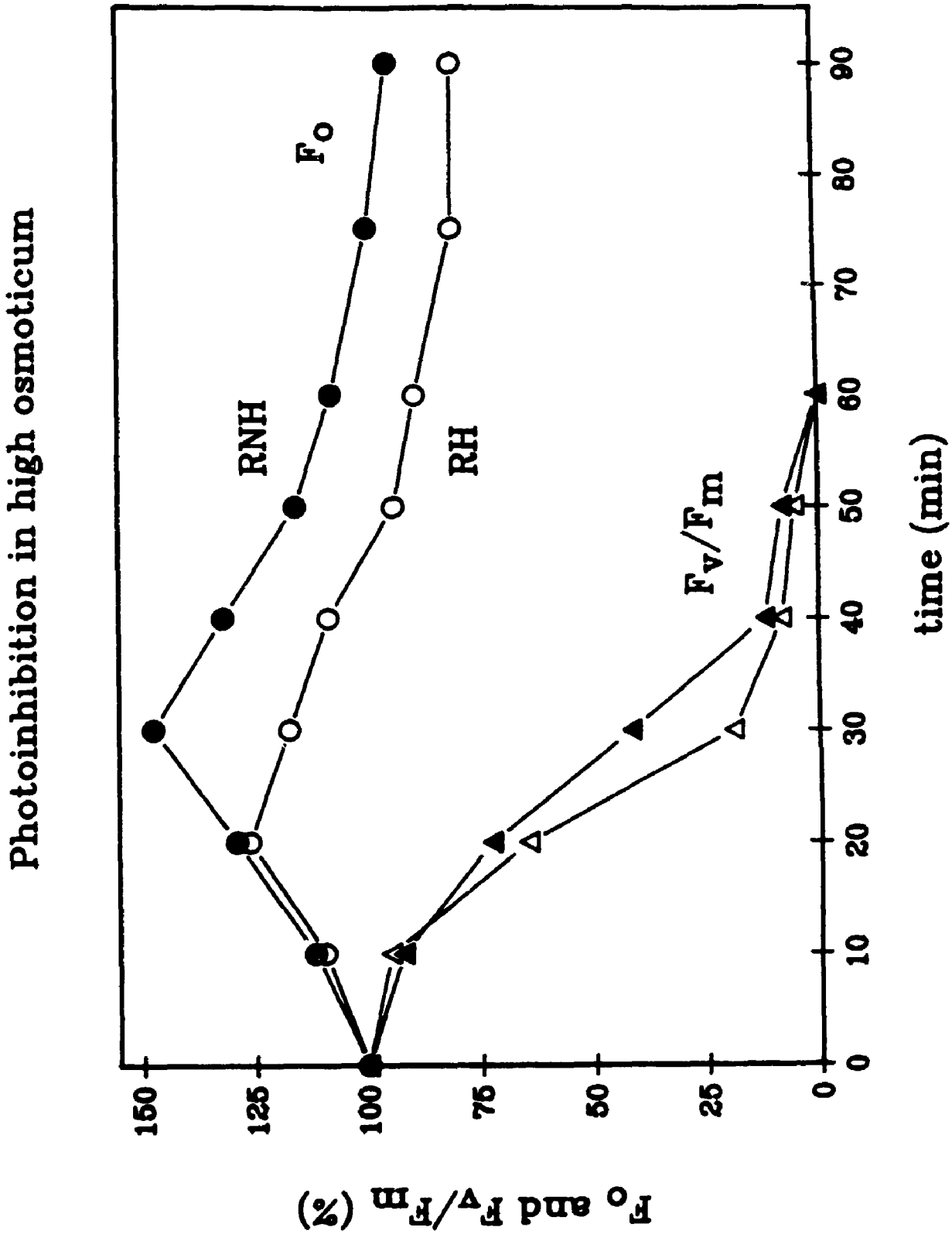
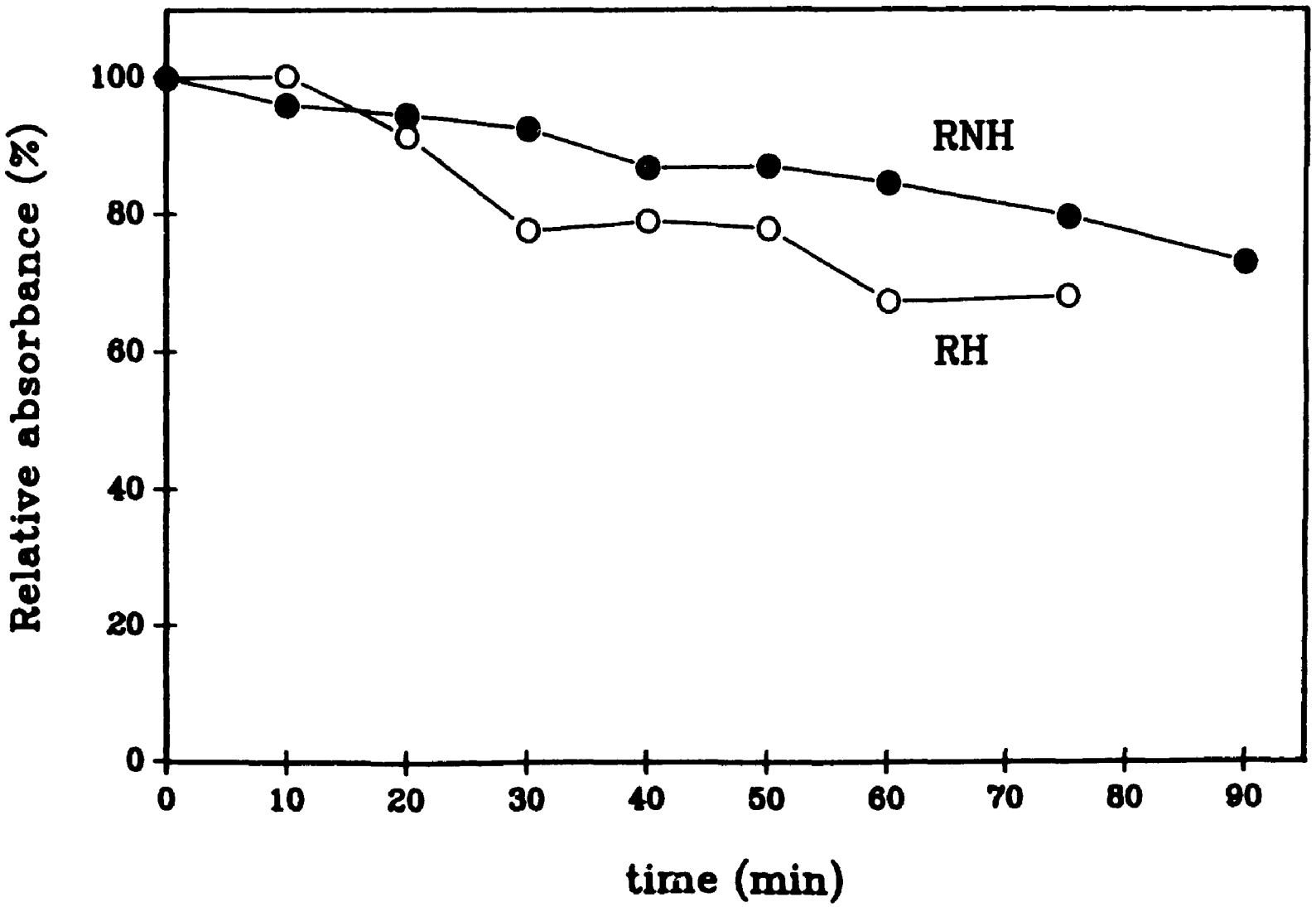

Photoinhibition in high osmoticum
F_o
RNH
RH
F_v/F_m
F_o and F_v/F_m (%)
time (min)
150
125
100
75
50
25
0
10
20
30
40
50
60
70
80
90

Figure 37: Relative absorbance at 680 nm of RH and RNH thylakoids during photoinhibition at 2600 μmol m^{-2} s^{-1}, 20°C. (○) RH, (●) RNH. Each point is the mean of two replicates.

RELATIVE ABSORBANCE DURING PHOTOINHIBITORY TREATMENT



3.7.3 Effect of photoinhibition on *in vitro* PSII activities

The photoinhibitory decrease in F_v/F_m of RH and RNH thylakoids implies a decrease in the photosynthetic efficiency of PSII (Baker and Horton, 1987). This was corroborated by independent measurements of PSII electron transport ($H_2O \rightarrow$ DCPIP) under saturating (Fig. 38A) and limiting PPF conditions (Fig. 38B). RNH thylakoids maintained higher PSII activities than RH thylakoids during high PPF treatment for both light saturated and light limited rates. The results for PSII activities are consistent with F_v/F_m ratios results (Fig. 33D) except that PSII activities decreased twice as fast as the F_v/F_m ratios.

PPF response curves for PSII electron transport were performed on control and 15 min photoinhibited samples. ϕ_{app} of PSII was determined from the initial slope of these curves (Table V). Control samples of RH and RNH thylakoids showed similar ϕ_{app} for PSII electron transport, but 15 min of high PPF treatment at 20°C caused a 2.5-fold decrease in the ϕ_{app} for PSII electron transport in RH thylakoids compared with a 1.4-fold decrease in RNH thylakoids. These results are again consistent with measurements on F_v/F_m.

3.7.4 Effect of photoinhibition on *in vitro* PSI measurements

I also measured the light saturated and the light limited rates of PSI activities during photoinhibition of isolated rye thylakoids (Fig. 39) because PSI has been shown to be susceptible to photoinhibition in isolated thylakoid systems (Cornic and Miginiac-

Figure 38: Changes in the initial A) light saturated (3000 μmol m^{-2} s^{-1}) and B) light limited (250 μmol m^{-2} s^{-1}) rates of PSII electron transport in RH and RNH thylakoids during exposure to 2600 μmol m^{-2} s^{-1} at 20°C. (○) RH, (●) RNH. Initial rates of PSII activities were 473 ±108 for RH and 399 ±53 μmol DCPIP reduced mg Chl^{-1} h^{-1} for RNH thylakoids when measured at 3000 μmol m^{-2} s^{-1} and 89 ±8 for RH and 84 ±11 μmol DCPIP reduced mg Chl^{-1} h^{-1} for RNH thylakoids when measured at 250 μmol m^{-2} s^{-1}. All measurements were done at 25°C

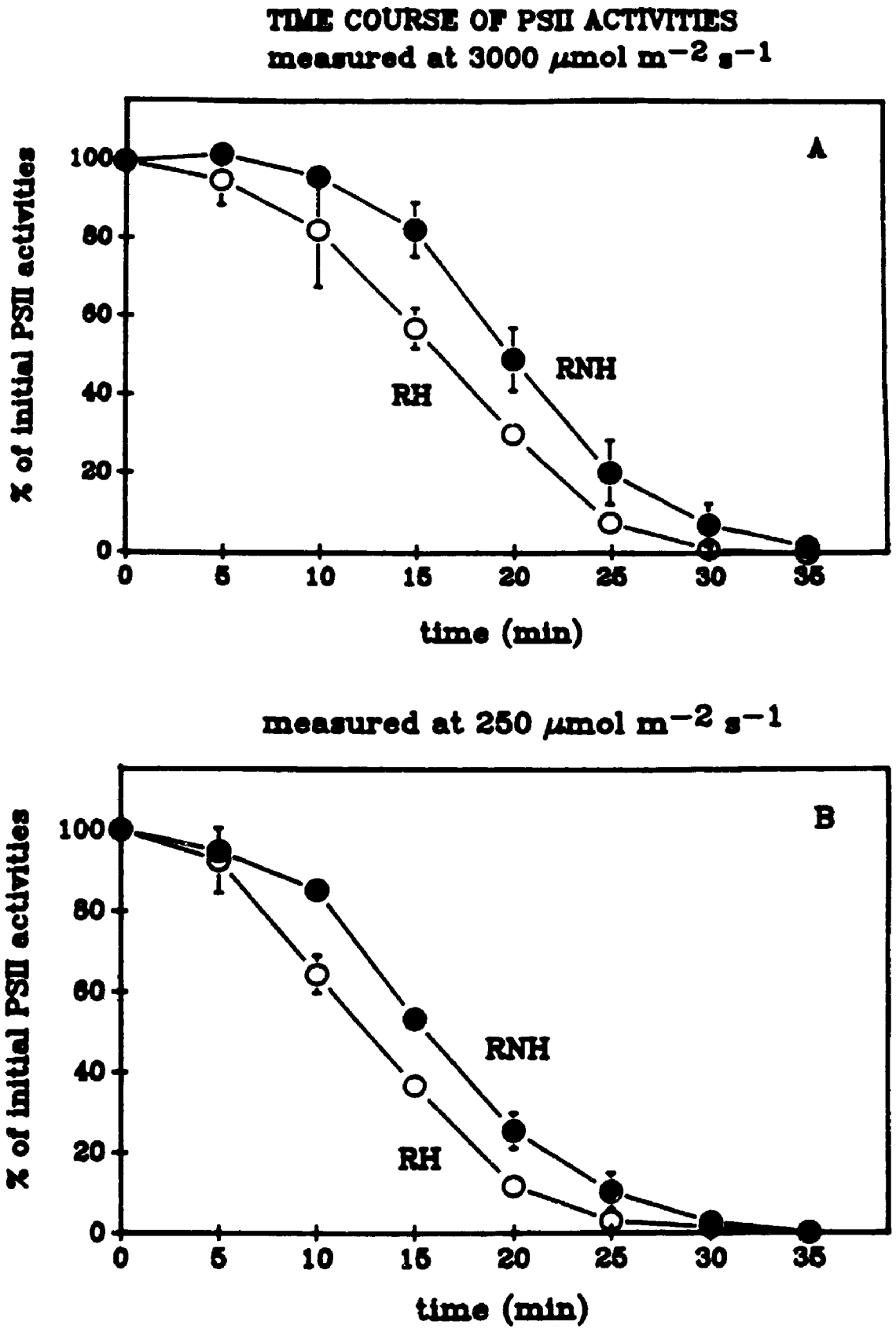

TIME COURSE OF PSII ACTIVITIES
measured at 3000 μmol m⁻² s⁻¹
A
% of initial PSII activities
100
80
60
40
20
0
RNH
RH
0
5
10
15
20
25
30
35
time (min)
measured at 250 μmol m⁻² s⁻¹
B
% of initial PSII activities
100
80
60
40
20
0
RNH
RH
0
5
10
15
20
25
30
35
time (min)

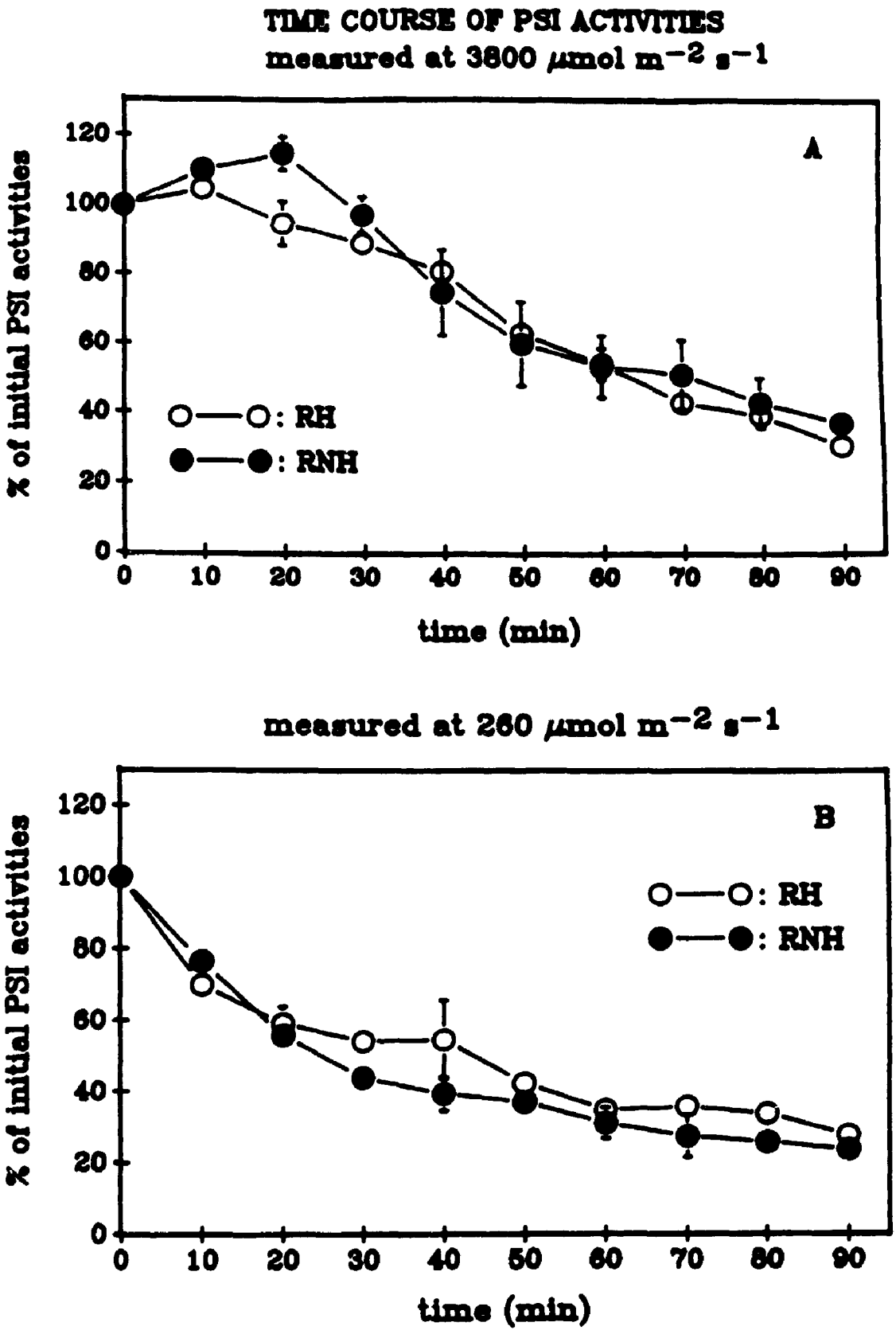
Table V. Initial slopes of PPF responses curves for PSII and PSI activities of control and photoinhibited RH and RNH thylakoids. Rye thylakoids were exposed to 2600 μmol m^{-2} s^{-1} at 20°C for 15 min (PSII) or for 30 min (PSI).

Conditions	PSII[1]		PSI[2]	
	RH	RNH	RH	RNH
Control	0.353 ±0.027	0.368 ±0.022	0.116 ±0.004	0.108 ±0.010
Treated	0.139 ±0.012	0.255 ±0.036	0.043 ±0.001	0.040 ±0.005

1 units: μmol DCPIP reduced·mg $chl^{-1}\cdot h^{-1}\cdot\mu$mol $photons^{-1}\cdot m^2\cdot s^1$

2 units: μmol O_2 consumed·mg $chl^{-1}\cdot h^{-1}\cdot\mu$mol $photons^{-1}\cdot m^2\cdot s^1$

Figure 39: Changes in the initial A) light saturated (3800 μmol m^{-2} s^{-1}) and B) light limited (260 μmol m^{-2} s^{-1}) rates of PSI electron transport in RH and RNH thylakoids during exposure to 2600 μmol m^{-2} s^{-1} at 20°C. (○) RH, (●) RNH. Initial rates of PSI activities were 289 ±24 for RH and 264 ±7 μmol O_2 consumed mg Chl^{-1} h^{-1} for RNH thylakoids when measured at 3800 μmol m^{-2} s^{-1} and 99 ±3 for RH and 103 ±4 μmol O_2 consumed mg Chl^{-1} h^{-1} for RNH thylakoids when measured at 260 μmol m^{-2} s^{-1}. All measurements were done at 20°C.


TIME COURSE OF PSI ACTIVITIES
measured at 3800 μmol m⁻² s⁻¹
A
% of initial PSI activities
120
100
80
60
40
20
0
○—○: RH
●—●: RNH
0
10
20
30
40
50
60
70
80
90
time (min)
measured at 260 μmol m⁻² s⁻¹
B
% of initial PSI activities
120
100
80
60
40
20
0
○—○: RH
●—●: RNH
0
10
20
30
40
50
60
70
80
90
time (min)

Maslow, 1985; Nedbal *et al.*, 1986). The light limited rates decreased rapidly during the initial 30 min of photoinhibitory treatment and then at a slower rate for the following 60 min (Fig. 39B). Conversely, light saturated rates did not show any significant decrease over the first 30 min then decreased steadily for the next 60 min of high PPF treatment (Fig. 39A). I noticed a stimulation of PSI activities in RNH thylakoids that had been characterized before (Huner and Reynolds, 1989). Otherwise, no difference was noticed between the RH and RNH PSI response to high PPF treatment. Some PSI activity remained even after 90 min of high PPF treatment of rye thylakoids; this could be an artefact of the method or an indication of a resistant population of PSI. In comparing the time course of photoinhibition of PSII (Fig. 38) and PSI activity (Fig. 39) it appeared that PSI showed less susceptibility to photoinhibition than PSII. Dark controls of rye thylakoids also showed a greater stability for PSI than for PSII at room temperature (data not presented).

PPF response curves were also done for PSI activities on control and on samples treated during 30 min under high PPF. RH and RNH thylakoids control samples showed similar PSI ϕ_{app} (Table V). Both RH and RNH photoinhibited samples showed a similar 63% decrease in their ϕ_{app} for PSI electron transport following 30 min of high PPF treatment.

3.7.5 PAS measurements

Photoacoustic measurements represent another independent means of assessing the photosynthetic capacity of PSII (Lasser-Ross *et al.*, 1980). ϕ'_r was monitored during photoinhibition of isolated thylakoids at room temperature (Fig. 40). ϕ'_r is calculated from the difference between the intensity of the PAS signal in presence (Q_m) and in the absence (Q_c) of the non-modulated saturating beam. It estimates the capacity of energy storage into the thylakoids during photosynthesis. As expected, photoinhibitory treatment decreased the energy storage capacity of PSII, that is, decreased the capacity for PSII electron transport. The curves showed a biphasic decrease in ϕ'_r: an initial fast decrease between 0 and 30 min followed by a much slower decrease after 30 min of high PPF treatment. After 90 min of treatment, 16% (RNH) and 20% (RH) of the initial capacity of energy storage was still present in the thylakoids. RH thylakoids exhibited a greater decrease in energy storage capacity than RNH thylakoids over the first 20 min of photoinhibitory treatment. Thus the photoacoustic measurements are consistent with the fluorescence and PSII electron transport data and show that isolated RH thylakoids are more sensitive than RNH thylakoids to photoinhibition.

I also plotted the maximum values of energy released (Q_m) of rye thylakoids during the high PPF treatment (Fig. 41). Despite large variability between samples, RNH thylakoids showed Q_m values significantly higher than the Q_m values of RH thylakoids ($F(1,47)= 4.63$, $p\leq 0.05$) between 10 and 60 min of high PPF treatment. RH thylakoids maintained constant Q_m values during the whole treatment, while RNH thylakoids increased their Q_m values by as much as 13% when submitted to high PPF

Figure 40: Changes in the initial ϕ'_r of RH and RNH thylakoids during exposure to 2600 μmoi m^{-2} s^{-1} at 20°C. (○) RH, (●) RNH. Initial values of ϕ'_r were 16.5 ±1.5% for RH and 15.9 ±0.6% for RNH thylakoids. The modulated measuring beam had an energy flux of 0.49 W^{-2} at a frequency of 35 Hz. The non-modulated saturating beam had an energy flux of 186 W^{-2}. All measurements were done at room temperature.

EFFECT OF PHOTOINHIBITORY TREATMENT ON THE CAPACITY OF ENERGY STORAGE

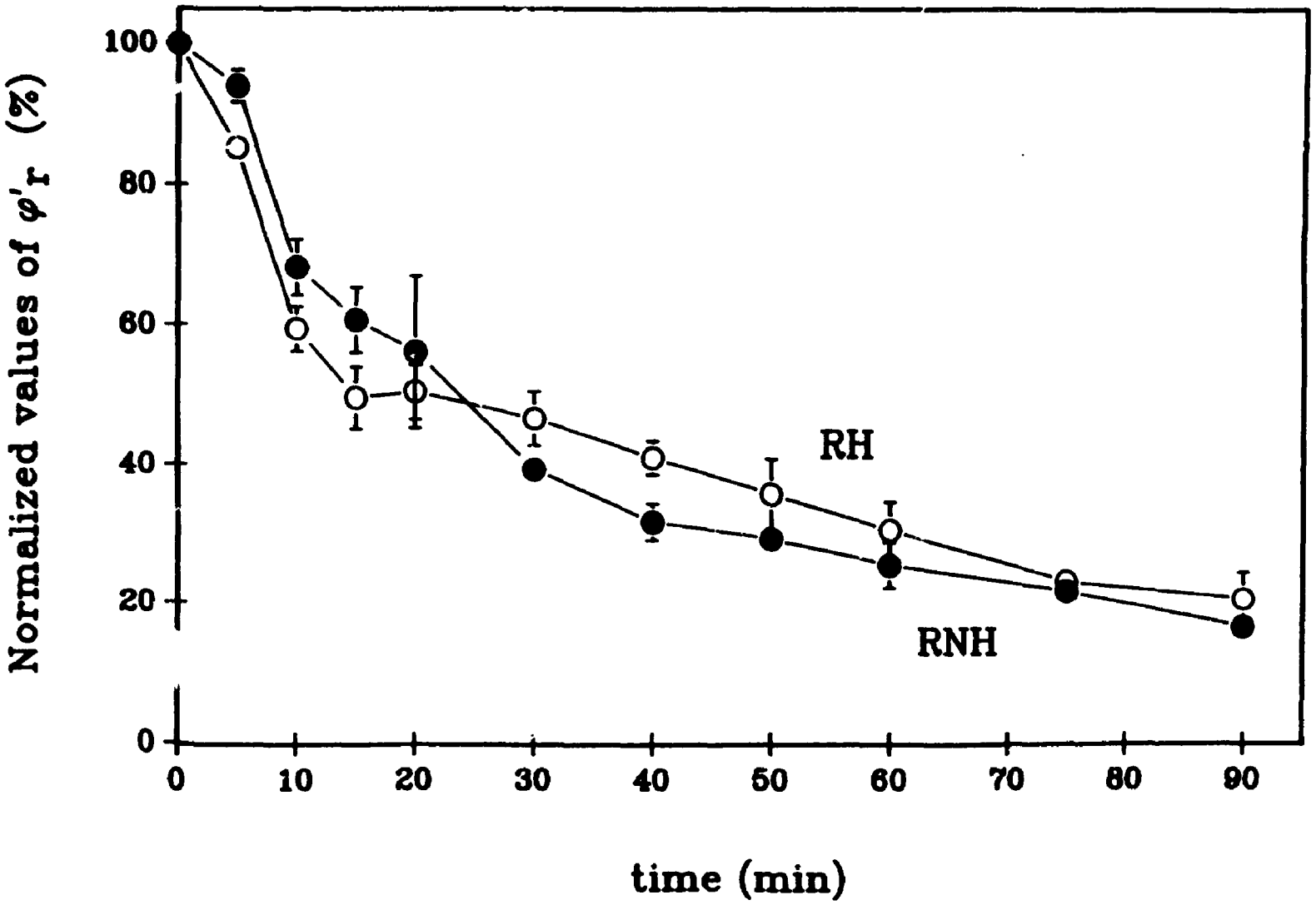
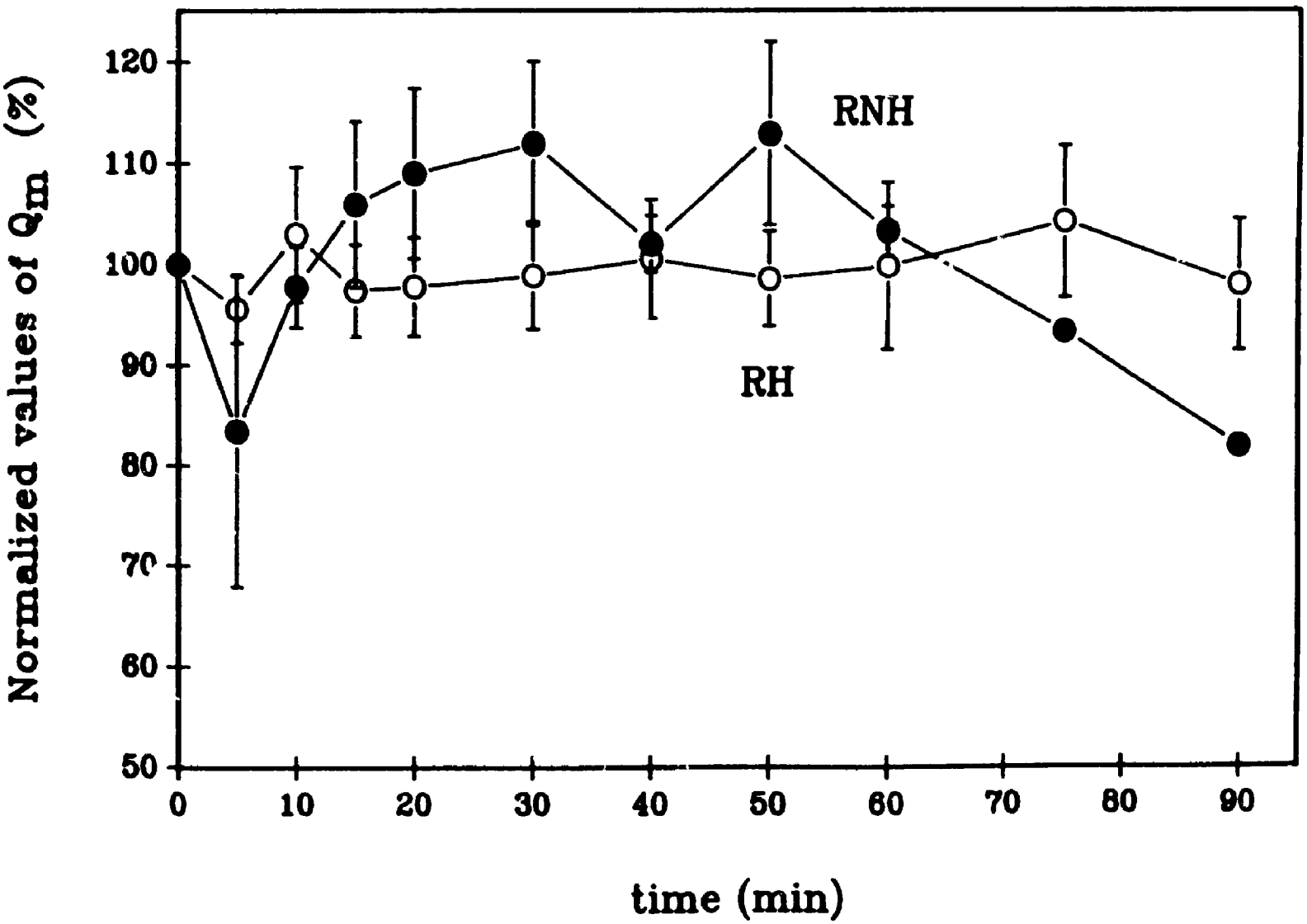


Figure 41: Changes in the Q_m values of RH and RNH thylakoids during exposure to 2600 μmol m^{-2} s^{-1} at 20°C. (○) RH, (●) RNH. All measurements were done at room temperature.

EFFECT OF PHOTOINHIBITORY TREATMENT ON Q_m



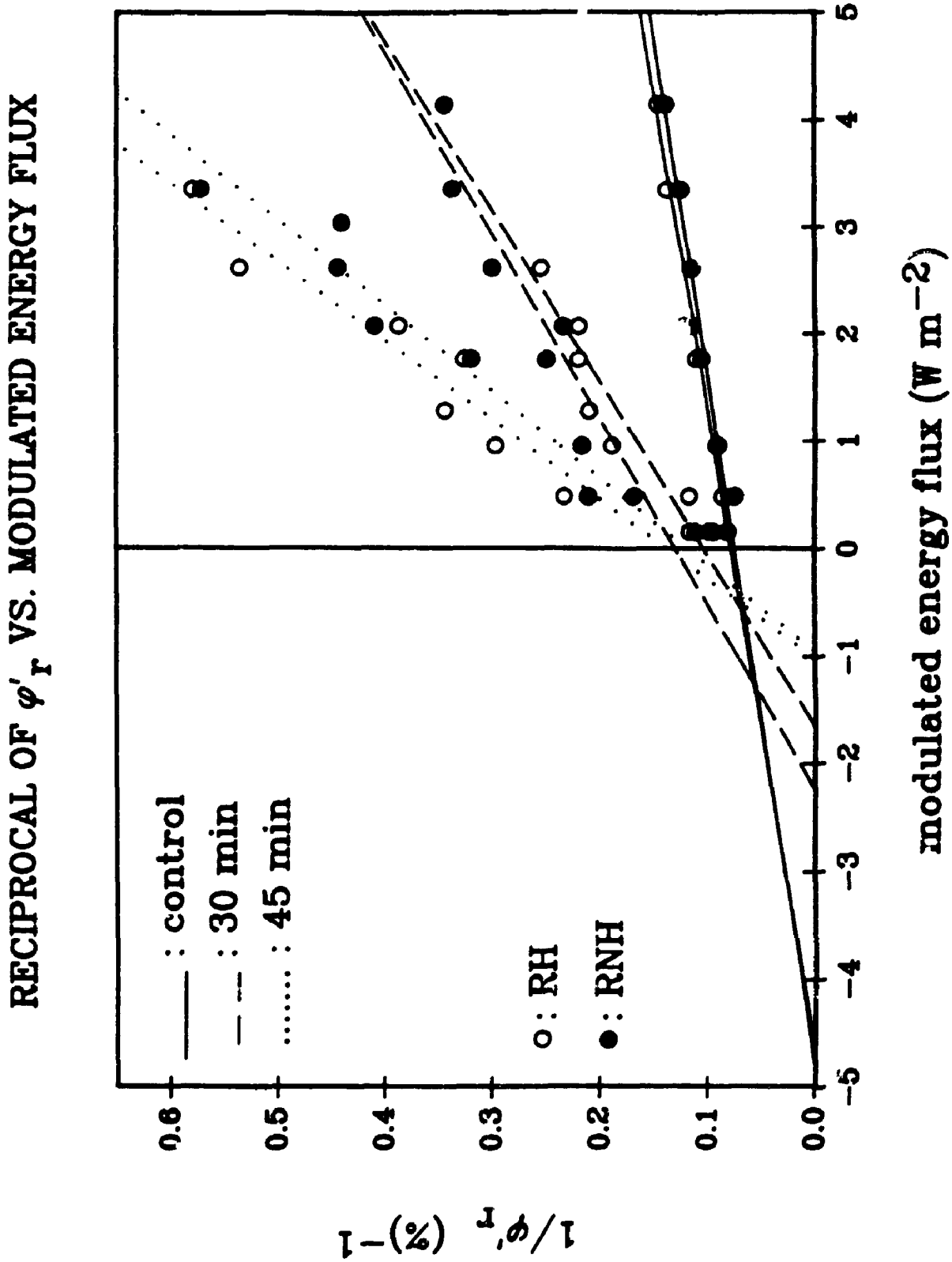
treatment. Thus, RNH thylakoids appeared to give off more heat than RH samples during photoinhibition.

Other PAS parameters can be estimated by measuring ϕ'_r under different energy fluxes of the modulated beam (Fig. 42). Using reciprocal plots, ϕ'_{r0} is measured as the inverse of the intercept on the ordinate and I_{50} as the intercept on the abscissa. ϕ'_{r0} (maximal energy storage yield) estimates the maximal energy thylakoids can store when the modulated energy flux tends toward zero, and I_{50} (half-saturation energy flux) represents the energy flux at which 50% of maximal ϕ'_r is reached. It has been shown that I_{50} is proportional to the electron transport rates as estimated by O_2 evolution in isolated thylakoids (Carpentier *et al.*, 1988). I_{50} values decreased following high PPF treatment from 5 W m^{-2} in control samples to 1 W m^{-2} after 45 min of high PPF treatment. Both thylakoid populations showed similar I_{50} values in control and in treated samples. Maybe I_{50} is not as sensitive a parameter as F_v/F_m or PSII activities are or it may measure something slightly different. The maximal capacity of energy storage also dropped after high PPF treatment, but there was no difference between 30 min and 45 min treated samples. Both RH and RNH thylakoids showed similar ϕ'_{r0} in control and in high PPF treated samples.

3.7.6 Atrazine binding study

A decrease in the F_v/F_m or the ϕ_{app} for PSII electron transport could be due to damage to PSII reaction centres or to an increased capacity for the non-radiative de-

Figure 42: Reciprocal plot of $1/\phi'_f$ as a function of the energy flux of the modulated beam for control (solid lines), 30 min (broken lines) and 45 min (dotted lines) photoinhibited RH and RNH thylakoids. Thylakoids were exposed to 2600 μmol m^{-2} s^{-1} at 20°C. (○) RH, (●) RNH. All measurements were done at room temperature. Each point is the mean of 3 replicates.


RECIPROCAL OF φ'_r VS. MODULATED ENERGY FLUX
—— : control
— — : 30 min
..... : 45 min
○ : RH
● : RNH
$1/\varphi'_r$ (%)$^{-1}$
0.0
0.1
0.2
0.3
0.4
0.5
0.6
−5
−4
−3
−2
−1
0
1
2
3
4
5
modulated energy flux (W m^{-2})

excitation of absorbed light energy (Björkman, 1987b). A principal site of photoinhibitory damage to PSII is at the level of the D1-protein Q_B site (Kyle *et al.*, 1984). To determine whether the photoinhibitory treatment caused damage to the Q_B-binding site of PSII, RH and RNH thylakoids were assayed for their capacity to bind labelled atrazine as a function of photoinhibitory treatment (Fig. 43). Atrazine is a competitive inhibitor of Q_B binding to the D1 protein (Vermaas *et al.*, 1983). Preliminary results showed that 1 μM was sufficient to saturate the Q_B sites in both RH and RNH thylakoids. Exposure of rye thylakoids to photoinhibition for 60 min caused a 70% reduction in the capacity to bind [^{14}C]atrazine (Fig. 43). The rate of loss of functional Q_B sites was slower than the loss of PSII activity or F_v in rye thylakoids during photoinhibition. The kinetics for the inhibition of [^{14}C]atrazine binding were similar for RH and RNH thylakoids, thus they exhibited similar susceptibilities to photoinhibitory damage at the Q_B binding site of PSII.

3.8 Thylakoids isolated from photoinhibited rye cells

Thylakoids isolated from photoinhibited cells were assayed to characterize the photoinhibition of thylakoids *in vivo*. After rye cells were photoinhibited for 20 min, both cells and thylakoids isolated from these cells showed a similar decrease in F_v/F_m (Fig. 44A & B). Light saturated PSII activities of the thylakoids showed a decrease always somewhat greater than the F_v/F_m decrease. This is consistent with the results obtained during the photoinhibition of isolated thylakoids where PSII activities (Fig. 38B) decreased faster than F_v/F_m ratios (Fig. 33D). During recovery at 20°C, F_v/F_m of

Figure 43: Effect of photoinhibition on [^{14}C]atrazine binding. RH and RNH thylakoids were exposed to 2600 μmol m^{-2} s^{-1} at 20°C. (○) RH, (●) RNH. RH control samples bound 6.3 ±1.7 nmol atrazine mg Chl^{-1} and RNH control samples bound 4.5 ±0.3 nmol atrazine mg Chl^{-1}.

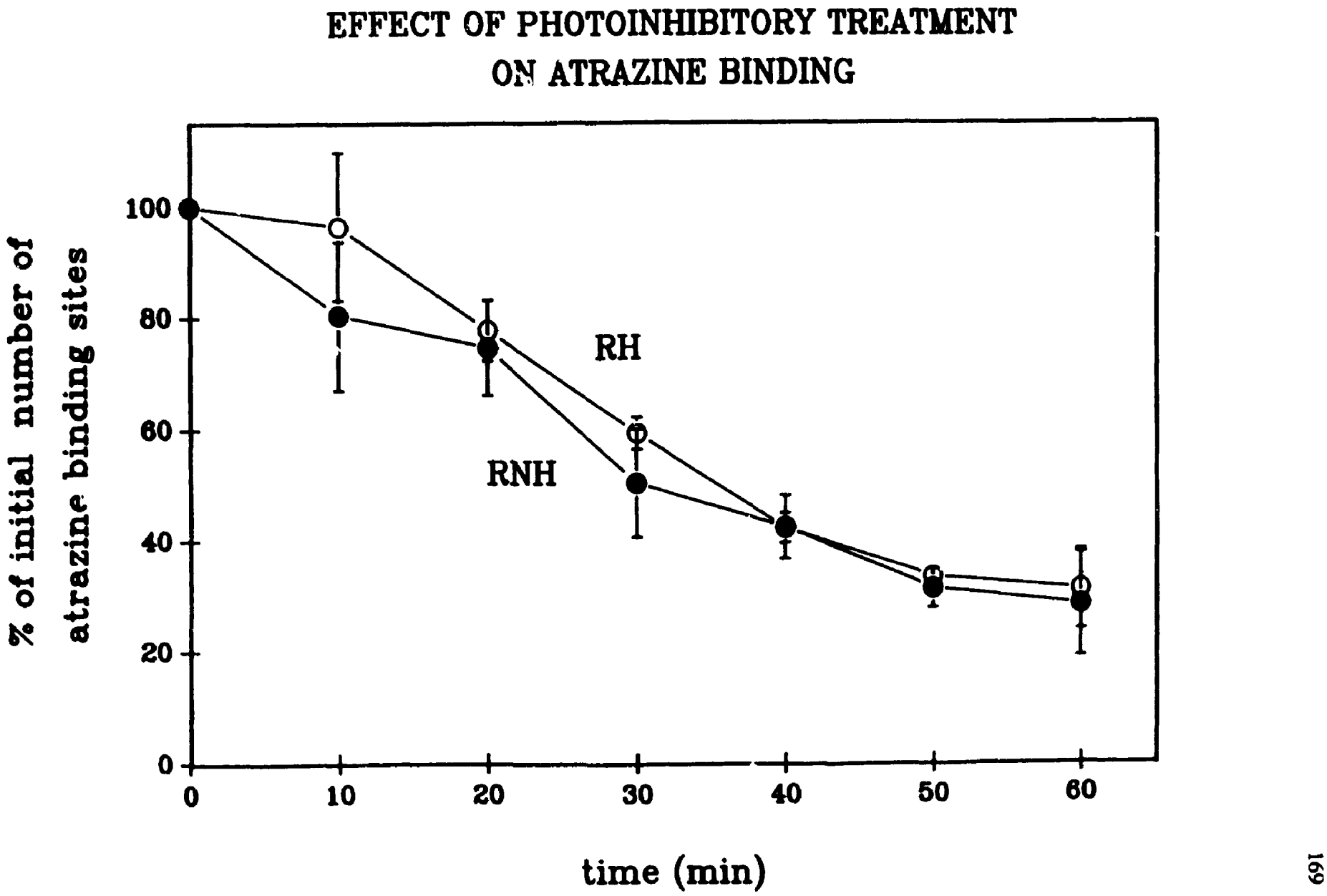
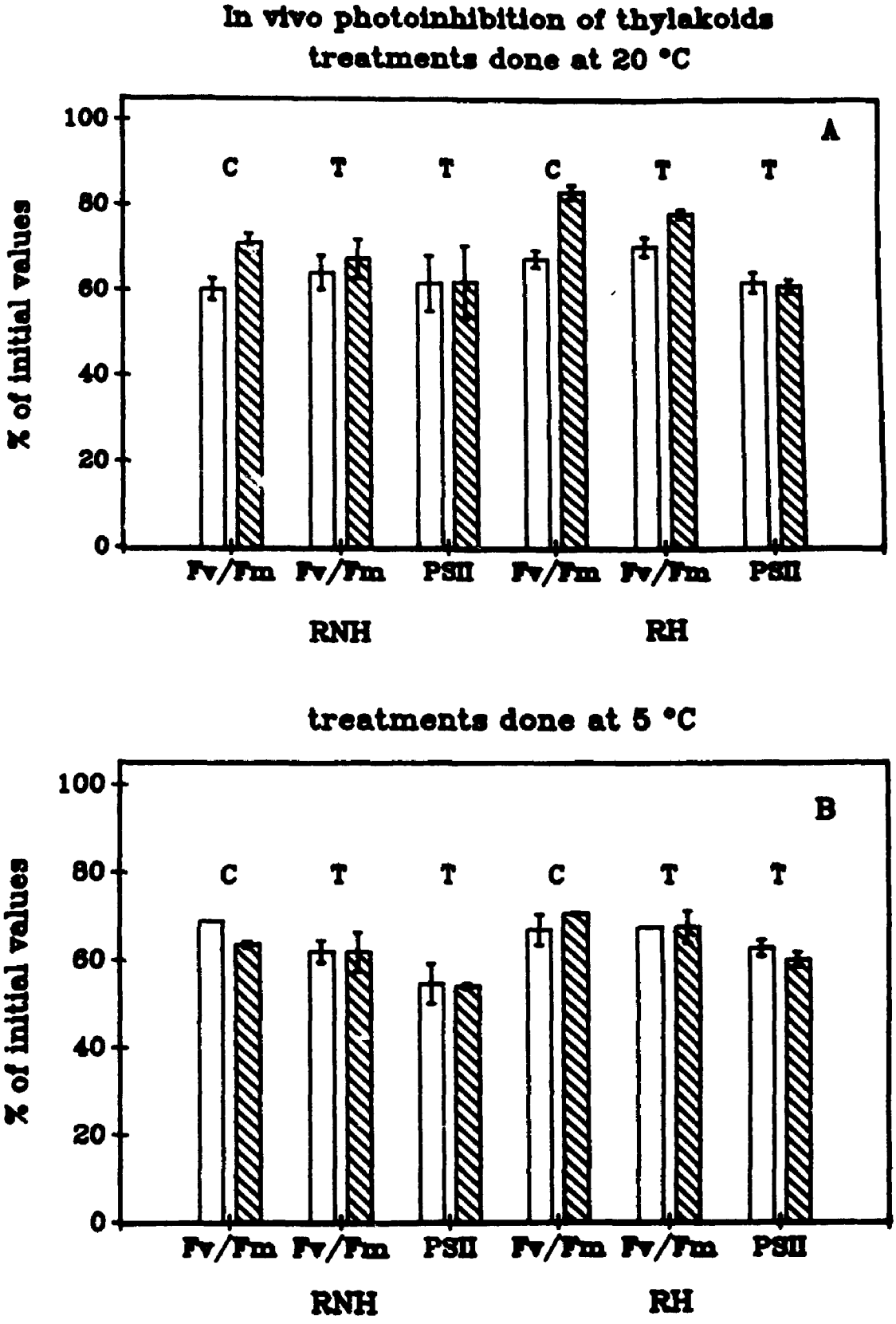

EFFECT OF PHOTOINHIBITORY TREATMENT
ON ATRAZINE BINDING
% of initial number of
atrazine binding sites
100
80
60
40
20
0
0
10
20
30
40
50
60
RH
RNH
time (min)

Figure 44: F_v/F_m and light saturated PSII activities of thylakoids isolated from photoinhibited RH and RNH cells. Cells were exposed to 2600 μmol m^{-2} s^{-1} at A) 20°C and at B) 5°C for 20 min. Recovery of rye cells occurred at 20°C (A) or at 5°C (B) under 30 μmol m^{-2} s^{-1} for 2 h. Open bars and crossed bars represent photoinhibited and recovered samples respectively, C= cells and T= thylakoids. Control F_v/F_m were 0.67 ±0.01 for RH and 0.66 ±0.01 for RNH cells. Control F_v/F_m were 0.56 ±0.01 for RH and 0.55 ±0.02 for RNH thylakoids. Initial rates of PSII activities were 393 ±10 for RH and 273 ±19 μmol DCPIP reduced mg Chl^{-1} h^{-1} for RNH thylakoids. Fluorescence measurements were done at room temperature and PSII activities were measured at 25°C at 3000 μmol m^{-2} s^{-1}.


In vivo photoinhibition of thylakoids
treatments done at 20 °C
A
% of initial values
100
80
60
40
20
0
C
T
T
C
T
T
Fv/Fm
Fv/Fm
PSII
Fv/Fm
Fv/Fm
PSII
RNH
RH
treatments done at 5 °C
B
% of initial values
100
80
60
40
20
0
C
T
T
C
T
T
Fv/Fm
Fv/Fm
PSII
Fv/Fm
Fv/Fm
PSII
RNH
RH

rye cells and F_v/F_m of their thylakoids increased to similar extent, while PSII activities showed no change. At 5°C, no change was detected in F_v/F_m of cells and of thylakoids or in the PSII activities of the thylakoids following recovery. Similar trends were obtained when comparing F_v/F_m of RH and RNH cells or F_v/F_m of their thylakoids.

CHAPTER 4

DISCUSSION

4.1 Photoinhibitory response of intact rye leaves

Decreases in F_v/F_m ratios of rye leaves during high PPF treatments are in accordance with results obtained at lower PPF (400 μmol m^{-2} s^{-1}) by Öquist and Huner (1991). Therefore, intact RH leaves appear to be more resistant to photoinhibition regardless of the PPF used during the treatment. At high PPF, photoinhibition could be obtained at low and at room temperature, while at 400 μmol m^{-2} s^{-1}, only the low temperature treatment gave rise to photoinhibition of rye leaves. Photosynthetic and metabolic rates might not be as important when the plants are submitted to very high PPF because photoinhibition develops rapidly, as suggested by Tyystjärvi *et al.* (1989). Part of the adaptation to low temperature induced photoinhibition can be related to increased recovery rates as shown in cold grown *Chlamydomonas reinhardtii* (Falk *et al.*, 1990). In the case of winter rye, RH and RNH leaves showed similar capacity to recover from photoinhibition at room temperature as already noticed by Öquist and Huner (1991). Similar recovery rates have also been obtained for intact leaves of cold hardened and non-hardened wheat and spinach cultivars (Hurry and Huner, unpublished; Boese and Huner, unpublished).

Initial lower F_v/F_m ratios in RH leaves could be essentially accounted for by F_o values that are higher than found in RNH leaves because F_v values were very similar

Figure 13: Corrected fluorescence emission spectra at 77K of RH and RNH cells. Samples contained 7 μg Chl ml^{-1}. The excitation wavelength was 440 nm.

(data not presented). Therefore the lower F_v/F_m ratios of RH leaves might not indicate slight photoinhibition under growing conditions as suggested by Öquist and Huner (1991). According to Demmig and Björkman (1987) an increase in F_o during photoinhibitory treatment reflects damage to PSII. RH leaves would thus be more damaged than RNH leaves following high PPF treatment. However, this hypothesis is not substantiated by my results. Although F_o measurements increased more in RH leaves during photoinhibition, F_v did not decrease faster in RH than in RNH leaves. Other components can affect F_o values. Those components appeared to maintain their effect even after 20 h of recovery, once F_v/F_m ratios had reached 89% (5°C) and 95% (20°C) of control values. In spinach, F_o increased more in non-hardened than in hardened spinach (Somersalo and Krause, 1989). Somersalo and Krause suggested that different photoinhibitory mechanisms are taking place in hardened and non-hardened spinach, based on a different response of F_o.

RH and RNH leaves exhibited similar steady-state q_p values: they thus have similar photochemical efficiency to oxidize Q_A even though RH leaves exhibited lower F_v/F_m. Under control conditions RH leaves showed lower q_N values suggesting that less energy was converted into non-radiative radiation in RH than in RNH leaves. However, upon exposure to photoinhibition, RH leaves showed a greater capacity to increase the amount of energy re-emitted as non-radiative decay than RNH leaves, especially at 5°C, where RH leaves increased their q_N considerably. This could reflect a protective mechanism that diverts absorbed light energy from photochemistry to non-radiative decay during photosynthesis (Björkman, 1987a). In both RH and RNH leaves q_o increased

slightly during photoinhibition, another indication of an increase in non-radiative decay, and q_o decreased back to initial level after recovery following the recovery of q_N. In contrast, q_p was stable in RNH leaves while it decreased significantly in RH during photoinhibition at 5°C. Furthermore, after a high PPF treatment at 5°C, RH was unable to recover its initial q_p values even though q_N recovered completely. According to Foyer *et al.* (1990) q_p is usually dampened against changes in the reduction level of the electron transport chain while q_N changes with incident PPF. Therefore a significant decrease in q_p might indicate that dissipation of energy through q_N is overcome (Weis and Lechtenberg, 1989). Thus a low q_p in photoinhibited and recovered RH leaves coupled with an increase in F_o suggests that these leaves may be more damaged than is evident from F_v/F_m measurements. Clearly, measurements of F_v/F_m alone may not be sufficient to elucidate the photoinhibitory response of intact leaves.

4.2 Photoinhibitory response of rye cells

Following treatment at 400 μmol m^{-2} s^{-1}, isolated RH and RNH cells showed a differential sensitivity to photoinhibition similar to the difference observed at the intact leaf level. This trend was not evident at high PPF (2600 μmol m^{-2} s^{-1}). Isolated mesophyll cells from RH leaves can thus exhibit an increased resistance to photoinhibition, but only when the PPF used during the treatment is not too high. At higher PPF, any difference between RH and RNH cells is probably obscured by the speed at which photoinhibition occurred.

The decrease in F_o values during the 400 μmol m^{-2} s^{-1} treatment for both RH and RNH cells suggested the presence of some protective mechanisms (Demmig and Björkman, 1987). During the treatment at 2600 μmol m^{-2} s^{-1}, the F_o values tended to be higher than the controls which suggested damage to PSII (Barényi and Krause, 1985). With isolated cells, I could not see a difference in the extent of the increase in F_o between RH and RNH samples. In contrast, isolated thylakoids showed a greater increase in the F_o of RNH thylakoids than of RH thylakoids. However, for intact leaves, a greater increase in the F_o of RH than of RNH samples was observed. Maybe the net effect of photoinhibition on F_o is masked by great variability in rye cells.

The extent of photoinhibition of rye cells is PPF dependent. This reflects a relationship between the number of quanta absorbed by the cells and the extent of photoinhibition as shown with rye thylakoids and with maize thylakoids (Habash and Baker, 1990) and with algae cells (Samuelsson *et al.*, 1985). However, the extent of photoinhibition is not necessarily temperature dependent as is also shown in rye leaves. High PPF treatments did not differentiate between 5 and 20°C treatments, while lower PPF treatments (400 μmol m^{-2} s^{-1}) showed greater decreases in CO_2 fixation rates at 5°C. Treatments at 2600 μmol m^{-2} s^{-1} possibly decrease photosynthetic activities too fast to observe a differential response and thus such high PPF may overcome possible protective mechanisms that could be used by RH and RNH cells. Treatments at 2600 μmol m^{-2} s^{-1} thus reflect the preponderance of biophysical phenomena, while the 400 μmol m^{-2} s^{-1} treatment implicates biochemical events as well. At 400 μmol m^{-2} s^{-1}, dissipative mechanisms as well as CO_2 fixation rates could play a role in protecting the

cells against photoinhibition. The rates of CO_2 fixation at 400 μmol m^{-2} s^{-1} being about 4 times higher at 20 than at 5°C could explain the greater photoinhibition seen at 5°C in both groups of rye cells as suggested by Öquist *et al.* (1987). Even after treatments at 2600 μmol m^{-2} s^{-1}, CO_2 fixation rates have shown small but significant differences between 20 and 5°C treatment for RNH cells as non-acclimated *Lemna gibba* thalli (Ögren *et al.*, 1984) and barley protoplasts (Horton *et al.*, 1987) have shown. However, RH cells were as resistant to the 5°C as to the 20°C treatment during the treatment at 2600 μmol m^{-2} s^{-1}. In contrast, F_v/F_m ratios did not show any difference between 5 and 20°C after a photoinhibitory treatment at 400 μmol m^{-2} s^{-1}. It is possible that the difference in response observed between CO_2 fixation and fluorescence is not related to photoinhibition *per se*, but may be associated with regulatory mechanisms or a slow down of certain key enzymes of the Calvin cycle during treatments at 400 μmol m^{-2} s^{-1} at 5°C (Ortiz-Lopez *et al.*, 1990).

The results of F_v/F_m measurements indicate a lower degree of photoinhibition of rye cells than light limited CO_2 fixation rates during both 2600 and 400 μmol m^{-2} s^{-1} treatments. A similar differential response of F_v/F_m and light limited CO_2 fixation rates has also been shown in tomato leaves (Yakir *et al.*, 1985). These two parameters are difficult to compare because F_v/F_m is measured in dark adapted plants and assesses the maximum ϕ_{app} for PSII, while CO_2 fixation rates are measured under steady-state conditions and can be regulated by many factors such as PPF, temperature, utilization of reducing power for other metabolic purposes and regulatory mechanisms that balance the electron transport rates and the CO_2 fixation rates. Thus, it should not be surprising

that F_v/F_m and CO_2 fixation provide a different picture of the photoinhibitory response.

Effect of photoinhibition on the quenching mechanisms measured under steady-state conditions should be more comparable with the effect of photoinhibition on the CO_2 fixation rates than F_v/F_m is. After photoinhibition at 2600 μmol m^{-2} s^{-1}, q_N had increased and q_p had decreased in rye cells. These two changes could induce a decrease in CO_2 fixation rates. But interestingly, q_p increased slightly during photoinhibition at 400 μmol m^{-2} s^{-1} as well as during the first 30 minutes of photoinhibition at 2600 μmol m^{-2} s^{-1}, while F_v/F_m and light limited CO_2 fixation rates decreased steadily. Similarly, in intact leaves, q_p did not show an important decrease except for the 5°C treated RH leaves. Therefore q_p seems to be buffered against decreases in ϕ_{app} of PSII and Q_A remains relatively oxidized even though the pool of PQ might become largely reduced due to important increases in q_N (Foyer *et al.*, 1990). And, as shown in isolated rye cells photoinhibited at 2600 μmol m^{-2} s^{-1}, q_p would decrease only when q_N can no longer increase (Weis and Lechtenberg, 1989). Therefore, a decrease in q_p might reflect damage to PSII, but q_N appears to be a better indicator of the ϕ_{app} of PSII as already shown by Demmig and Winter (1988a).

The decrease in ϕ_{app} of PSII could be caused by inactivation of PSII reaction centres or damage to the Q_B site, but also by an increase in non-radiative quenching such as the one caused by sustained increased level of zeaxanthin in the thylakoid membranes (Demmig *et al.*, 1987b). Increases in q_N and q_o during photoinhibition of rye cells suggest that more energy is lost as non-radiative decay under steady-state conditions

once the samples have been photoinhibited. These increases were especially important during the treatment at 2600 μmol m^{-2} s^{-1}. Demmig-Adams *et al.* (1989d) have shown that q_N and zeaxanthin formation are much slower at 5 than at 20°C in soybean and in *Rhizophora mangle*. However, q_N levels were similar during photoinhibition at 20 and at 5°C for rye cells. The capacity of rye cells to develop q_N during photoinhibition at 5°C might be related to the intrinsic cold resistance of rye or simply to the fact that Demmig-Adams *et al.* (1990d) measured q_N formation at 5°C while all fluorescence measurements on rye cells were done at 25°C. During the present study I did not discriminate between the different possible components of q_N that have been described in the Introduction section. But independent of the mechanism, an increase in q_N could lead to a further decrease in the rates of CO_2 fixation because less energy is transmitted through the electron transport chain once steady-state of electron transport is established.

One possible explanation for the lack of differentiation between the photoinhibitory resistance of RH and RNH cells when measured by CO_2 fixation rates after the treatment at 400 μmol m^{-2} s^{-1} could be the greater increase in q_p in RNH than in RH cells. F_v/F_m was more affected in RNH cells at the end of a treatment at 400 μmol m^{-2} s^{-1}, but this treatment increases q_p to a greater extent in RNH cells than in RH cells. Higher increases in q_p could lead to CO_2 fixation rates higher than expected from F_v/F_m data. Unlike the light limited CO_2 fixation data, the response of both q_p and q_N were indistinguishable during the 5 and the 20°C treatment at 400 μmol m^{-2} s^{-1}; therefore, some of the photoinhibitory effects on CO_2 fixation rates appear to occur at

some other level than electron transport. Thus I conclude that steady-state measurements of Chl *a* fluorescence quenching parameters and light limited CO_2 fixation rates of rye cells reflect the presence of different regulatory mechanisms acting on the photosynthetic apparatus.

The light limiting rates of CO_2 fixation showed more photoinhibition than the light saturated rates for RNH cells, but showed similar levels of photoinhibition as the light saturated rates in RH cells. Increased photoinhibition under light limited conditions has been observed also with whole leaf CO_2 uptake by Ögren *et al.* (1984), and by Le Gouallec and Cornic (1988) and with O_2 evolution rates of barley protoplasts by Horton *et al.* (1987). This reinforces the fact that light saturated rates are limited by other factors besides quantum efficiency. RNH cells showed higher CO_2 fixation rates under light saturated conditions than RH cells at 20°C (Fig. 12); this could explain the decreased sensitivity of their light saturated rates during photoinhibition as compared with RH cells. But this implies that *in vivo* after return of the plant to PPF growth conditions (250 μmol m^{-2} s^{-1}) RNH mesophyll cells would show better CO_2 fixation rates than RH cells following photoinhibition.

Protein synthesis inhibitors had no detrimental effect on the photoinhibitory response of CO_2 fixation rates of rye cells. Lidholm *et al.* (1987) and Ohad *et al.* (1984) have shown that the presence of protein synthesis inhibitors during short periods of photoinhibitory treatments on *Chlamydomonas reinhardtii* accelerated photoinhibition of both O_2 evolution and F_v/F_m. Samuelsson *et al.* (1985) had measured light limited O_2

evolution rates of *Anacystis nidulans* and Greer *et al.* (1986) F_v/F_m of intact bean leaves and also noticed an extensive decrease in photosynthesis when photoinhibition was conducted in presence of chloramphenicol and no effect in presence of cycloheximide. A study with barley protoplasts (Horton *et al.*, 1987) has shown that chloramphenicol has no effect during a photoinhibitory time course at 2°C, while at 20°C the presence of chloramphenicol increases the extent of photoinhibition of O_2 evolution. In barley protoplasts a 2°C treatment at 2300 μmol m^{-2} s^{-1} induced more photoinhibition than a 20°C treatment. They explained these results by a lack of protein synthesis at 2°C. The results for isolated rye cells at 5°C did not show any effect of protein synthesis inhibitors in accordance with the barley protoplasts response at 2°C.

The absence of any effect of protein synthesis inhibitors on photoinhibition at 20°C could be explained by the fact that the ratio of photoinhibition rate (Fig. 17A) to recovery rate (Fig. 26) is very high for rye cells and therefore a change in the recovery rate might not be noticeable in the net photoinhibition rates. Furthermore, the presence of protein synthesis inhibitors could have affected the F_v/F_m differently from the CO_2 fixation rates of rye cells, because they possibly assess different sites of inhibition. According to the present data, however, there is no evidence that rye cells use any repair mechanism involving protein synthesis during their photoinhibitory treatment.

An important factor to consider when studying temperature effects is the assay temperature. A constant assay temperature allows one to eliminate the effect of that

factor. Historically, the effects of photoinhibition have always been assayed at 20 to 25°C even when the photoinhibitory treatment occurred at a different temperature (Van Hasselt and Van Berlo, 1980; Ögren *et al.*, 1984; Bongi and Long, 1987; Somersalo and Krause, 1989). In the case of low temperature induced photoinhibition, it is my contention that it is also important to assay the effects of photoinhibition at the same temperature as the photoinhibitory treatments in order to assess the importance of the temperature treatment on the actual metabolic rates at that temperature. As shown in the first figures (Fig. 9 to 12), photosynthesis in rye cells is slowed down 5-fold at 5°C compared with 20°C. Therefore, temperature *per se* limits CO_2 fixation to a great extent. During photoinhibition at 5°C, CO_2 fixation rates of rye cells were decreased but to a lesser extent when the rates were measured at 5 rather than 20 or 25°C (compare Fig. 23, 24 and 25), especially the light saturated rates. These results clearly show that rye cells can tolerate more photoinhibition at 5°C before their photosynthetic rates are affected because these rates are very much depressed by low temperature. Falk *et al.* (1990) have shown that O_2 evolution rates measured at the growth temperature are less affected in 12°C than in 27°C grown *Chlamydomonas reinhardtii*. They did not measure both groups of cells at the same temperature, and so it is difficult to estimate if 12°C grown cells are more resistant or if photosynthetic rates at 12°C are less sensitive to photoinhibition. A decreased sensitivity of photosynthesis to photoinhibition at low temperature is possible at the leaf level in rye even though their light saturated CO_2 fixation rates are similar at 10°C and at 20°C (Huner *et al.*, 1986). RH leaves could possibly tolerate some photoinhibition under low temperature growth conditions before their CO_2 fixation rates decrease. This should be monitored in the

future to assess the real effect of photoinhibition on photosynthesis of RH and RNH leaves.

4.3 Recovery of isolated rye cells from photoinhibition

Photoinhibitory recovery of rye cells is time dependent and temperature dependent. The recovery rates were faster at 20 than at 5°C, which suggests a possible metabolic requirement, such as protein synthesis. Greer (1988) and Greer and Laing (1988) have also shown an increase in the extent of recovery after photoinhibition with increased temperature in a cold sensitive plant. The implication of a metabolic requirement is also suggested by the protein synthesis inhibitor effect at 20°C on the recovery of CO_2 fixation. However, protein synthesis inhibitors had no effect on the recovery rates during the first 40 min of recovery at 20°C for rye cells, even though Lidholm *et al.* (1987) noticed an immediate inhibition of recovery of O_2 evolution in presence of chloramphenicol with *Chlamydomonas reinhardtii*. Greer *et al.* (1986) also noticed an immediate effect on F_v/F_m recovery of bean leaves when chloramphenicol was applied during recovery. Similar to the present study, Huse and Nilsen (1989) noticed a lag phase in the effect of chloramphenicol on the recovery rates of CO_2 fixation of *Lemna gibba*. They suggested that the time required for uptake and transport of the inhibitor caused the lag or that the plants may recover with material from a pool already present in the chloroplasts. These are possible explanations for the lag period but I think that this is neglecting the importance of other phenomena occurring during photoinhibition.

Repair mechanisms other than replacement of polypeptides damaged during photoinhibition are possibly involved in rye cells during the recovery process of CO_2 fixation. Those mechanisms are more obvious during the first minutes of recovery because, in presence of protein synthesis inhibitors, the recovery rates are not changed during the first 40 min of recovery. Furthermore, the rate of recovery is faster during the first 40 min than during the subsequent 80 min for both RH and RNH cells. This difference in recovery rate also suggests the presence of two different repair mechanisms having different physiological rates. The initial recovery could be due to a decrease in non-radiative decay once the stress is over (Buschmann, 1987; Havaux, 1989). In maize photoinhibited under chilling temperature, both F_v/F_m and ϕ_{app} for CO_2 uptake recovered faster than the number of atrazine binding sites which also suggests that other factors (e.g. a decrease in thermal dissipation) occur during recovery (Ortiz-Lopez *et al.*, 1990).

The second phase of recovery at 20°C implies the presence of protein synthesis as part of the repair mechanism for CO_2 fixation. When protein synthesis was blocked the recovery rates became negative after 1 h of recovery. Control samples treated with protein synthesis inhibitors for 2 h showed only a slight decrease in their photosynthetic rates, which strongly suggests that recovery requires more than the usual turnover of proteins. The apparent difference between RH and RNH cells in their sensitivity to chloramphenicol might be related to substantial variations between samples which can obscure significant difference between control recovery rates and the chloramphenicol recovery rates. Surprisingly, cycloheximide affected recovery rates at 20°C, which suggests a requirement for nuclear encoded protein synthesis. In previous studies the

presence of cycloheximide affected the recovery rates only slightly (Ohad *et al.*, 1984; Greer *et al.*, 1986). However, a study on tobacco protoplasts has shown that cycloheximide can affect also chloroplastic protein synthesis (36% depression) and chloramphenicol can affect cytoplasmic protein synthesis (36% depression) (Sakai and Takebe, 1970). Therefore the effect of these protein synthesis inhibitors might not be as precise as expected.

As with CO_2 fixation, recovery of F_v/F_m ratios appeared also to be a 2-phase process; this is consistent with the proposal that there are two separate recovery phases which differ by their rates and time of appearance after the photoinhibitory stress is over. However recovery of F_v/F_m was much slower than the recovery of light limited CO_2 fixation rates. I propose two possible explanations for this difference: first, some quenching mechanisms quench F_v for a long time after photoinhibition is over without affecting the rate of CO_2 fixation, or alternatively, part of the recovery of CO_2 fixation may be related to the modulation of light activated enzymes. One of the possible F_v quenching mechanisms could be q_I which has been observed in photoinhibited samples and appeared to relax with a $t_{1/2}$ of several hours (Quick and Stitt, 1989). Some evidence suggests that q_I might develop rapidly even during short term exposure to high PPF (Lee *et al.*, 1990). The presence of q_I could explain the difference in the extent of recovery between F_v/F_m and light limited CO_2 fixation rates after 2 h under low PPF. I do not know if q_I had developed in rye cells during photoinhibition nor if it could affect the rates of CO_2 fixation. Furthermore this argument tends to be circular because q_I is measured as one of the quenching mechanisms of F_v in photoinhibited samples, and

currently it cannot be monitored in any other way.

The modulation of light activated enzymes during photoinhibition and recovery appears to be a better candidate to explain the initial fast recovery of light limited CO_2 fixation rates. Light activated enzymes such as ribulose-5-P kinase, NADP malate dehydrogenase and NADP glyceraldehyde-3-P dehydrogenase (Buchanan, 1980) are controlled by the incident PPF level and the reduction level of NADP. When electron transport rates are depressed during photoinhibition by damage to PSII or by the presence of protective mechanisms that increase the non-radiative decay of light energy, less NADPH is then available to reduce these enzymes (Dujardyn and Foyer, 1989). Powles *et al.* (1982) have shown a decrease in activity of light activated enzymes in bean leaves during photoinhibition. The *in vitro* activity of these enzymes was fully recovered within 90 min, while CO_2 fixation rates recovered completely only after 2 to 4 h. Giersch and Robinson (1987) and Miginiac-Maslow *et al.* (1988) have also shown a reduction in activity of fructose-1,6-bisphosphatase and of NADP-malate dehydrogenase respectively during photoinhibition of intact spinach chloroplasts. These data are consistent with the initial fast recovery in rye cells which appeared to be independent of the extent of photoinhibition (Fig. 30). They could also explain the greater suppression of light limited CO_2 fixation rates during photoinhibition than of F_v/F_m of rye cells.

Recovery of F_v/F_m in rye cells appeared to be independent of the extent of photoinhibition. Other studies have shown an inverse relationship between the extent of photoinhibition and the rate of recovery of F_v/F_m (Bhogal and Barber, 1987; Greer and

Laing, 1988). This discrepancy might reflect the different processes that can induce a decrease in F_v/F_m during photoinhibition. The effect of the extent of photoinhibition on recovery rates suggests that the first phase of recovery represents different phenomena when studied with CO_2 fixation rates and with F_v/F_m ratios. If, as suggested, repair mechanisms are not implied during the first phase, quenching mechanisms responsible for the decrease in F_v/F_m would relax at the same rate independently of the extent of F_v/F_m decrease. Relaxation of these quenching mechanisms could also increase CO_2 fixation rates by allowing an increase in the rates of electron transport. However, q_p and q_N did not show a clear 2-phase recovery (Fig. 27) which suggests that the increase in F_v/F_m does not have a direct effect on the electron transport rates. If the inactivation of light activated enzymes during photoinhibition is partly responsible for the important decrease in light limited CO_2 fixation rates in rye cells, it is possible that reactivation of these enzymes bring the CO_2 fixation rates to similar rates during the first min of recovery, independently of the extent of decrease of CO_2 fixation. In this case, the extent of photoinhibition of CO_2 fixation rates would be correlated with the extent of inactivation of the light activated enzymes. Further work is required to test this hypothesis.

In leaves, q_N has been shown to recover slowly. Greer (1988) reported 50% recovery of q_N after 100 min in kiwifruit leaves and Björkman (1987a) also showed a slow recovery of q_N in *Monstera deliciosa*. In rye cells, q_N recovered faster than in these studies, with 72% (RH) and 84% (RNH) of q_N recovered at 20°C after 2 h at low PPF. This reflects that different mechanisms can give rise to q_N which also will exhibit

different relaxation rates.

At 5°C, the recovery rates are very slow even during the first 40 min of recovery as also shown for F_v/F_m of spinach leaf discs (Chow *et al.*, 1989). This is in contradiction with data for spinach leaves where similar initial rates of recovery of F_v/F_m occurred at 5°C and at 18°C (Somersalo and Krause, 1988). However, in rye cells, both recovery dependent upon protein synthesis and recovery dependent upon other processes are slowed down at low temperature. Havaux (1989) has shown using PAS that pea leaves subjected to photoinhibition at low temperatures were not able to increase their non-radiative decay, while at 20°C they increased it during the treatment and decreased it at a fast rate during the subsequent recovery period. Similarly, Adams *et al.* (1990a) have shown that the development of q_N was strongly inhibited in spinach leaves at temperatures lower than 13°C. But q_N data for rye cells showed no difference between 5 and 20°C treatment during photoinhibition and only a slightly greater relaxation of q_N at 20°C during recovery of rye cells. So, rye cells develop q_N during photoinhibition at 5°C and relax it to a certain extent during recovery. As mentioned previously, differential q_N values during photoinhibition at 5 and at 20°C could be masked by the use of a single assay temperature, 25°C. Nevertheless, both initial recovery of F_v/F_m and initial recovery of light limited CO_2 fixation rates were inhibited at 5°C even though both q_p and q_N changed at 5°C. From the present data, I cannot determine the factor responsible for the increase of F_v/F_m during the initial phase of recovery at 20°C, but I suggest that a possible quenching mechanism of F_v is relaxed and that this relaxation is inactivated at 5°C. If light activation of certain enzymes is

responsible for the initial recovery phase of light limited CO_2 fixation rates, this process would also be inactivated at 5°C, maybe via a slow electron transport rate which would maintain a low level of NADPH or via slow enzymatic processes.

The overall slow recovery rates at 5°C can be explained by slow metabolic rates that turn over the proteins much slower than at 20°C, as shown with L-[^{35}S]methionine incorporation studies. The effect of protein synthesis inhibitors on recovery rates at 5°C were therefore less noticeable than at higher temperatures as noticed by Greer *et al.* (1986). It is worth mentioning that Gong and Nilsen (1989) showed that Q_B was degraded faster when photoinhibition occurred at 20°C than at 5°C in *Lemna gibba*. From similar data in pumpkin leaves, Tyystjärvi and Aro (1990) suggested that at low temperature, photoinhibition inactivates PSII but leaves the D1 protein undegraded with the possibility of spontaneous reactivation of PSII activity following photoinhibition (Aro *et al.*, 1990). A recent study on *Chlamydomonas reinhardtii* also showed that in presence of chloramphenicol, the irreversible decrease in F_v/F_m was more important at 23°C than at 5°C, where part of the decrease was rapidly reversible upon return to room temperature and was related to a large increase in F_o (Kirilovsky *et al.*, 1990). Therefore recovery at 5°C might not be as dependent on protein synthesis as recovery at 20°C.

Light limiting rates recover faster than light saturated rates at 20°C as shown in cucumber and maize by Long *et al.* (1987). However, Horton *et al.* (1987) have found that light saturated rates of barley protoplasts recovered faster than light limiting rates at

20°C following photoinhibition at 2°C. Light limiting rates recover as soon as the quantum efficiency is improved, but the light saturated rates can be slowed down by other factors such as regulatory mechanisms.

A mild photoinhibitory treatment at 20°C induced some photoinhibition of RNH cells, but after recovery, higher CO_2 fixation rates than the control rates were observed. This was correlated with higher q_p in RNH cells after recovery than before the treatment. Samuelsson *et al.* (1987) have also found an increase in photosynthetic rates during recovery to more than 100% of the control after a 40% photoinhibition treatment in high PPF grown *Anacystis nidulans* cells. Maybe these cell systems require long exposure to low PPF in order to develop maximum photosynthetic activity.

RNH cells recovered both F_v/F_m and light limited CO_2 fixation rates at the same rate as RH cells at both 20°C and at 5°C. They are thus probably capable of similar repair mechanisms and relaxation of quenching mechanisms. The data from q_N and q_o suggest in fact that both RH and RNH cells relaxed their non-photochemical quenching mechanisms to the same extent. But L-[^{35}S]methionine uptake and incorporation have shown that RNH cells synthesized proteins at a faster rate than RH cells at both 5 and 20°C. Therefore the replacement of damaged proteins in rye cells after photoinhibition does not appear to be limited by protein synthesis rates *per se*. Similar recovery rates in rye cells is consistent with the recovery of F_v/F_m in rye leaves after high PPF treatment at 2600 μmol m^{-2} s^{-1} (Fig. 6) and at 400 μmol m^{-2} s^{-1} (Öquist and Huner, 1991). Therefore the differential response of RH and RNH leaves to photoinhibition does not

reside at the recovery level.

4.4 Photoinhibitory response of isolated thylakoids

Rye thylakoids submitted to different high PPF treatments suffered from photoinhibition as indicated by the decrease in their F_v/F_m, PSII activities and in the number of atrazine binding sites. These decreases were essentially due to high PPF treatment because dark controls kept at room temperature showed a much slower decrease of their fluorescence parameters (Fig. 34). Photoinhibitory effects on rye thylakoids varied with PPF and temperature during the treatment. Lower PPF slowed down the decrease in F_v and the increase in F_o; this reflects a relationship between the number of quanta absorbed by the thylakoids and the extent of photoinhibition as seen with isolated rye cells. Rye thylakoids were less sensitive to photoinhibitory damage when treated at low temperature than at room temperature. This temperature dependence has also been noticed by Satoh (1970) and by Nedbal *et al.* (1986). This is different from the results found in rye leaves and in isolated mesophyll cells where high PPF treatments induced similar decrease in F_v/F_m at 20 and at 5°C. Possible structural changes could occur at low temperature when thylakoids are isolated and explain the differential temperature effect during high PPF treatment. Furthermore, the activity of proteases could be slowed down at low temperature and somehow reduce the damage caused by photoinhibition (Richter *et al.*, 1990a). Low temperature high PPF treatment of rye thylakoids showed a different relationship between F_v and F_o changes compared with room temperature treatments. This suggests a partial independence in the changes

of those two parameters during high PPF treatment or that low temperature affects F_o and F_v differently from room temperature.

Thylakoids exposed to photoinhibitory conditions at either 5°C or 20°C exhibited an increase in F_o. F_o increases have been associated with damage to PSII (Demmig and Björkman, 1987). However, RNH thylakoids exhibited greater increases in F_o even though their F_v/F_m decreased more slowly than in RH thylakoids. An increase in F_o may reflect a decrease in the efficiency of energy transfer from LHCII to the PSII reaction centres due to a physical dissociation of LHCII from the PSII core (Barényi and Krause, 1985). This mechanism could offer some protection to the thylakoids during photoinhibition. The differential effects of photoinhibition on F_o may be the result of the organizational differences between RH and RNH LHCII-PSII units (Huner *et al.*, 1987).

Two additional observations support the hypothesis of LHCII dissociation to account for F_o increase: first, the increase in Q_m, and second, the initial lower F_o values in RNH thylakoids. Q_m values of RNH thylakoids increased during high PPF treatment; this could be interpreted as a greater capacity for non-radiative decay in RNH thylakoids once submitted to high PPF. The same phenomenon has been observed with photoinhibited *Raphanus sativus* cotyledons (Buschmann, 1987) and with photoinhibited pea leaves (Havaux, 1989). RH thylakoids maintained similar Q_m values during the whole treatment; this appears to correlate with the limited increase in F_o in RH thylakoids. The difference in the initial F_o values of RH and RNH thylakoids could

reflect also a different organization in the two populations of thylakoids. Lower F_o values in RNH thylakoids could be explained by a closer association between LHCII and PSII in RNH than in RH thylakoids; this close association would allow higher quantum efficiencies as indicated by higher F_v/F_m in RNH thylakoids. Earlier study on fluorescence emission spectra and induction kinetics of isolated rye thylakoids have already suggested some uncoupling between LHCII and PSII units in RH thylakoids as well as a better connectivity between PSIIα units in RNH thylakoids (See section 1.1 and Griffith *et al.*, 1984b). Leaves showed the same trend as isolated thylakoids: RNH leaves had lower F_o and higher F_v/F_m than RH leaves.

The decrease in light saturated PSII activities and in ϕ_{app} of PSII confirmed that the loss of F_v in rye thylakoids is due to PSII inactivation. These inhibited reaction centres may become quenchers of Chl fluorescence (Barényi and Krause, 1985). However, PSII activities decreased at a faster rate than F_v/F_m as noticed by Cleland and Critchley (1985) and by Cornic and Miginiac-Maslow (1985).

Contrary to the results found *in vivo* where PSI is little affected during photoinhibition (Powles and Björkman, 1982), isolated thylakoids showed significant PSI inactivation. Therefore, the presence of electron transport activities around PSI (linear, cyclic and/or pseudocyclic) *in vivo* protects the PSI against inactivation. Nedbal *et al.* (1986) have suggested that inactivation of PSI reflected impairment of functional integrity of the thylakoid membranes at higher temperature since PSI activity of isolated thylakoids was stable during photoinhibition at low temperature. It is possible also that

PSI becomes photoinhibited (Cornic and Miginiac-Maslow, 1985; Barényi and Krause, 1985). RH and RNH thylakoids exhibited a similar decrease in PSI activities and in ϕ_{app} of PSI; the differential response to photoinhibition between RH and RNH thylakoids resides thus at the PSII level only.

PAS measurements have been used to monitor energy storage, also called photochemical loss, in thylakoids during photosynthesis (Lasser-Ross *et al.*, 1980). At a modulated frequency of 35 Hz, the relative energy storage yield in isolated thylakoids has been proposed to reflect the energy accumulated in the PQ pool (Malkin and Cohen, 1979; Carpentier *et al.*, 1985). Therefore, one should expect the PAS results to be similar to PSII measurement results because active electron transport from water to Q_B are required in both types of measurements. The initial decrease in ϕ'_r was fast and followed the decrease in PSII activities. Once all PSII activity was lost (35 min), the thylakoids were still able to store 40% of the initial energy level and ϕ'_r decreased slowly with time under high PPF treatment resembling the kinetics of PSI decrease. After all PSII activity is lost, cyclic electron transport around PSI could store energy as shown in *Anacystis nidulans* (Carpentier *et al.*, 1984, 1986) and in *Chlamydomonas reinhardtii* (Canaani *et al.* 1989). However, Carpentier *et al.* (1990) have shown that in isolated thylakoids poisoned with DCMU, which blocks linear electron transport from PSII to PSI, no cyclic PSI can be measured as a PAS signal. This is probably caused by the absence of NADP in isolated thylakoids; O_2 becomes the final electron acceptor, but it does not induce cyclic electron transport around PSI (Egneus *et al.*, 1975). Furthermore, when an electron acceptor like MV was added to DCMU poisoned

thylakoids, no effect was noticeable, which suggested that cyclic PSI was not involved in energy storage (Carpentier *et al.*, 1990). Therefore the remaining PAM signal after PSII activity is lost has to come from another component of the electron transport chain. One other possibility is a cyclic electron transport around PSII (Heber *et al.*, 1979; Thompson and Brudvig, 1988). This cycle possibly works through cytochrome b_{559}, a component of PSII reaction centres (Fig. 1), which receives electrons from PQ and reduce P_{680} (Thompson and Brudvig, 1988). Jansen *et al.* (1989) have also suggested that ϕ'_r reflects linear as well as cyclic electron transport around PSII and PSI to explain that ϕ'_r was less sensitive to photoinhibition than O_2 evolution in leaves. If a cyclic electron cycle stores energy in PSII, the energy could be stored in a different intermediate than the PQ pool as suggested by a different ϕ'_{r0} in 30 and in 45 min photoinhibited samples compared with the ϕ'_{r0} of control samples. Further work is required to show this definitively.

The atrazine binding study suggests that at least part of the decrease in the PSII activity and in F_v/F_m ratios is due to damage to the Q_B site. Although the overall kinetics for the decrease in F_v/F_m and atrazine binding were similar, the [^{14}C]atrazine binding data showed no significant differences between RH and RNH thylakoids, whereas Chl *a* fluorescence and PSII electron transport data showed that RNH thylakoids were less susceptible to photoinhibition than RH thylakoids. Thus, the differential effect of photoinhibition on F_v in RH and RNH thylakoids could not be accounted for by differential damage to the Q_B binding site of the PSII reaction centres. Other possible sites of damage may be at the level of the water splitting side of PSII

(Theg *et al.*, 1986) or at the reaction centre proper, P_{680} (Cleland, 1988). We have insufficient data to discriminate between these two possibilities.

The relationship between PSII activity, F_v, ϕ'_r and the number of atrazine binding sites is complex. After 35 min of photoinhibition, rye thylakoids have lost all their PSII activity, with 30% of F_v, 40% of ϕ'_r and 50% of [^{14}C]atrazine binding sites left in these thylakoids. From similar data, Virgin *et al.* (1988) have suggested that photoinhibition primarily destroys the photochemical reaction and that protein degradation is a subsequent event. The PSII reaction centres that still have a functional Q_B site are not capable of stable charge separation and this would block all electron transport and quench fluorescence (Virgin *et al.*, 1988). After 60 min of photoinhibition, all F_v is quenched but 30% of ϕ'_r and 30% of the [^{14}C]atrazine binding sites are still present in rye thylakoids. The PSII reaction centres not inactivated after 60 min of photoinhibition appeared to be very resistant as shown by the slow decrease in ϕ'_r between 60 and 90 min. As suggested by Robert Carpentier (personal communication), these reaction centres could be PSIIß, which have Q_B sites but do not participate in the linear electron transport chain and have a small Chl antenna (Melis and Anderson, 1983). According to Melis and Anderson (1983), PSIIß represents about 20-25% of the initial number of PSII reaction centres, but this could possibly vary from species to species and with growth conditions (Neale and Melis, 1986). PSIIß could show a great resistance to photoinhibition because they have small Chl antennae (Cleland and Melis, 1987; Farineau, 1990). According to the PAS data, they could store energy, but the fluorescence data suggest that the PSIIß do not fluoresce. Under

control conditions, Neale and Melis (1990) calculated that PSIIß fluorescence represents about 15% of the total F_v in *Chlamydomonas reinhardtii*. An explanation for the lack of F_v from PSIIß in isolated rye thylakoids could be that the F_o emitted from LHCII during photoinhibition masks the F_v emitted from PSIIß. Regardless of the state of PSII reaction centres after 60 min of photoinhibition, they are still capable of storing some energy, possibly through cyclic electron transport, and some still have intact Q_B sites.

4.5 Comparison of the three levels studied

According to F_v/F_m data, RH leaves are more resistant to photoinhibition than RNH leaves. This can also be shown in rye cells with F_v/F_m data. Clearly, whatever is causing an increase in resistance to photoinhibition in RH leaves is also present in isolated cells. However, when isolated thylakoids are photoinhibited, the resistance is actually higher for RNH than for RH thylakoids. Therefore, the resistance to photoinhibition in RH leaves either does not reside at the level of the thylakoids or it requires intact chloroplasts in order to be expressed. This narrows down the possible mechanisms responsible for the increased resistance to photoinhibition in RH leaves. One possible explanation would be the presence of more O_2 radical scavengers in RH leaves and cells which would protect the thylakoids against damage. These scavengers are probably lost during thylakoid isolation and would explain the similar loss in Q_B binding during photoinhibition of isolated thylakoids. Increased levels of O_2 radical scavengers following cold acclimation has been shown in spinach (Schöner and Krause, 1990). A second possibility is an increased level of zeaxanthin formation during

photoinhibition of RNH leaves and cells compared with RH leaves and cells. This would decrease the F_v/F_m in RNH relative to RH leaves and cells, but partially protect PSII against damage (Demmig *et al.* 1987b). According to Demmig-Adams *et al.* (1990), the violaxanthin/zeaxanthin cycle components are lost during thylakoid isolation and would not quench F_v nor F_o in isolated thylakoid system.

Recovery studies in both leaves and cells showed similar capacity to recover from photoinhibition for RH and RNH leaves. Even though some recovery can occur during photoinhibition as suggested by Greer *et al.* (1986), this factor cannot be responsible for the increased resistance to photoinhibition in RH leaves.

According to data obtained with thylakoids isolated from photoinhibited leaves (Lapointe *et al.*, submitted), F_v/F_m of RNH leaves were much more depressed than the F_v/F_m of thylakoids isolated from these leaves. As expected from a photoinhibitory gradient across the leaf, F_v/F_m of thylakoids was higher than F_v/F_m of photoinhibited leaves. This has been shown also in spinach (Somersalo and Krause, 1990) and in pumpkin (Tyystjarvi *et al.*, 1989). The decrease in F_v/F_m of leaves was also greater than the decrease in the number of atrazine binding sites, as previously observed in spinach by Chow *et al.* (1989). However, the difference between F_v/F_m of thylakoids and leaves was much greater in RNH than in RH samples. Similar losses of [^{14}C]atrazine binding sites were shown in RH and RNH thylakoids from leaves exhibiting different levels of photoinhibition (as monitored by F_v/F_m data). These data would be consistent with an increase in zeaxanthin formation in RNH leaves, which would protect the photosynthetic

apparatus during photoinhibition. RNH would therefore have a greater capacity to increase its zeaxanthin level during photoinhibition than RH leaves. Cells and thylakoids isolated from these cells showed a similar decrease in F_v/F_m for both RH and RNH samples (Fig. 44), but the cells were treated at 2600 μmol m^{-2} s^{-1} where no significant difference was measured between the resistance to photoinhibition of RH and RNH cells. An increased level of O_2 radical scavengers in RH leaves could not explain the data obtained with thylakoids isolated from photoinhibited leaves because in this case thylakoids and leaves should present similar level of photoinhibition. Thus, the apparent increased resistance of RH leaves to photoinhibition should still be considered with suspicion and a more thorough study of non-photochemical quenching during photoinhibition is required to assess its importance in the overall response of F_v/F_m of RH and RNH leaves.

More importantly, CO_2 fixation rates failed to show any difference in resistance to photoinhibition between RH and RNH cells. Furthermore the recovery of CO_2 fixation rates and of F_v/F_m of rye cells appeared as though they were quite independent phenomenon. Therefore, I question the use of F_v/F_m alone to quantify the effect of photoinhibition on plants. What F_v/F_m measures is the extent of photoinhibition on ϕ_{app} of PSII. However, to conclude that plants are more resistant to photoinhibition requires that the whole photosynthetic process shows more resistance to photoinhibition. This can only be assessed by measuring ϕ_{app} for CO_2 fixation rates in intact systems. Even though photoinhibition has been usually assessed as a decrease in ϕ_{app} for PSII, I think that in order to evaluate the importance of photoinhibition for overall plant

photosynthesis and growth it is necessary to show an impact on CO_2 fixation at the PPF that the plants grow. This is not usually done. My data show clearly that photoinhibition of PSII as measured by F_v/F_m is quite distinct from photoinhibition at the level of CO_2 fixation. Both aspects of the photosynthetic process must be monitored to assess the true impact of photoinhibition on photosynthesis.

Another point of interest in this study is the effect of photoinhibition on F_o at different levels of biological organization as previously noticed by Somersalo and Krause (1989). F_o appears to be controlled by a lot of factors. F_o values increased more in RH than in RNH leaves during photoinhibition. They showed similar changes during photoinhibition of RH and RNH cells at either 2600 μmol m^{-2} s^{-1}, where they tend to be higher than the controls, and at 400 μmol m^{-2} s^{-1}, where they decreased during the treatment. F_o values increased to a greater extent in RNH than in RH thylakoids. F_o changes were more important in isolated thylakoids than in intact systems, as previously noticed by Barényi and Krause (1985) who were comparing spinach intact and broken chloroplasts. In isolated thylakoids, F_o values seem to be related to the extent of cooperation between LHCII and PSII units. Yet in rye cells and leaves, some quenching mechanisms could decrease F_o, other quenching mechanisms could have no effect on F_o (Adams *et al.*, 1990a), while damage to PSII reaction centres could tend to increase F_o (Demmig and Björkman, 1987). F_o values show the net results of these divergent effects (Winter and Königer, 1989). It is therefore more difficult to analyze the significance of F_o changes in cells and in leaves.

The different quenching mechanisms measured at steady-state showed similar effects in leaves and in cells. Since leaves and cells did not have the same q_N, q_p and q_o levels under control conditions, the comparisons are limited to examining the relative changes occurring during photoinhibition. As expected, q_N and q_o increased, suggesting that non-photochemical quenching is built up to try to dissipate the excess energy (Krause and Behrend, 1986). Under saturating but non photoinhibitory conditions, high q_N can maintain Q_A in an oxidative state (Demmig and Winter, 1988b; Weis and Lechtenberg, 1989), but excessive PPF could induce both high q_N and low q_p (Demmig and Winter, 1988b) as measured after high PPF treatment in rye cells. Van Wijk and Van Hasselt (1990) have suggested that during photoinhibition the large build-up of q_N could decrease the electron transport rates as expressed by a decrease in q_p. However, decreases in q_p can also reflect damage to the photosynthetic apparatus once the non-radiative decay mechanisms are overcome as suggested by Foyer *et al.* (1990) and as supported by the photoinhibitory and recovery study in isolated rye cells.

4.6 Characterization and usefulness of the isolated cell system

Several factors appeared to be important to give a good yield during the cell isolation procedure. The presence of high M_r potassium dextran sulfate increased the yield considerably even though Baumann and Günther (1986) have found that low M_r potassium dextran sulfate was more efficient than high M_r in the isolation of *Chenopodium album* cells. The yield was higher when maceration occurred at 7.3 rather than at pH lower than 6 (data not presented), as already observed by Baumann and

Günther (1986). Finally, stirring the leaf segments and circulating the isolated cells and the maceration medium sped up the isolation procedure. The technique of Servaites and Ogren (1977a) has been an important improvement over the usual maceration techniques where the leaf segments were soaked in maceration medium for a few hours before isolated cells were separated from the maceration mixture.

Most studies on photosynthetic activity of isolated mesophyll cells do not indicate the percentage of viable cells in their preparation. When indicated, viability ranged between 55 (Rees *et al.*, 1985) and 95% (Singh, 1981) with an average value of 84 ± 8% (n= 9). The viability of RH and RNH cell samples are thus slightly lower than the average viability obtained from different species, and lower than what has been obtained previously with winter rye (Singh, 1981). From the studies indicating the yields of cells or protoplasts released on a Chl basis, many had yields varying between 10 and 30%, while two studies had much higher yield between 50 and 70% (Colman *et al.*, 1979; Servaites and Ogren, 1980). Most of these studies required a long period of maceration, while the technique used in this study isolated cells within 30 min. Furthermore, yield is probably very dependent upon species. Two studies on isolated rye cells (Singh, 1981) and rye protoplasts (Schulz and Weissenböck, 1986) indicated a yield of 0.5- 1 x 10^6 cells and of 4- 5 x 10^6 protoplasts per g of fresh weight of leaves, which is comparable to what has been obtained in this study.

Many studies have measured CO_2 fixation rates on isolated cells. About half of the studies surveyed indicated CO_2 fixation rates of 29 ±15 μmol mg Chl^{-1} h^{-1} (n= 9)

and the other studies indicated much higher CO_2 fixation rates at about 109 ± 28 μmol mg Chl^{-1} h^{-1} (n= 12). Other studies have measured O_2 evolution rates and showed a similar trend some with low photosynthetic rates (37 ±12) and some with high photosynthetic rates (121 ±17 μmol O_2 evolved mg Chl^{-1} h^{-1}). All of these photosynthetic rates were light saturated, CO_2-saturated and measured at room temperature (20- 25°C). Studies on cells or protoplasts isolated from cereals showed a wide range of results from 103 ±5 μmol CO_2 fixed mg Chl^{-1} h^{-1} (Sarhan and Cesar, 1988) to 100- 150 μmol O_2 evolved mg Chl^{-1} h^{-1} (Edwards *et al.*, 1978), 288 nmol O_2 evolved 10^5 $cells^{-1}$ h^{-1} (Watanabe *et al.*, 1988), and 1800 nmol CO_2 fixed 10^5 $cells^{-1}$ h^{-1} (De Filippis, 1986). The photosynthetic rates of rye cells reported here and expressed on a Chl basis are comparable with the low photosynthetic rates obtained by many others. However, when the rates are expressed on a viable cell basis, they are as high as the other studies surveyed (n= 5) with the exception of the study of De Filippis (1986).

Photosynthetic rates of leaves are more accurately expressed on a leaf area basis, but when these rates are expressed on a Chl basis, a more direct comparison can be made with photosynthetic rates of isolated cells. RH samples showed similar rates of CO_2 fixation between leaf and cells (48 and 53 μmol CO_2 fixed mg Chl^{-1} h^{-1} at 20°C, respectively). RNH cells showed lower rates than RNH leaves (28 and 69 μmol CO_2 fixed mg Chl^{-1} h^{-1}, respectively). This discrepancy could be related again to a differential extraction of Chl between RH and RNH cells. Other studies have also measured lower photosynthetic activity in the isolated cells than in leaves (Rees *et al.*, 1985; Plaut *et al.*, 1989; Colman and Mawson, 1978; Morris *et al.*, 1981), which suggests that cells are

under stress after isolation or that aqueous conditions are not optimal for gas exchange during photosynthesis of mesophyll cells.

The Chl *a* emission spectra of isolated rye cells are different from the emission spectra of isolated rye thylakoids. In rye cells, the PSII peaks were slightly blue-shifted to about 680 nm with a shoulder at 685 nm, while in isolated rye thylakoids the major peak was measured at 685 nm with a small shoulder at 695 nm present in RH samples only (Griffith *et al.*, 1984b; Huner and Reynolds, 1989). Furthermore, the spectra of rye thylakoids exhibited PSII peaks that were higher than PSI peaks, while rye cells showed higher PSI peaks. Low temperature fluorescence emission spectra of spinach or pea thylakoids exhibited two separate peaks at 685 and at 695 nm and a similar peak height for all 3 peaks (Cleland and Critchley, 1985) or higher PSI peak (Barényi and Krause, 1985; Horton and Lee, 1985). Higher PSI peaks can be caused by reabsorption of PSII Chl fluorescence within the samples when the concentration of Chl is high or not homogenously distributed like in cells or in leaves (Nedbal *et al.*, 1986).

Chl *a* fluorescence induction curves differed between rye cells and rye leaves. Rye cells did not show the secondary M peaks typically observed in leaves (Krause and Weis, 1984). RNH cells quenched F_m faster than RH cells, while RH leaves quenched F_m faster than RNH leaves (data not shown). Faster quenching of F_m in hardened than in non-hardened material has been shown already by Hurry and Huner (1991) in spring and winter wheat cultivars and by Boese (1990) in spinach. This could be related to a faster processing of light energy by RH PSIIα units as suggested by Griffith *et al.*

(1984b). Both RH and RNH leaves tended to quench F_m faster than rye cells and to a greater extent under the same actinic PPF. This is reflected in lower q_p and higher q_N for cells than for leaves. The actinic PPF of 80 μmol m^{-2} s^{-1} possibly saturates the photosynthetic capacity of cells to a greater extent than for the leaves and could explain the higher q_N in cells, because q_N increases with PPF (Bilger and Schreiber, 1986). Demmig-Adams *et al.* (1990) have also measured lower q_p and higher q_N in chloroplasts than in leaves of spinach. At a PPF similar to what has been used during this study, barley protoplasts (Horton and Hague, 1988) showed higher q_p but similar q_N as shown by rye cells. Furthermore barley protoplasts showed a q_o of about 0.2 which is higher than what rye cells showed. However, the non-photochemical quenching parameters are dependent upon the PPF and therefore upon the Chl concentration of the samples. The low q_p in rye cells reflects a decreased capacity to oxidize Q_A but the reason is unknown.

Some variation in the uptake and incorporation of amino acids was found among studies surveyed. On a Chl basis, spinach cells have shown 0.04 to 0.1 nmol leucine incorporated mg Chl^{-1} h^{-1} (Nishimura and Akazawa, 1978) and tobacco cells 0.075- 0.15 nmol leucine mg Chl^{-1} h^{-1} (Francki *et al.*, 1971). RNH and RH cells incorporated 0.1 and 0.07 nmol methionine mg Chl^{-1} h^{-1} respectively. On a cell or protoplast basis, Sakai and Takebe (1970) estimated an incorporation rate of 2 x 10^{-4} μmol leucine 10^5 $protoplasts^{-1}$ h^{-1} in tobacco, while Fuchs and Galston (1976) have estimated 8.6 x 10^{-9} μmol leucine 10^5 $protoplasts^{-1}$ h^{-1} in oat. Again RH and RNH cells showed intermediate rates of incorporation on a cell basis (2.2- 5.8 x 10^{-7} μmol methionine 10^5 $cells^{-1}$ h^{-1}).

Comparison with these studies can be only relative because the proportion of leucine and methionine in proteins can be different. The extent of incorporation of leucine taken up by the cells varied from 36% in tobacco protoplasts (Sakai and Takebe, 1970) to 45% of total uptake in tobacco cells (Francki *et al.*, 1971), which is similar to what has been estimated for RH and RNH cells at 20°C.

Rye cells incorporated some of the ^{35}S-labelled proteins into thylakoids. Since these membranes have been washed, the labelled proteins were probably functional proteins integrated into the membranes. Over a 2 h period, many proteins did not turn over significantly. The exception is the Q_B protein which has been shown to turn over very rapidly even under low PPF conditions (Ohad *et al.*, 1984; Mattoo *et al.*, 1984). *Chlamydomonas reinhardtii* and rye cells synthesized similar thylakoid polypeptides with the exception that the algae synthesized also some of the LHCII polypeptides during their 15 min incubation with $^{35}SO_4$ (Ohad *et al.*, 1984). Polypeptides of M_r higher than 45 000 appeared to be synthesized abundantly in rye cells and in algae, but they did not show up on SDS polyacrylamide gels and therefore have not been identified.

In summary, isolated rye cells have proved to be photosynthetically competent and capable of synthesizing proteins. Even though their metabolic rates are slower than what has been measured in intact leaves, rye cells can be useful as a homogenous system to study a phenomenon such as photoinhibition. More importantly, RH and RNH cells exhibit similar photosynthetic characteristics and can therefore be used to compare RH and RNH responses to photoinhibition and subsequent recovery.

CHAPTER 5

CONCLUSION

Several conclusions can be drawn from this project. The importance of assessing the effect of photoinhibition on CO_2 fixation became obvious from the data obtained with isolated rye cells. The Chl *a* fluorescence parameter F_v/F_m has been used extensively to monitor photoinhibition, but its usefulness in assessing the effect of high PPF treatments on the overall photosynthetic responses of plants is limited. Other parameters such as Chl fluorescence quenching mechanisms under steady-state conditions can monitor in a more complete manner the effect of photoinhibition on photosynthesis and can be very useful to discern the different phenomena occurring during photoinhibition and subsequent recovery. According to the present study, CO_2 fixation rates have to be monitored to assess the effect of photoinhibition on photosynthesis because regulatory mechanisms can amplify or diminish the effect of high incident PPF on the electron transport components.

Study of the kinetics of recovery from photoinhibition appears as important as the kinetics of photoinhibition to elucidate the different phenomena occurring during photoinhibition. Since recovery takes place more slowly, it might discriminate better between damage requiring protein synthesis and relaxation of the different protective mechanisms. Again, both CO_2 fixation and Chl *a* fluorescence must be monitored during recovery because they present different kinetics.

Isolated rye cells showed the same trends as intact leaves during photoinhibition and recovery when monitored with F_v/F_m. This indicates that the differential morphology between RH and RNH leaves (Huner, 1985b) is not implicated in the possible increased resistance of RH leaves to photoinhibition. It also proves the usefulness of the isolated cell system in such studies.

Isolated rye thylakoids have shown that the structural differences observed at the thylakoid level between RH and RNH leaves induced a differential response to photoinhibition. However, RNH thylakoids were less susceptible to photoinhibition than RH thylakoids. Therefore, the differential structural changes occurring at the thylakoid level during cold hardening of rye is not implicated in its possible increased resistance to photoinhibition.

The isolated rye thylakoid study has suggested a possible role for the differential organization of rye thylakoids following cold hardening. According to F_o and F_v/F_m data, the transfer of energy between LHCII and PSII would be more efficient in RNH than in RH plants and this might be related to the different ratios of monomeric to oligomeric forms of LHCII found in RH and RNH plants. Further work on the transfer of energy by the different LHCII populations as well as on their physical organization in the thylakoid membrane is required before any conclusions can be drawn.

Protein synthesis inhibitor studies on isolated rye cells have shown that photoinhibition induced some damage to the thylakoid membrane and that recovery

partially relied on synthesis of new proteins. But part of the decrease in photosynthetic activity of rye cells during photoinhibition is related to the increase in non-radiative dissipation of light energy at steady-state. A recent study of thylakoids isolated from photoinhibited rye leaves (Lapointe *et al.*, submitted) also suggested an important role for non-radiative dissipation of light energy during photoinhibition. Their data further suggested that RNH leaves might have a greater capacity to re-emit light in excess as heat and questioned the increased resistance of RH leaves based on F_v/F_m data. However, non-radiative decay mechanisms affecting F_v/F_m are probably different from non-radiative mechanisms taking place under steady-state photosynthesis.

Further work is certainly required to elucidate the phenomenon of increased resistance to photoinhibition in RH leaves. The different Chl *a* fluorescence non-photochemical quenching mechanisms should be assessed to see their importance during photoinhibition of both RH and RNH leaves. As suggested by data from isolated cells, q_N should be assessed at the temperature of the treatment. Furthermore, the level of zeaxanthin during and following photoinhibition should be monitored to determine its importance in the quenching of F_v/F_m of RH and RNH leaves. More importantly, CO_2 fixation of RH and RNH leaves should be measured during and following photoinhibition before any major conclusions can be drawn in relation to the initial hypothesis that the cold hardening process implies a greater resistance to low temperature photoinhibition in winter rye.

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